Degradation of Azaglycinamido Residues in Model Tripeptides Derived from Goserelin

MARNIX A. HOITINK, JOS H. BEIJNEN, AUKE BULT, J. MIRJAM A. DAMEN, OEDS A. G. J. VAN DER HOUWEN, JOHN A. W. KRUIJTZER, MATTHIJS M. TIBBEN, GERARD WIESE, WILLY J. M. UNDERBERG

Department of Pharmaceutical Analysis, Faculty of Pharmacy, Utrecht University, Sorbonnelaan 16, NL-3584 CA Utrecht, The Netherlands

Received 6 July 1999; accepted 26 October 1999

ABSTRACT: Three model tripeptides, *N*-acetyl-Tyr-Pro-azaGly-NH_2 (NYPaG), Tyr-Pro-azaGly-NH_2 (YPaG), and Tyr-Pro-Gly-NH_2(YPG), were subjected to a systematic degradation study to get information about the degradation of the azaglycinamido residue. The degradation products were characterized with LC-MS. Main degradation products of NYPaG possess partially or totally eliminated azaglycinamido residues, while YPaG and YPG are exhibit cyclo(Tyr-Pro) formation, a diketopiperazine. The influence of the pH on the degradation rate constant k_{obs} was investigated for NYPaG and YPaG in the pH range 0.4–11. An U-shaped profile with an inflexion around pH 9 was found for NYPaG while the degradation rate of YPaG was independent of the pH. NYPaG apparently was subject to proton-, solvent-, and hydroxyl-catalyzed degradation reactions whereas YPaG only underwent solvent-catalyzed reactions. Some influence of acetate and phosphate ions on k_{obs} was found for YPaG. Arrhenius plots of NYPaG and YPaG were found to be linear. © 2000 Wiley-Liss, Inc. and the American Pharmaceutical Association J Pharm Sci 89: 108–114, 2000

INTRODUCTION

Peptides are gaining importance as medicinal compounds. A traditional disadvantage for therapeutical purposes is that these peptides are sensitive to relatively fast hydrolysis by ubiqutuous human enzymes. To overcome this disadvantage, peptidomimetics can be developed in which some or all amino acid residues are replaced by unnatural derivatives that are less susceptible to enzymatic hydrolysis. A good example of a medicinal peptidomimetic compound is the gonadorelin analogue goserelin, in which the C-terminally located glycinamido moiety in gonadorelin has been replaced by an azaglycinamido moiety. In this way, the plasma half-life in humans is increased from 15 min to more than 4 h.[1]

Correspondence to: M. A. Hoitink (E-mail: M.A.Hoitink@pharm.uu.nl)

Journal of Pharmaceutical Sciences, Vol. 89, 108–114 (2000)

However, besides pharmacokinetics, the pharmaceutical characteristics are also changed. In studies on the stability of goserelin,[2,3] degradation of the azaglycinamido moiety was observed at pH > 4. Not all degradation products could be identified because in goserelin more degradation pathways occur and the yield of individual products is limited. In order to get more insight into both qualitative and quantitative degradation processes of the the azaglycinamido moiety, small model peptides were synthesized containing this functional group and these peptides were subjected to a systematic stability study.

EXPERIMENTAL SECTION

Chemicals

All chemicals used were of analytical grade.

N-Acetyl-Tyrosine-Proline-aza-Glycine-amide (NYPaG) and Tyrosine-Proline-aza-Glycine-amide (YPaG) were synthesized. Every coupling

step was followed by an extraction procedure: the compound was brought into ethylacetate and subsequently extracted with 1 M $KHSO_4$, 1 M $NaHCO_3$, and a saturated NaCl solution. The ethylacetate solution was dried with anhydrous Na_2SO_4 and filtered, and the solvent was removed under vacuum.

