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Qualitative and quantitative aspects of the degradation of several tripeptides derived from the antitumour peptide antagonist [Arg^6, D-Trp^{7,9}, MePhe^8] substance P{6–11}

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Abstract

The tripeptides Arg–Trp–Phe, Arg–Trp–Phe–NH_2, Phe–Trp–Arg and Phe–Trp–Arg–NH_2 were subjected to a degradation study to get a more detailed insight into the degradation processes of the antitumor hexapeptide antagonist [Arg^6, D-Trp^{7,9}, MePhe^8] substance P {6–11} which was investigated in earlier research. Degradation kinetics as well as identities of degradation products of the tripeptides emerging in alkaline and acidic media were studied. The amidated forms (Arg–Trp–Phe–NH_2, Phe–Trp–Arg–NH_2) appear to be less stable than the carboxylic forms (Arg–Trp–Phe, Phe–Trp–Arg). Deamidation of the amide C-terminus, racemization of the Phe and Arg residues, ornithine formation, hydrolysis of the peptide backbone and diketopiperazine formation with elimination of the N-terminal fragments were the major degradative processes. Comparing these reactions with the reactions of antagonist [Arg^6, D-Trp^{7,9}, MePhe^8] substance P{6–11} it appeared that racemization of Phe and Arg, hydrolysis of the peptide backbone and diketopiperazine formation did not occur in detectable amounts in the hexapeptide, probably due to lower reaction rates of these reactions compared to the overall degradation rate of antagonist [Arg^6, D-Trp^{7,9}, MePhe^8] substance P{6–11}.

1. Introduction

Antagonist [Arg^6, D-Trp^{7,9}, MePhe^8] substance P{6–11} (antagonist G, Fig. 1) is a peptide antitumor agent with activity against small cell lung cancer cells [1]. It consists of six amino acids. During earlier research the degradation kinetics of the compound and the structures of the degradation products were elucidated [2]. Considering the amino acid composition of antagonist G a large variety of degradation reactions could be expected. However, actually, only a few reactions take place during the time that antagonist G is

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degraded for at least 75%. This observation is made in acidic media as well as in alkaline media. [3].

Better insight into the details of the degradation processes of antagonist G may be obtained by studying the decomposition reactions of different model peptide structures derived from the hexapeptide. Several tripeptides have been synthesized for this purpose: Arg–Trp–Phe, Arg–Trp–Phe–NH_2, Phe–Trp–Arg and Phe–Trp–Arg–NH_2 (Fig. 2). The tripeptides Arg–Trp–Phe and Arg–Trp–Phe–NH_2 are part of the antagonist G sequence. In Phe–Trp–Arg and Phe–Trp–Arg–NH_2 the N- and C-termini are reversed in comparison with antagonist G. The rationale for this choice is based on the expectation that the basic function of Arg may play a pivotal role in both the degradation mechanism and kinetics. Also the differences between amidated and free carboxylic termini may influence the degradation processes. Studying the differences in degradation mechanism and kinetics between the peptides with the similar and reversed amino acid sequence compared to antagonist G may provide information about the role of each individual amino acid in (de)stabilization of the various structures.

The possible degradations of the synthesized tripeptides are oxidation of Trp [4] and Phe [5], deamidation of the amidated C-terminus [3,6–8], formation of ornithine from Arg [9], racemization of Arg, Trp and Phe [10–12], formation of diketopiperazine products [8] and hydrolysis of the amide bonds [6,8].

In this paper the degradation kinetics as well as the structural elucidation of the degradation products of the tripeptides are discussed. A comparison is made with the degradation pattern of antagonist G. The dipeptides Trp–Phe, Phe–Trp, Arg–Trp and Trp–Arg have been synthesized to facilitate the identification of the degradation products of the tripeptides and to clarify the degradation mechanisms.

2. Materials and methods

2.1. Chemicals

Trp–Phe, Phe–Trp, Arg–Trp, Trp–Arg, Arg–Trp–Phe–NH_2, Arg–Trp–Phe, Phe–Trp–Arg–NH_2 and Phe–Trp–Arg were synthesized by the Dutch Cancer Institute (NKI, Amsterdam, The Netherlands). Purity of these samples was higher than 95%, checked with reversed-phase high performance liquid chromatography (RPHPLC) and mass spectrometry (MS) as described below. All other chemicals used were of analytical grade and deionized water was used throughout the study.

