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Review

Peptide and Amino Acid Glycation: New Insights into the Maillard Reaction

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Abstract: Nonenzymatic glycation of proteins, peptides and other macromolecules (the Maillard reaction) has been implicated in a number of pathologies, most clearly in diabetes mellitus, but also in the normal processes of aging and neurodegenerative amyloid diseases such as Alzheimer's. In the early stage, glycation results in the formation of Amadori-modified proteins. In the later stages, advanced glycation end products (AGE) are irreversibly formed from Amadori products leading to the formation of reactive intermediates, crosslinking of proteins, and the formation of brown and fluorescent polymeric materials. Although, the glycation of structural proteins has been attributed a key role in the complications of diabetes, recent attention has been devoted to the physiological significance of glycated peptide hormones. This review focuses on the physico-chemical properties of the Amadori compounds of bioactive peptides of endogenous and exogenous origin, such as Leu-enkephalin and morphiceptin, investigated under different conditions as well as on novel pathways in the Maillard reaction observed from investigating intramolecular events in ester-linked glycopeptides. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: glycation; Amadori; Leu-enkephalin; morphiceptin; glycosylamine; fluorescence; AGE; mass spectrometry; opioid; imidazolidinone; Maillard

INTRODUCTION

The chemistry and consequences of the reaction between reducing sugars, such as glucose, and reactive amino groups in proteins, lipids and nucleic acids, has received considerable attention in recent years, both in food and health science. The complex network of secondary reactions following these initial reactions, first reported in 1912 [1], is known as the Maillard reaction. It may be subdivided into the three main stages summarized in Figure 1. The early stage, consisting of the formation of a Schiff base which, through rearrangements, gives rise to an 'Amadori product' (ketoamine), if the reducing sugar is an aldose. The latter, an important intermediate in Maillard chemistry, is then degraded to a variety of carbonyl compounds, being much more reactive than the original sugar. The main degradation pathway of Amadori products is through dehydration of the sugar moiety to form deoxyglucosones. In the late stage, these highly reactive carbonyl compounds lead to the formation of a group of substances known as advanced glycation end products or AGEs, often coloured, fluorescent and prone to produce crosslinks in proteins; two representative AGE products are illustrated in Figure 1. Over the past decade, significant progress has been made in the unravelling of the individual steps of the Maillard reaction. For comprehensive information the reader is referred to published review articles covering all [2–4] and specific [5–9] aspects of this chemistry.

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BIOGRAPHIES

Dr Štefica Horvat Štefica Horvat currently holds a position of Senior Research Scientist in the Division of Organic Chemistry and Biochemistry at the Ruđer Bošković Institute (RBI) in Zagreb, Croatia. She was born in Zagreb where she graduated in chemistry (1971) from the Faculty of Natural Sciences, University of Zagreb. After receiving a PhD in organic chemistry (1976), under the supervision of Dr Dina Keglević, she joined RBI. In 1978–1979 she conducted postdoctoral studies under the direction of Dr W. Korytnyk in Roswell Park Memorial Institute, Buffalo, USA. Since 1991 she has been head of the Laboratory for Carbohydrate, Peptide and Glycopeptide Research at RBI. Her research interests are focused on the synthesis and analysis of glycoconjugates of bioactive peptides and on nonenzymatic glycation processes, collectively known as the Maillard reaction.

Dr Andreja Jakas Andreja Jakas was born in Zagreb, Croatia (1965), were she graduated in chemistry (1991) from the Faculty of Natural Sciences, University of Zagreb. She received a PhD in chemistry (2001), under the supervision of Dr Štefica Horvat for the synthesis and study of reactivity of Amadori compounds of biologically active peptides. Currently she is working in the Division of Organic Chemistry and Biochemistry at the Ruđer Bošković Institute in Zagreb, Croatia, as Senior Assistant.