YPaG was synthesized by coupling tertiary butyloxycarbonyl(boc)-tyrosine(benzyl) with proline methyl ester using benzotriazolyl-*N*-oxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) and diisopropylethylamine (DIPEA) in dichloromethane (DCM). The methylester group was hydrolyzed with NaOH in methanol. This dipeptide was then coupled to semicarbazide with BOP/DIPEA in DCM. The benzyl moiety was removed by hydrogenation with palladium on carbon in methanol. The boc moiety was eliminated with HCl in ether.

NYPaG was synthesized similarly to YPaG except that the boc-tyrosine(benzyl) was first treated with HCl in ether and then N-acetylated with acetic anhydride and triethylamine in dioxane.

YPaG was purified with a gradient RP-HPLC procedure using an 250 × 20 mm i.d. econosphere C_8 column (Alltech, Breda, The Netherlands) at a flow of 11 mL/min. Mobile phases A and B consisted of 1 mL trifluoro acetic acid (TFA)/1,000 mL water and 1 mL TFA/500 mL water/500 mL methanol, respectively. The gradient was run from 0 to 10% B in 30 min.

NYPaG was purified with an isocratic RP-HPLC procedure using an 250 × 10 mm i.d. Chromspher C_{18} column (Chrompack, Bergen op Zoom, The Netherlands) at a flow of 4 mL/min. The mobile phase consisted of 1.42 mL acetic acid/0.936 mL 25% w/w ammonia/800 mL water/200 mL methanol. After purification, the methanol was removed under a nitrogen stream and the remaining solutions were freeze-dried. Yields were in the order of magnitude of 20%.

YPG (Tyrosine-Proline-Glycine-amide) was synthesized at the Netherlands Cancer Institute (Amsterdam, The Netherlands) utilizing a peptide synthesizer.

The identities of all three peptides were confirmed with MS and ^{1}H-NMR, and the purities were verified with RP-HPLC and found to be more than 95%.

pH Measurement

All pH measurements were performed on a Consort P514 pH meter (Salm & Kipp, Breukelen, The Netherlands) equipped with a Slim-trode electrode (Hamilton, Darmstadt, Germany). The pH was measured before and after degradation. All pH values were determined at the appropriate degradation temperature.

Liquid Chromatography-Mass Spectrometry (LC-MS)

The liquid chromatograph consisted of a LC-10 AD liquid chromatograph (Shimadzu, Tokyo, Japan) provided with a 7125 injector (Rheodyne, Cotati, CA). The column used was a Superspher 100 RP-18 (5 μm) 119 × 2 mm i.d. (Merck). As mobile phase 0.1% v/w TFA in mixtures of methanol and water was used. For YPaG-containing samples the mixture consisted of 12% w/w methanol/88% w/w water, while 16% w/w methanol/84% w/w water was used for all other compounds. The flow was set at 0.1 mL/min.

This LC system was directly coupled to a VG Platform II mass spectrometer (MicroMass, Altrincham, U.K.), operated in the positive ion mode. Calibration was performed with an aqueous 4 mg/mL sodium iodide solution. The scanned mass range is 100–500 *m/z,* scan time is set at 1 s, and low and high mass resolutions are 12.5 and 15 (instrumental units), respectively. Cone voltage was 25 V, and the probe temperature was kept at 120°C. Data acquisition and calculation of the *m/z* values was done with MassLynx version 2.3 (MicroMass).

RP-HPLC Systems for NYPaG, YPaG, and YPG

A Gynkotech Model 300 precision pump (Separations, H.I. Ambacht, The Netherlands) was connected to a ISS 100 sampling system (Perkin-Elmer Corporation, Norwalk, CT). Mixtures of acetonitrile or methanol, water, and TFA were used as mobile phases. For the analysis of NYPaG-, YPaG-, and YPG-containing samples, the composition of the mobile phase was 0.1% v/w TFA in 12% methanol/88% water w/w, 4% methanol/96% water w/w, and 6% acetonitrile/94% water, respectively. The column was a Lichrospher 100 RP-18 (5 μm) 125 × 4 mm i.d. (Merck, Darmstadt, Germany). Detection was performed using an Applied Biosystems 785A programmable absorbance detector (Separations, H.I. Ambacht, The Netherlands) operating at 214 nm, and a 40 μL sample was injected.