2.2. Reversed-phase high performance liquid chromatography

The gradient RPHPLC system consisted of a Gynkotek Model 480 pump with gradient controller, a Gynkotek Model 300 CS pump and an Applied Biosystems 785A programmable absorbance detector (all from Separations, H.I. Am-

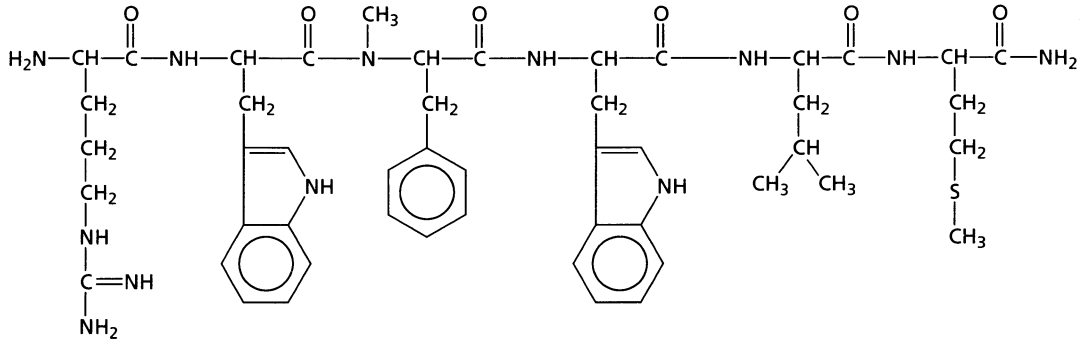

Fig. 1. Chemical structure of antagonist G.

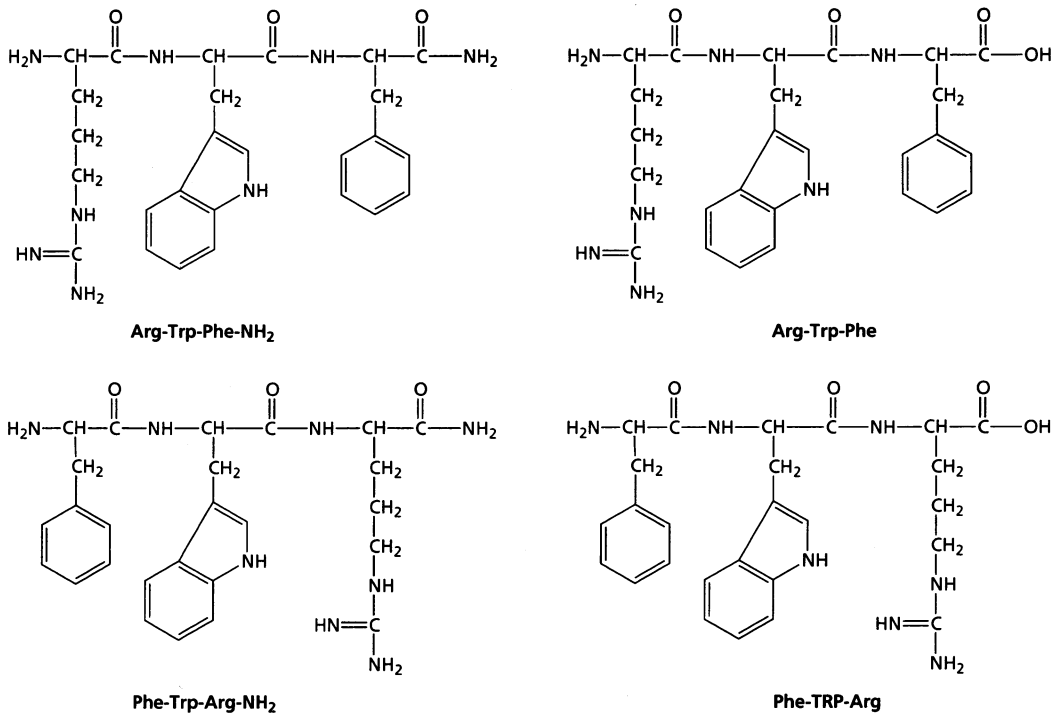

Fig. 2. Chemical structures of the tripeptides.

bacht, The Netherlands), a Model U6K Injector (Waters, Milford, MA) or a WISP 717 with Autoinjector (Waters Associates, Milford, MA) and a LiChroCART 125-2 RP-18 column (MERCK, Darmstadt, Germany). For the kinetic studies the mobile phases consisted of *A*: 10% acetonitrile in water (w/w) containing 10 mM perchloric acid and 100 mM sodium perchlorate (pH 2) and *B*: 40% acetonitrile in water (w/w) containing 10 mM perchloric acid and 100 mM sodium perchlorate (pH 2). Separation was achieved using a linear gradient from 0 to 100% mobile phase *B* in 12 min and back to 0% mobile phase *B* within 1 min. The injection volume was 10–25 μl, the flow was 1.0 ml min^{-1} and the detection was performed at 205 nm.