The Maillard reaction takes place in nearly all food, producing changes in colour and flavour, loss in nutritional quality, and formation of bioactive compounds [10,11]. These compounds include kidney-damaging Maillard reaction products [12,13], mutagens [14], carcinogens [15] as well as beneficial products having antimicrobial and/or antioxidant activity [16,17]. There is increasing evidence that AGEs formed in heated foods while ingested in bioreactive forms could exacerbate diabetic injury by inducing oxidative stress and promote inflammatory signals [18–20].

In vivo, the physical changes in colour, fluorescence, solubility and elasticity of proteins during ageing were detected long before the identification of the underlying changes in chemistry. During the 1970s and 1980s, it was realized that the Maillard reaction, involving nonenzymatic glycation reactions of carbohydrates and lipids, is implicated in causing complications primarily via adventitious crosslinking of proteins [21–23]. Different glycation (end)products are known to accumulate with age in tissue proteins and the acceleration of protein ageing in disorders such as diabetes, renal failure and amyloidoses is apparently driven by the increase in circulating levels of oxidizable substrates, carbohydrates and lipids [24–27]. Most of the primary effects of AGEs leading to the complications of diabetes and ageing are due to AGE formation on long-lived proteins. Collagen is a prime target, especially damaging are the effects of glycation on vascular collagen. AGE accumulation results in vascular thickening with loss of elasticity, hypertension and endothelial dysfunction, thus, contributing to atherosclerosis and coronary disease, kidney damage, retinal pathology and poor peripheral circulation [28–33]. Many studies confirmed the presence of a broad family of AGE-binding receptors (AGE-R1-3, RAGE; scavenger receptors ScR-II and CD-36) on a range of different cell types, including haemopoietic/phagocytic, vascular, renal and neuronal cells [34–36]. AGE interaction with these receptors results in triggering of cellular signal transduction pathways closely involved in the inflammatory response, cellular proliferation, tumour growth and metastasis [37–39]. In this context, under the cumulative burden of AGE-deposits occurring in ageing and diabetic tissues, cellular AGE-receptors are upregulated leading towards exaggerated cellular dysfunction typically seen throughout the vasculature and the kidneys of the diabetic patient [40]. There is also evidence that AGEs play a role in abnormal amyloid aggregation in age-related diseases, including neurodegenerative diseases (Alzheimer's and Parkinson's disease) [41] as well as prion diseases such as Creutzfeldt–Jakob disease [42]. Recently, an interesting hypothesis was raised that neurodegenerative diseases, such as scrapie in sheep and goats and bovine spongiform encephalopathy in cattle (BSE) originate from the consumption of glycated proteins contained in their feed, obtained during the production of feed by heating meat, blood and bones with ubiquitous reducing carbohydrates [43].
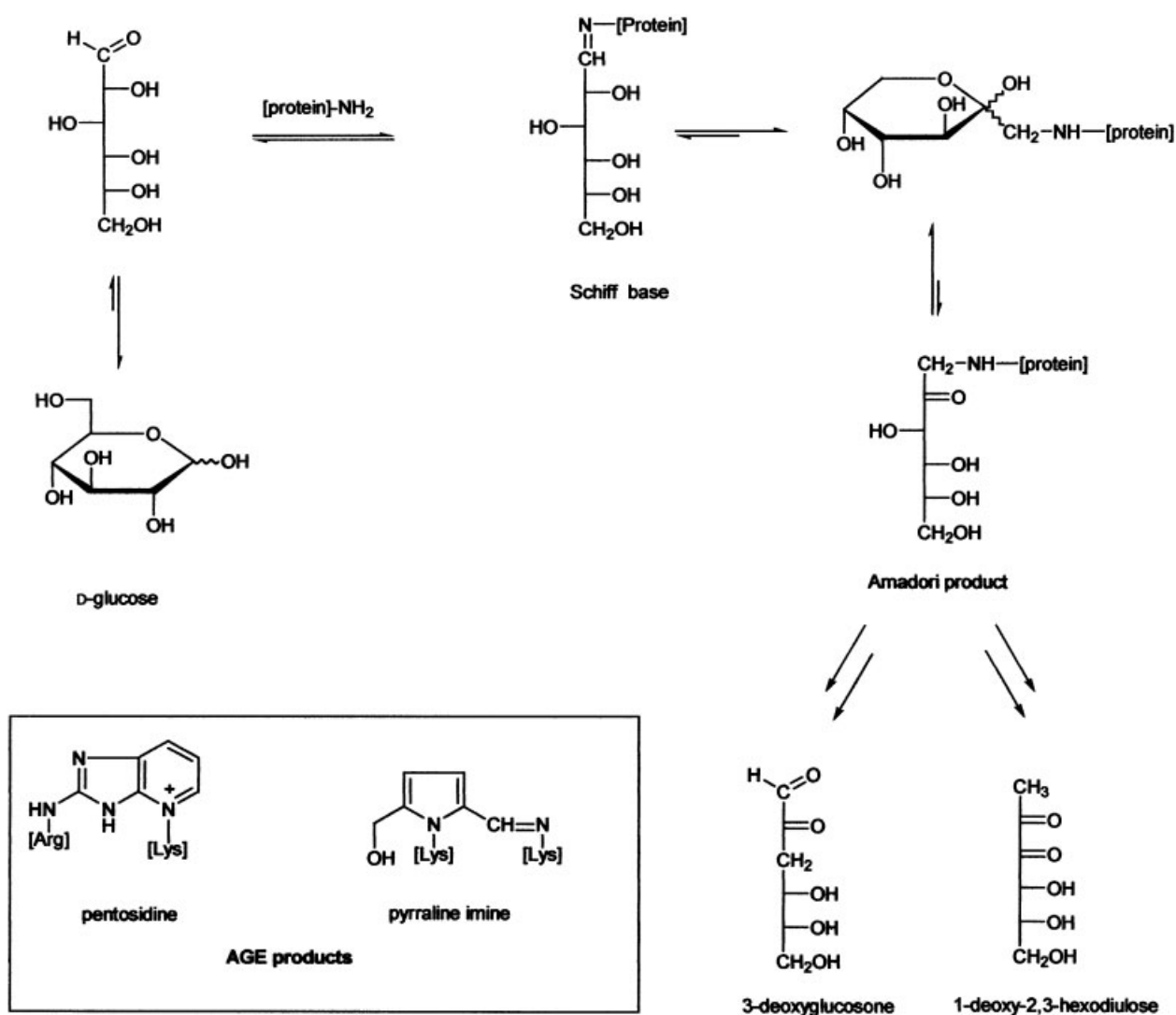
Figure 1   Reactive intermediates resulting from the early stages of the Maillard reaction.