Preparation of Degraded Samples for LC-MS

For LC-MS analysis the initial tripeptide concentration was 500 μg/ml. For NYPaG, samples were prepared at pH 2, 5, 9, and 10, for YPaG at pH 2, 5, 7, 9, and 10, and for YPG at pH 9 and 10. A carbonate buffer was used for pH 10 instead of the standard buffer. NYPaG pH 5 was degraded at 80°C, all other samples at 70°C. The typical degradation time was two estimated half-lives.

Standard Degradation Conditions

Degradation experiments were executed in flame-sealed 1 mL glass ampoules, that were kept in a thermostated water bath at the appropriate temperature. Sampling of the ampoules with NYPag, YPaG, and YPG was done over a range of at least twice the estimated half-life. For each degradation curve, 10 measurement points were used and all experiments were performed in duplicate. Standard degradation conditions were 70°C, a tripeptide concentration of 20 μg/mL, a buffer concentration of 25 mM, and an ionic strength of 0.1, adjusted with sodium perchlorate. Buffers used were perchloric acid below pH 2.5, acetate in the pH range 4–6, phosphate in the pH range 6–7.5, borate in the pH range 8–9.5, triethylamine in the pH range 9.5–10, and sodium hydroxide above pH 10. All experiments were done under standard degradations conditions, unless otherwise stated.

Calibration Curves and Standard Deviation

Calibration curves of NYPaG, YPaG, and YPG were constructed in the concentration range 5–40 μg/mL.

Standard deviations in k_{obs} for NYPaG and YPaG were determined at pH 8.4 and 8.6, respectively. For each determination 6 degradation curves were used.

Influences of Buffers and pH on the Degradation Rate Constant k_{obs} of NYPaG, YPaG, and YPG

The influence of acetate and phosphate buffer ions on k_{obs} of YPaG was determined at pH 4.9 and pH 6.9, respectively. The buffer concentration range was 25–100 mM and 12.5–50 mM for acetate and phospate, respectively.

The influence of the pH for NYPaG and YPaG was determined in the pH range 1–11, and that for YPG in the pH range 8–11.

Influence of the Temperature on k_{obs} of NYPaG and YPaG

The influence of the temperature on the degradation of NYPaG and YPaG was determined at pH 1, 5, and 8.7, and at pH 1.5, 5, and 8.6, respectively. The temperature range was 70–90°C for low and middle pH values, and 50–80°C for pH 8.6–8.7.

RESULTS AND DISCUSSION

The selected model compounds are NYPaG, YPaG, and YPG, all tripeptides (Figure 1). Since