2.3. Liquid chromatography–mass spectrometry (LC–MS)

For LC–MS experiments the mobile phases consisted of *C*: 0.1% trifluoroacetic acid (TFA) in 10% acetonitrile in water (w/w, pH 2) and *D*: 0.1% TFA in 40% acetonitrile in water (w/w, pH 2) in a linear gradient ranging from 0 to 75% mobile phase *D* in 12 min, and back to 0% mobile phase *D* in 1 min. Chromatography was performed on a Superspher 100 RP-18 column with 2 mm internal diameter (MERCK). The flow was reduced to 200 $\mu l\ min^{-1}$.

MS detection was performed using a VG Platform Benchtop LC–MS (Fisons Instruments, Altricham, UK). An electrospray interface in the positive ion mode was used to ionize the molecules. The nebulizing gas had a flow of 25 l h^{-1}, the drying gas had a flow of 300 l h^{-1}. The applied voltage to the capillary was 3.4 kV, and a low cone voltage (22 V) was applied to prevent extensive fragmentation. The MS was calibrated from 102 to 2000 Da with a mixture of sodium iodide and triethylamine. Measurements were carried out from 100 to 600 Da.

2.4. Chiral gas chromatography (chiral GC)

The racemization of amino acids in the tripeptides was investigated by chiral GC with a Chirasil-L-Val fused silica column (25 m × 0.25 mm) (Chrompack, Bergen op Zoom, The Netherlands). The carrier gas was helium (flow 2.0 ml min^{-1}) with flow detector gasses: make up gas helium at 35 ml min^{-1}, air at 350 ml min^{-1} and hydrogen at 35 ml min^{-1}. The temperature program started at 75°C to achieve 200°C with a speed of 5°C min^{-1} after which the temperature was held at 200°C for 10 min. Both the injector and detector temperature were set at 250°C. Detection was carried out with a nitrogen/phosphorous (N/P) detector and the injection volume was 1 μl with a split ratio 1:5.

For the GC experiments the tripeptides were degraded at pH 13 for approximately two half-lives. These samples and the parent solutions were hydrolyzed to single amino acids as described by Creighton [13] with 6 M HCl for 24 h at 110°C under nitrogen in a sealed vial. After hydrolysis, the samples were evaporated to dryness under nitrogen at 50°C. Hydrolyzed products and L- and D-amino acids were derivatized in 2 M HCl with isopropanol at 110°C (carboxyl esterification) and with trifluoroacetic acid anhydride/ethylacetate in a 4:1 ratio at 110°C (amino acylation) under nitrogen. Samples were injected directly.

2.5. Degradation conditions

A solution of 0.02 mg ml^{-1} peptide in water was freshly prepared. To 100 μl of this solution 100 μl of buffer of the appropriate pH was added in 1 ml ampoules or 1 ml vials. For the construction of the pH–log k_{obs} profile, rate constants, k_{obs}, were measured in the pH range 0–13. Buffer solutions used were perchloric acid for the pH/H_0 range 0–2; 150 mM acetate for the pH range 3–6; 150 mM phosphate for the pH range 6–8; 150 mM carbonate for the pH range 8–11, while for pH/H values > 11 sodium hydroxide solutions were used [14]. H_0 and H values were calculated according to Hammett et al. [15]. All buffer solutions were brought to an ionic strength (μ) of 0.6 with sodium chloride. With this procedure the final reaction solutions contain 0.01 mg ml^{-1} peptide and 75 mM buffer with an ionic strength of 0.3. The temperature was set at 80°C.

For qualitative measurements the procedure as described above is used with a final peptide concentration of 0.1 mg ml^{-1}.

Degradation of the dipeptides Phe–Trp and Trp–Phe was carried out only at pH 1.