Although the glycation of structural proteins has been attributed a key role in the complications of diabetes, recent attention has been devoted to possible glycation and impaired function of relatively short-lived important peptide hormones. A novel radioimmunoassay and immunocytochemical techniques revealed that glycated insulin is stored and secreted from pancreatic β-cells of diabetic patients as well as from β-cells of diabetic animals, representing a significant portion of total circulating insulin in type 2 diabetes [44]. Also, chemically induced and spontaneous animal models of diabetes were found to possess significantly greater levels of glycated glucagon-like peptide-1(7–36)amide (tGLP-1) than controls, corresponding to 24%–71% of the total [45]. Peptide hormones glycated by *in vitro*

incubation with D-glucose under reductive conditions, demonstrated significant changes in the functional activities of insulin [46,47], cholecystokinin-8 [48,49], glucagon-like peptide 1(7–36)amide [50] and gastric inhibitory polypeptide [51]. Recent studies also revealed that advanced glycation products, formed spontaneously by addition of glucose to synthetic β-amyloid peptide monomers as well as to islet amyloid polypeptide, enhance peptide aggregation *in vitro* [52,53] and suggest a model for amyloid deposition in Alzheimer's disease, and for pancreatic islet amyloid formation associated with ageing and type 2 diabetes mellitus.