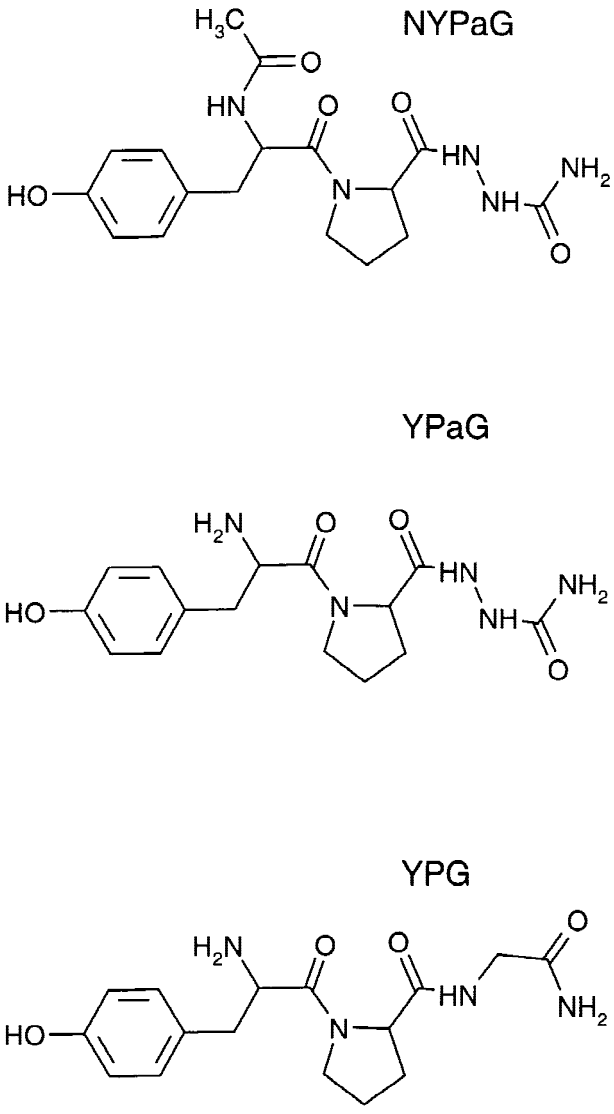

Figure 1. Chemical structures of the tripeptides NYPaG, YPaG, and YPG.

azaglycinamido-containing peptides cannot be synthesized with commercially available reagents using a peptide synthesizer, an in-solution synthesis method was developed for NYPaG and YPaG. The N-terminal residue is tyrosine that gives the necessary UV absorbance for RP-HPLC detection and is sufficiently stable in solution. The second and the third residues in NYPaG and YPaG are proline and azaglycinamide, respectively, being identical with the two C-terminally located residues in goserelin. This is likely to ensure similar degradation behavior of the model peptides compared to goserelin. A free N-terminus has both advantages and disadvantages. The advantages are satisfactory solubility in water and high sensitivity in an LC-MS system. However, an important disadvantage is the reactivity, which might cause interfering degradation reactions. This is why the N-acetylated tripeptide NYPaG is included in this study. The solubility and LC-MS sensitivity of NYPaG were still found to be sufficient for our purposes. YPG is used to determine whether any unwanted reactions in YPaG are exclusively the result of the free N-terminus or if also the azaglycinamidogroup is involved.

Identification of the Degradation Products of NYPaG, YPaG, and YPG

Degradation samples of the tripeptides were prepared at varying pH values and with different buffers. The samples were analyzed with a straightforward LC-MS method using TFA as acidifying and ion-pairing reagent. Instead of a sensitivity-reducing flow splitter, a 2 mm column was used, operating at 0.1 mL/min. The 0.1 mL/min represents the best compromise between analysis time and ion spray mass spectrometer performance on a 2 mm column. In Table I, the LC-MS results are presented as *m/z* values with their intensities, related to the intensity of the strongest peak. From these relative intensities, the amount of product can be estimated under the condition that the number of basic groups is unchanged. In all NYPaG samples, degradation products with *m/z* values of 321 and 335 are found, products B and A in Figure 2, respectively, which are similar to goserelin.[2] Since these products are found at all pH values, the mechanism is probably solvent-catalyzed. Only at pH 2, are *m/z* values of 261 and 336 found, and these are likely cyclo(Tyr-Pro) (Figure 2C) and YPaG, respectively. Apparently, the *N*-acetyl group undergoes proton-catalyzed hydrolysis, and the resulting tripeptide YPaG undergoes cyclization as the result of a nucleophilic attack by the free N-terminus on the carbonyl group of proline. Products with *m/z* values of 305 and 360 are found at pH 2, 9, and 10. The identities of these products are unknown, but the mass difference of *m/z* 360 with the parent is identical to that of an unknown product of goserelin. Speculative structures are presented in Figure 2D and E for *m/z* values 360 and 305, respectively. In conclusion, the degradation of NYPaG is mainly located at the azaglycinamido and this makes NYPaG a good model compound.