3. Results and discussion

3.1. Kinetic studies

The degradation curves show (pseudo) first order kinetics. The k_{obs} values for the degradations have been calculated from the slopes of these degradation curves.

pH–log k_{obs} profiles were constructed for all tripeptides. In Fig. 3 the pH–log k_{obs} profiles of the tripeptides are shown. It is clear that the amidated tripeptides are more susceptible to

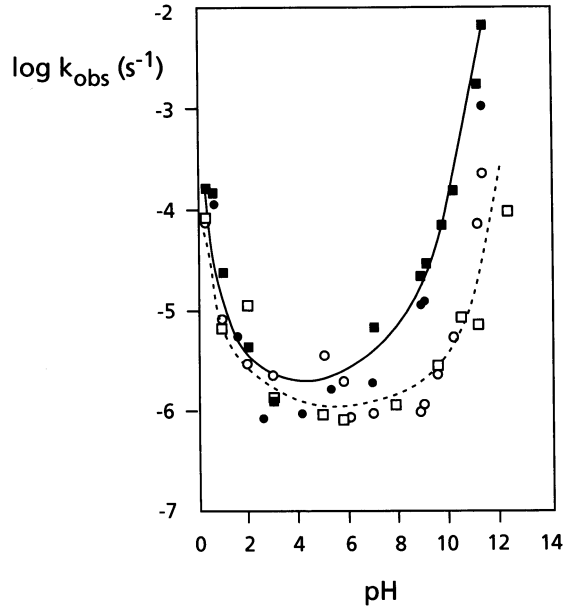

Fig. 3. pH–log k_{obs} profiles of the degradation of the tripeptides. Phe–Trp–Arg (○), Phe–Trp–Arg–NH_2 (●), Arg–Trp–Phe (□), Arg–Trp–Phe–NH_2 (■). pH–log k_{obs} profiles of the amidated forms (——) and of the carboxylic acid forms (- - -) are combined.

Table 1
m/z values for the degradation products of the tripeptides Arg–Trp–Phe–NH_2, Arg–Trp–Phe, Phe–Trp–Arg–NH_2 and Phe–Trp–Arg in acidic and alkaline media

Arg–Trp–Phe–NH_2	Arg–Trp–Phe–COOH	Phe–Trp–Arg–NH_2	Phe–Trp–Arg–COOH
Degradation products in alkaline media			
507[a]	508[a]	352[b]	254[c]
507[d]	508[d]	466[d,e]	352[b]
507[d]		466[d,e]	466[d,e]
508[f]		466[d,e]	466[d,e]
		507[a]	508[a]
		508[d,f]	508[d]
		508[d,f]	508[d]
Degradation products in acid media			
361[b]	166[b]	352[b]	136[g]
507[a]	343[h]	423[g]	166[b]
508[f]	361[b]	507[a]	346[h]
	421[g]	508[f]	352[b]
	508[a]	514[g]	352[b]
	561[g]	521[g]	508[a]

[a] Parent,
[b] Hydrolysis,
[c] Double charged m/z,
[d] Racemization,
[e] Ornithine formation,
[f] Deamidation,
[g] Unidentified,
[h] Diketopiperazine formation.

degradation in the pH region 5–12 than the carboxylated tripeptides. No clear differences are observed between the tripeptides in the low part of the pH region.

3.2. Qualitative analysis

Qualitative analysis of the degradation products with LC–MS reveals that similarities as well as differences occur in products arising from the various peptide degradations. In Table 1 an overview is given of m/z values of the degradation products.

Similarities are seen in alkaline degradation media. All tripeptides seem to racemize since products are formed with the same values for m/z as the parent but a different retention time in RPHPLC. This indicates the formation of diastereomers. These results agree with data obtained for degradation products of antagonist G where also diastereomer formation was observed.

In case of the amidated tripeptides in both acidic and alkaline media deamidation of the C-terminal amide takes place ($\Delta m/z = +1$ a.m.u.). This is, similar to antagonist G, the main degradation reaction.

Hydrolysis of the peptide backbone occurs in all peptides in acidic media: formation of dipeptide Arg–Trp ($m/z = 361$) from Arg–Trp–Phe (both carboxylic and amidated form) and formation of Phe–Trp ($m/z = 352$) from Phe–Trp–Arg (both carboxylic and amidated form). In case of Arg–Trp–Phe and Phe–Trp–Arg the free amino acid Phe ($m/z = 166$) is detected. In the other peptides neither Trp–Arg nor the free amino acid Arg is detected. The observed hydrolysis in alkaline media of Phe–Trp–Arg [both carboxylic and amidated form) is not seen in the peptides with a reversed amino acid order. From the three-dimensional structure of Arg–Trp–Phe it is feasible that hydrogen bridge formation may occur: one between the side chain of Phe and the adjacent