It is clear that the challenge for the next decades will be to unravel structure and function of the bioreactive glycation products related not only to

structural proteins but also to endogenous peptide hormones and/or neuropeptides in biological systems as well as to understand the adverse effects of both exogenous and endogenously derived products implicated in the pathogenesis of diabetic complications and changes associated with ageing including atherosclerosis, renal, eye and neurological diseases. Further insight into the underlying chemistry may lead to the development of preventive measures or treatment strategies that may be of relevance to the growing population of diabetic patients and to the elderly in general.

This article presents some of our recent achievements in the field of Maillard chemistry and consists of the three main parts. The first part summarizes results that describe the preparation of defined Amadori compounds structurally related to opioid peptides of either endogenous or exogenous origin along with an evaluation of the effect of glycation on the physico-chemical properties of the parent peptides. The second part focuses on intramolecular Maillard reaction of selected carbohydrate-peptide or amino acid esters and on the information that can be gained from investigating such chemical processes. Finally, the utility of MS techniques for differentiation between early Maillard products of sugars is demonstrated.

## SYNTHESIS AND PROPERTIES OF AMADORI COMPOUNDS RELATED TO THE OPIOID PEPTIDES

The preparation of well-defined Amadori compounds of bioactive peptides has emerged as a major focus in the field of Maillard chemistry, and this is no doubt a result of the growing awareness that molecules of this kind are formed *in vivo* [44,53] are implicated in reactions affecting peptide pharmaceuticals in the solid-state [54–56], or are investigated for targeted imaging of different receptors in ongoing clinical trials [57].

As a result of the increased understanding of the important roles that endogenous and exogenous peptides play in fundamental life-sustaining processes, and in an effort to better understand the reactivity of Amadori products and the mechanism of their further reactions, glycated products **2–6**, structurally related to the opioid peptide of endogenous origin known as Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) and to the milk-derived opioid tetrapeptide morphiceptin (Tyr-Pro-Phe-Pro-NH$_2$)

were prepared [58,59]. The peptide hormone Leu-enkephalin belongs to an important group of opioid peptides producing a wide range of central and peripheral effects, which, in addition to analgesia, include tolerance and physical dependence, respiratory depression, and effects on gastrointestinal motility, cardiovascular and immune functions [60]. Morphiceptin is the most active of opioid agonistic peptides which are encrypted in intact milk protein sequences but can be released and thus activated by enzymatic proteolysis during gastrointestinal digestion or during food processing and then reach the endogenous opioid receptors [61]. It is also well documented that hyperglycaemia induces impaired functioning of the endogenous opioid system thus contributing to the worsening of diabetic complications [62]. On the other hand, several pieces of evidence suggest that cow's milk proteins may provide mimicry proteins relevant to the development of diabetes and other immune-mediated or neurological diseases [63].

Amadori compounds **2–6** were prepared by using two major synthetic strategies outlined in Figure 2. In one approach, the Amadori products (1-deoxy-D-fructos-1-yl derivatives) were synthesized by refluxing the amino compounds in the presence of an excess of reductive free sugar, followed by isolation and purification of the products. The second strategy was based on organic synthesis with suitably protected starting materials. This route involved the reductive amination of the fully protected hexodiulose **1** by the amine component, usually in the presence of sodium cyanoborohydride. In the next step, removal of the acetonide protecting groups with aqueous acid furnished the target Amadori compounds in acceptable yields.