The main degradation product of YPaG has a *m/z* value of 261, probably cyclo(Tyr-Pro) depicted in Figure 2C. The free N-terminus is now responsible for a degradation reaction not found for goserelin, and this obscures the observation of the

Table I. *m/z* Values and Relative Intensities of Compounds Resulting From the Degradation of NYPaG, YPaG, and YPG*

Peptide/pH	Found *m/z* values (relative intensity)
NYPaG pH 2	261(16%),305(3%),321(4%),335(8%),335(5%),336(30%),360(7%),**378**(100%)
MYPaG pH 5	321(42%),335(10%),335(77%),**378**(100%)
NYPaG pH 9	305(7%),321(13%),335(13%),360(14%),**378**(100%)
NYPaG pH 10	305(6%),321(7%),335(9%),360(13%),**378**(100%)
YPaG pH 2	261(67%),**336**(100%)
YPaG pH 5	261(33%),318(5%),**336**(100%)
YPaG pH 7	261(100%),318(36%),336(6%),**336**(88%)
YPaG pH 9	261(35%),261(22%),318(74%),336(3%),**336**(100%)
YPaG pH 10	261(34%),261(16%),318(20%),336(<5%),**336**(100%)
YPG pH 9	261(25%),335(20%),**335**(100%),336(35%)
YPG pH 10	261(2%),335(7%),**335**(100%),336(24%)

* The parent compounds are written in bold.

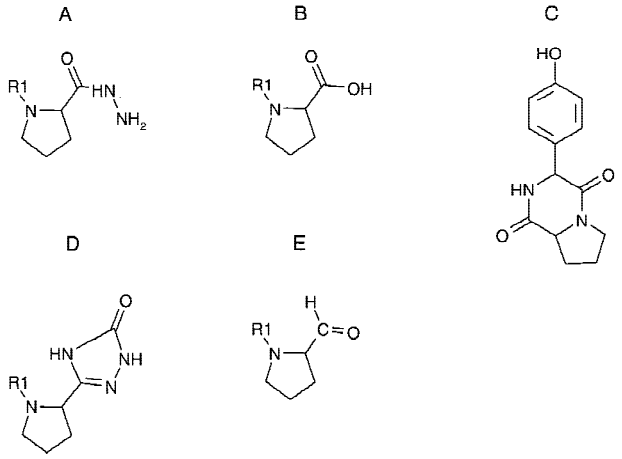

Figure 2. Degradation products of NYPaG (R1 = *N*-acetyltyrosine) and YPaG (R1 = tyrosine).

azaglycinamido degradation. One product involving the azaglycinamido group at *m/z* 318 can still be found. It is similar to *m/z* 360 in degraded NYPaG samples. A structure as in Figure 2D can therefore be postulated. The cyclization reaction of YPaG might be brought about by the azaglycinamido group, because it is the leaving group. To assess the influence of the azaglycinamido moiety on the mechanism and kinetics of the cyclization, a third tripeptide was included in the study, YPG. The difference between YPG and YPaG is substitution of the glycine residue in YPG for the azaglycinamido residue in YPaG. If YPG undergoes a similar cyclization reaction as YPaG, the free N-terminus is the driving force of this reaction. If YPG does not undergo this cyclization, the azaglycinamido group in YPaG is likely to be involved.

In the degraded YPG samples, cyclic products with *m/z* 261 are found. The cyclization reaction is indeed found, and yields the same product as with YPaG. The free N-terminus is the driving force in the cyclization reaction, so in small model peptides the N-terminus can best be blocked through, for example, N-acetylation. Parallel to cyclization deamidation of YPG occurs yielding a product with *m/z* 336. The conclusion can be drawn that in the development of model peptides, a C-terminal primary amido group might easily be a disturbing factor because of its sensitivity for deamidation. In contrast with the primary amido groups, secondary amido groups are not sensitive to hydrolysis, as demonstrated for buserelin,[3] and are a better alternative. Epimers of some compounds were found especially at pH values above 5 with one exception: the product of NYPaG with a *m/z* value of 335 was also found to epimerize at all pH values. The free C-terminal -CO-NH-NH_2 possibly promotes a solvent-catalyzed epimerization reaction of the proline residue.