secondary amine of the Trp residue in the peptide backbone and one between the guanidino side chain of Arg and its carbonyl in the peptide backbone. In Fig. 4(a) representation of the three-dimensional structure is given concerning the hydrogen bridge between the side chain of Phe and the peptide backbone. These hydrogen bonds stabilize the structure [16,17]. This is not the case in Phe–Trp–Arg. Due to this hydrogen bond formation carbonyl groups in the peptide backbone of Arg–Trp–Phe become less reactive due to a lower positive charge on the carbon in the carbonyl group. The first step in hydroxyl ion-catalyzed hydrolysis is a nucleophilic attack at the electrophilic carbon atom in the carbonyl moiety. For proton-catalyzed hydrolysis the first step in the hydrolysis mechanism is protonation of the doubly bound oxygen in the carbonyl group. In antagonist G hydrolysis of the peptide backbone in alkaline media is not observed during 2–3 half lives. Although the overall degradation velocities in antagonist G are similar to that of the tripeptides, obviously, degradation reactions like deamidation, racemization, oxidation and ornithine formation are reactions with higher velocities than hydrolysis in antagonist G. The amino acid sequence of the N-terminus of antagonist G resembles the sequence of Arg–Trp–Phe, therefore formation of hydrogen bridges in antagonist G could also be an explanation for the higher stability of the peptide backbone.

In alkaline media Phe–Trp–Arg (both carboxylic and amidated form) are also exclusive in

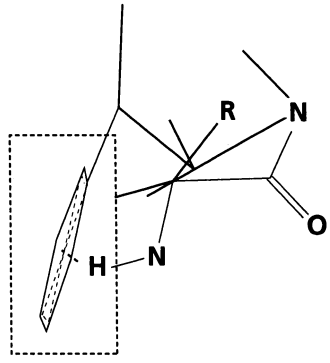

Fig. 4. Hydrogen bridge formation between Phe and the peptide backbone.

the formation of ornithine containing products ($\Delta m/z = -42$ a.m.u.). This is not observed in the reversed amino acid order. Remarkably, antagonist G with the N-terminus sequence Arg–Trp–Phe showed ornithine formation. Possible explanation for this might be that the position of the bulky side chain of D-Trp in antagonist G causes less sterical hindrance in this reaction. The tripeptides contain L-Trp instead of D-Trp.

Remarkably, the formation of cyclic dipeptide intermediates (diketopiperazine) is only found during proton-mediated degradation of the carboxylic forms of the tripeptides. Diketopiperazine formation always occurs at the N-terminus of the peptide and results in the formation of a cyclic product consisting of the two N-terminal amino acids [8]. In the Arg–Trp–Phe degradation sample besides the dipeptide Arg–Trp ($m/z = 361$) also a product with m/z 343 ($\Delta m/z = -18$) is formed. This is probably a cyclic product of Arg–Trp. Although in the Phe–Trp–Arg degradation the cyclic intermediate is not found, the dipeptide Phe–Trp as well as the dipeptide Trp–Phe (both m/z 352) have been detected in this chromatogram, however, the latter in smaller quantities. Since conversion of Phe–Trp into Trp–Phe might proceed via the cyclic intermediate, the dipeptides Trp–Phe and Phe–Trp have been degraded in acidic media separately. In both cases a compound with m/z 334, m/z of the putative cyclic product, is detectable. Possibly out of this cyclic intermediate the dipeptides Trp–Phe and Phe–Trp emerge. Pilot experiments revealed that Phe–Trp forms lower amounts of cyclic intermediate than Trp–Phe. This, together with the fact that Phe–Trp is probably more susceptible to hydrolysis without formation of cyclic intermediates, might be the cause for not detecting the cyclic intermediate in the degradation of the tripeptide Phe–Trp–Arg.

The fact that a cyclic peptide or a dipeptide is only formed with the N-terminal amino acids is in accordance with the reaction mechanism for diketopiperazine formation as proposed by Powell [8]. Formation of diketopiperazine products is not detected in the degradation pattern of antagonist G nor in an amide at the C-terminus. This is not due to steric hindrance of side chains otherwise

the reaction would not have taken place in the tripeptide Arg–Trp–Phe. More likely is that diketopiperazine formation, if any, is much slower than other degradation reactions. To investigate if diketopiperazine formation takes place in antagonist G, also longer periods of degradation needs to be regarded than 2–3 half-lives.