The influence of *N*-glycation on the tautomeric distribution of Amadori compounds **2–5**, explored by NMR analysis in D$_2$O [58], revealed the presence of β-pyranose, α-furanose and β-furanose forms in solution, the β-pyranose tautomer being the most abundant at equilibrium (67%–75%). The α-pyranose form and the open chain keto form were not detected. In contrast, in DMSO-d_6 solution, the equilibrium compositions of **2–5** were markedly shifted towards a higher proportion of furanose forms, amounting to two-thirds of the mixture, which also contained a surprisingly high proportion of the acyclic hydrate form (∼10%) (Figure 2). NMR investigation of morphiceptin-related Amadori compound **6** [59] revealed the presence of multiple conformers in D$_2$O and DMSO-d_6 solutions arising from *cis/trans* isomerization about the Tyr^1-Pro^2
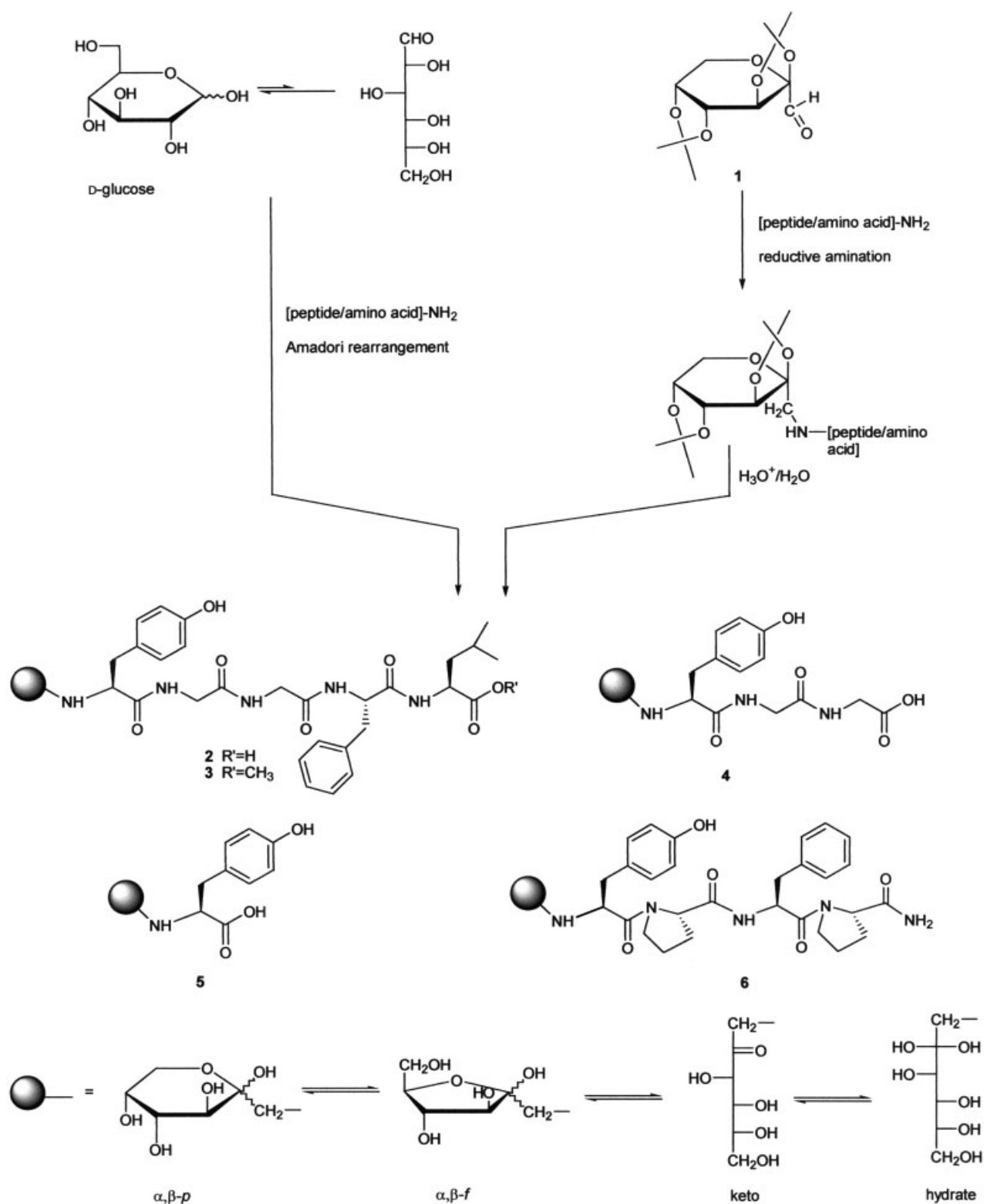
Figure 2   Synthetic pathways to Amadori products **2**–**6**.