RP-HPLC systems with UV detection, based on the LC-MS methods described earlier, were developed for all three model peptides for the determination of degradation rate constants. Calibration curves are linear with correlation coefficients of 0.998 and higher. The stability-indicating properties of the RP-HPLC systems can be derived from the LC-MS data, since co-eluting products can be distinguished by their *m/z* ratios. No interfering products were found.

All three peptides were found to degrade according to (pseudo)first-order kinetics. The observed degradation rate constant k_{obs} was calculated from the slopes of plots of the natural logarithm of the residual parent compound concentration versus degradation time. The relative standard deviations in k_{obs} of NYPaG and YPaG were found to be 16% and 9%, respectively. These values are in the expected order of magnitude.

Influence of the pH on the Degradation Rate Constant k_{obs} of NYPaG

The pH–log(k_{obs}) data of NYPaG are depicted in Figure 3. Data were fitted with a model developed

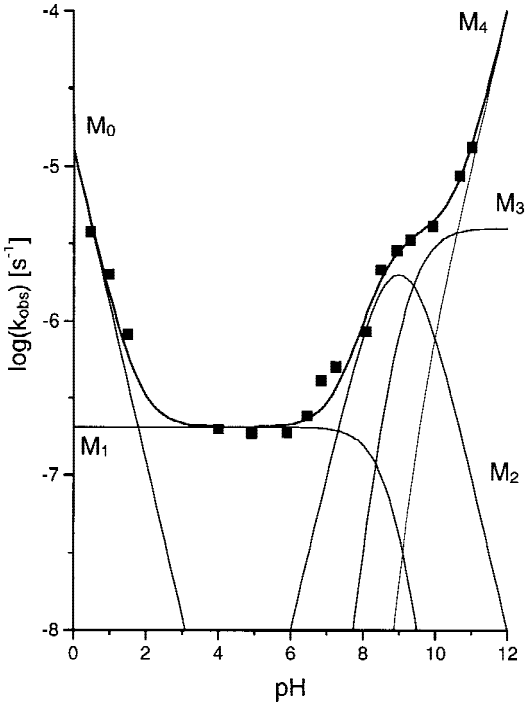

Figure 3. pH–log(k_{obs}) profile of NYPaG at 70°C and an ionic strength of 0.1.

Table II. Macro Constants of the pH–log(k_{obs}) Fit of NYPaG Using One- and Two-pK_a Models

Ionizable groups in model	log(M_0)	log(M_1)	log(M_2)	log(M_3)	log(M_4)	pK_{a1} (aza-glycinamide)	pK_{a2} (tyrosine)
2	–4.9 ± 0.1	–6.69 ± 0.04	–14.0 ± 0.1	–23.4 ± 0.3	–34.0 ± 0.2	8.5 ± 0.2	9.50[a]
1	–4.9 ± 0.1	–6.69 ± 0.05	–14.0 ± 0.1	–24.5 ± 0.2	—	8.5 ± 0.1	—
1	–4.9 ± 0.1	–6.64 ± 0.08	–14.6 ± 0.1	–26.0 ± 0.4	—	—	9.50[a]

[a] Fixed.

by Van der Houwen et al.[4] which accounts for the influence of the ionizable groups on k_{obs}. The pK_a value of the side chain of tyrosine in peptides was determined at 70°C in an earlier study and was found to be 9.5.[5] The macro constants of these fits are presented in Table II. The resulting fit of an one pK_a model with a value fixed at 9.5 is not acceptable, the line does not include most points. If the fit is performed with a second variable pK_a value using eq 1, a reasonable profile is obtained with a calculated pK_a of 8.5.