Powell [8] also states that the nature of the amino acid residue on the third position is important for the diketopiperazine formation, indicating that small changes in this amino acid residue can cause large differences in reactivity. Also the apparent non-reactivity of the amidated forms of the tripeptides needs further investigation.

Chiral GC experiments show that, although experiments were carried out under nitrogen, Trp can not be detected. However, L-/D-Phe and L-/D-Arg could be detected. This is probably caused by the sample pretreatment necessary for the GC experiments since in other experiments the presence of Trp is obvious. It might be that, despite the precautions taken to keep the samples oxygen-free, Trp is oxidized. In Arg–Trp–Phe–NH_2 the Arg residue does not racemize in a detectable amount during two half-lives. In Arg–Trp–Phe only a small fraction of the Arg residue racemizes during the same degradation time. However, the Arg residue in Phe–Trp–Arg–NH_2 and Phe–Trp–Arg racemizes to a larger extent than the Arg residue in the former tripeptides. In all tripeptides the Phe residue racemizes substantially. It is worth noticing that these amino acids do not racemize in a detectable amount in antagonist G. It seems that racemization of Arg and Phe in antagonist G occurs at a much lower rate. To investigate if racemization of these two amino acids in antagonist G occurs the hexapeptide degradation probably needs to be followed for a longer period than 2–3 half-lives, as done in the stability study of Reubsaet et al. [2].

The difference in stability (Fig. 3) between the amidated and the free carboxylic acid form of the tripeptides can be explained by the presence of the amide group on the C-terminus of Arg–Trp–Phe–NH_2 and Phe–Trp–Arg–NH_2. This group is susceptible to hydrolysis. In the neutral/alkaline region of the profile (pH > 5) the amidated forms are less stable. Clear differences in stability between the tripeptides are not observed in the lower part of the pH-region.

Comparing theoretically possible degradation reactions with the obtained data it is obvious that racemization, hydrolysis and deamidation takes place in all tripeptides. Formation of diketopiperazine is only observed in the tripeptides with a carboxylic C-terminus. Formation of ornithine from Arg is only detectable in the tripeptides Phe–Trp–Arg (both carboxylic and amide termini). Oxidation is not detected in the tripeptides.

4. Conclusions

Comparing the kinetic data of the degradation of the tripeptides with each other it can be seen that the pH–log k_{obs} profiles of Arg–Trp–Phe and Phe–Trp–Arg on one hand and the profiles of Arg–Trp–Phe–NH_2 and Phe–Trp–Arg–NH_2 on the other hand show a high degree of similarity. It was expected that the pH–log k_{obs} profiles of the carboxylic and amidated forms differ. The amidated forms are more susceptible to degradation. The shape of the pH–log k_{obs} profiles is the same, however, the value for log k_{obs} is higher in the case of the amidated tripeptides in the pH-region 5–12.

Identification of degradation products makes clear that various degradation products are formed. In case of an amidated form of the tripeptide deamidation occurs. Also racemization takes place in all tripeptides. However, in alkaline media ornithine formation is only detected in both forms of the Phe–Trp–Arg tripeptides. Further investigation is needed to explain this phenomenon. Also hydrolysis of the peptide backbone in alkaline media occurs only in Phe–Trp–Arg tripeptides. Hydrogen bond formation may be the possible explanation for the absence of hydrolysis in Arg–Trp–Phe. Hydrolysis in acidic media occurs in all tripeptides. Formation of diketopiperazine is only observed in the carboxylic forms of the tripeptides.

Comparing these observations to those in antagonist G shows that reactions like racemization, deamidation, hydrolysis and ornithine formation occur in both cases. However, racemization of the

Phe and Arg residue occurs in the tripeptides but is not detectable in the antagonist G degradation. Conversion into ornithine is not seen in the tripeptides with the similar amino acid sequence as the N terminus of antagonist G. Diketopiperazine formation is not detected at all in antagonist G.

Although there are similarities in the degradation of antagonist G and the tripeptides, the resemblance is not striking. Probably, partly the reactions that take place in the tripeptides also take place in antagonist G but with a so low velocity that the degradation products are not detectable within two half-lives of the antagonist G degradation.

The approach chosen to get more insight into the degradation of antagonist G shows that procedure to extrapolate results from small model peptides to larger peptides has its uncertainty: small modifications in structure can cause large differences in reactivity which makes extrapolation to a vulnerable step. Experimental confirmation is necessary.

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