$$k_{obs} = \frac{M_0[H^+] + M_1 + \frac{M_2}{[H^+]} + \frac{M_3}{[H^+]^2} + \frac{M_4}{[H^+]^3}}{1 + \frac{K_1}{[H^+]} + \frac{K_1K_2}{[H^+]^2}} \quad (1)$$

in which M_0–M_4 represent the macroconstants and K_1 and K_2 are the dissociation constants of the glycinamido and tyrosine residue, respectively. Apparently, a second ionizable group is involved with a pK_a of 8.5, presumably located in the azaglycinamido moiety since the sequence *N*-acetyl-Tyrosine-Proline only has one prototropic function, the phenol moiety in tyrosine. In the compound semicarbazide, structurally identical to the azaglycinamido group, an acid group with a pK_a value of 10.8 occurs.[6] Because in NYPaG the extra electron-withdrawing CO group of proline is present and the degradation temperature is 50°C higher, the pK_a of the azaglycinamido group might well have a lower value. The fit with a two pK_a model with one pK_a fixed at 9.5 results in the most appropriate fit (Figure 3).

Influence of the pH on the Degradation Rate Constant k_{obs} of YPaG and YPG

The pH–log(k_{obs}) profile (Figure 4) of YPaG differs from NYPaG in two ways: (1) The average log(k_{obs}) values are higher and (2) there is no U-shape indicating, neither proton- nor hydroxyl-catalyzed mechanisms. The main degradation product of YPaG is cyclo(Tyr-Pro), as described above, and, apparently, this cyclization reaction is solvent-catalyzed. The variations in the log(k_{obs}) values are probably induced by the ionizable groups in YPaG, the free N-terminus and the phenolic moiety in tyrosine, and buffer ion catalysis. The influences of acetate and phosphate ions on k_{obs} was investigated (Table III). Indeed, buffer ion influences were found. Since the same products are found using different buffer ions, these buffers apparently catalyze the already relevant reaction. Data used in the pH-log(k_{obs}) profile are corrected for this buffer influence. The pH–log(k_{obs}) profile is fitted as a horizontal line with a k_{obs} value of 1.52×10^{-5} s^{-1}. The fifth datapoint, which results from extrapolation of several phosphate buffer concentration to zero buffer con-

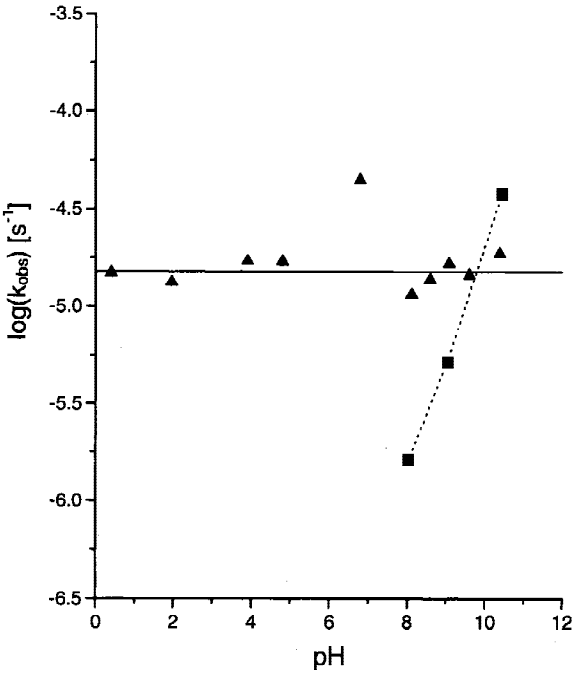

Figure 4. pH–log(k_{obs}) profiles of (▲) YPaG and (■) YPG at 70°C a and an ionic strength of 0.1.

Table III. Influence of Acetate and Phosphate Buffer Ions on k_{obs} of YPaG

Buffer	pH (70°C)	Buffer concentration range [mM]	Slope	Intercept	Correlation coefficient (R^2)
Acetate	4.8	25–100	1.6×10^{-4}	1.7×10^{-5}	0.98
Phosphate	6.8	13–50	4.8×10^{-4}	4.5×10^{-5}	0.90

centration, is omitted from the calculation of this horizontal line since there is a large deviation from most data points (Figure 4). The correction for the phosphate buffer influence is possibly less precise. If the limited data of YPG are compared with the YPaG results, the main feature of the degradation of YPG is hydroxyl catalysis (Figure 4). YPG, like YPaG, is subject to cyclization but in contrast with YPaG, YPG also undergoes deamidation. In earlier studies the hydroxyl-catalysis of the deamidation process was established.[3] Another difference in the degradation of YPG compared to YPaG is the lower k_{obs} between pH 8 and 10. The cyclization reaction of YPG seems to be slower compared to YPaG and it looks like the azaglycinamido residue of YPaG increases the cyclization rate when compared to glycinamido moiety.

Influence of the Temperature on k_{obs} of NYPaG and YPaG

The Arrhenius equation parameters are presented in Table IV. The variations in the energies of activation (E_a) of the degradation of NYPaG are to be expected since both proton-, solvent-, and hydroxyl-catalyzed mechanisms are involved and are not likely to have equal E_a values. However, for YPaG similar E_a values are likely since there is only one main solvent-catalyzed degradation mechanism over the total pH range studied. However, similar E_a values are not found. Possible explanations are the influence of the deacetylation reaction at pH 2 and ionization effects at pH 8.7.

Table IV. Arrhenius Plot Parameters of NYPaG and YPaG

Peptide/pH	Energy of activation [$\times 10^4$ J/mol]	ln(A)	Correlation coefficient (R^2)
NYPaG pH 1.0	8.1	15.3	0.998
NYPaG pH 5.0	10.9	23.1	0.994
NYPaG pH 8.7	9.0	18.9	0.996
YPaG pH 1.5	10.3	25.0	1
YPaG pH 5.0	9.7	23.1	0.9997
YPaG pH 8.6	8.8	19.7	0.9998

CONCLUSIONS

NYPaG is a good model peptide for studying the degradation of the azaglycinamido residue present, for example, in goserelin. YPaG is not a good model, since the the free N-terminus induces a relatively fast cyclization reaction. Model peptides for the investigation of degradation mechanisms other than cyclization and deamidation preferably should not have a free N-terminus or C-terminal primary amido group.

The azaglycinamido group can give rise to the following degradation reactions: elimination of CHNO yielding a C-terminal hydrazide derivative, elimination of the azaglycinamido group, and H_2O elimination. These degradation reactions are specific for the azaglycinamido residue.

REFERENCES AND NOTES

1. Dutta AS, Furr BJA, Hutchinson FG. 1993. Zoladex: Discovery, pharmacodynamics and formulation. Drug News Perspect 6:325–332
2. Hoitink MA, Hop E, Beijnen JH, Bult A, Kettenes-van den Bosch JJ, Underberg WJM. 1997. Capillary zone electrophoresis as a tool in the stability research of the luteinising hormone-releasing hormone analogue goserelin. J Chromatogr A 776: 319–327.
3. Hoitink MA, Beijnen JH, Boschma MUS, Bult A, Hop E, Nijholt J, Versluis C, Wiese G, Underberg WJM. 1997. Identification of the degradation products of gonadorelin and three analogues in aqueous solution. Anal Chem 69:4972–4978.
4. Van der Houwen OAGJ, Beijnen JH, Bult A, Underberg WJM. 1988. A general approach to the interpretation of pH degradation profiles. Int J Pharm 45:181–188.
5. Hoitink MA, Beijnen JH, Bult A, Van der Houwen OAGJ, Nijholt J, Underberg WJM. 1996. Degradation kinetics of gonadorelin in aqueous solution. J Pharm Sci 85:1053–1059
6. 1976. Merck Index, 9th ed. Rahway: Merck & Co. p 1093.