and Phe[3]-Pro[4] peptide bonds and tautomerization of the sugar moiety. By comparing ^{13}C NMR data in D_2O, a lower proportion of the α-furanose tautomer (7%) was found in solution of Amadori compound **6** related to the exogenous, milk derived, opioid peptide, than for the *N*-glycated endogenous opioid peptide Leu-enkephalin **2** (18%), whereas comparison of the data obtained in DMSO solution showed a higher content of the α-furanose fraction in **6** (40% vs 33% in **2**). In compound **6** no signals were observed which could be attributed to either C=O of the open chain or to the hydrate form. This observation suggests that the structure of the peptide backbone influences the tautomeric distribution in the Amadori compounds studied.

CD and FTIR spectroscopy have been used to investigate the conformational effects of glycation on the secondary structure of Leu-enkephalin and on structurally related peptides in TFE solution [64]. CD analysis of Leu-enkephalin-related Amadori compounds revealed that attachment of the protected or free sugar has a marked spectral effect suggesting a significantly different conformer distribution in the peptide part of the molecule. The presence of the open-chain sugar form, detected in the NMR spectra of Amadori compounds **2**–**5**, is also supported by the FTIR spectra measured in DMSO which show, for compounds **2** and **4**, the component band at ca. 1730 cm^{-1}, indicative of the presence of the keto form of the D-fructose moiety in solution.

Pharmacological evaluation *in vitro*, using both GPI and MVD assay, revealed that *N*-glycation of Leu-enkephalin was of major consequence to the bioactivity of the parent peptide [65]. Amadori compound **2** displayed a drastic decrease in potency in both assay systems, in accordance with the known requirement of the free *N*-terminal tyrosine residue in enkephalins for opioid activity. This finding is not only of theoretical interest since, as found for some other peptide hormones [44], similar adducts with altered interactions with endogenous opioid receptors could be formed during exposure of opioid peptides to glucose *in vivo*. Consequently, the study of glycated peptides in a variety of solvent media at different temperatures are likely to provide insights into the stability of the early glycation products and their metabolites and should also contribute to better understanding of the processes of peptide/protein modifications during heat treatment of biopharmaceuticals in the presence of carbohydrate excipients.

The degradation of Amadori compounds related to Leu-enkephalin and to structurally smaller fragments as well as their ability to develop characteristic Maillard fluorescence was investigated under oxidative conditions in methanol and phosphate buffer, pH 7.4, at two different temperatures [66]. At 37 °C, *N*-glycated Leu-enkephalin **2** degraded slowly in methanol ($t_{1/2} \sim 13$ days) and phosphate buffer ($t_{1/2} \sim 9$ days) producing the parent peptide compound as a major product, whereas incubation at 70 °C resulted in fast degradation of compounds **2** and **3** accompanied with intensive browning of the reaction mixture (Figure 3). The increase of absorbance in the ultraviolet and visible region, browning, and the formation of fluorophores have been found associated with the Maillard product formation [67]. Fluorescence spectroscopy of Amadori compounds **2**–**5** incubated in phosphate buffer at both 37 °C and 70 °C showed, in addition to fluorescence associated with the tyrosine residue, the increase in advanced glycation end product (AGE)-associated fluorescence (excitation 320 nm/emmision 420 nm). Figure 4 shows relative Maillard fluorescence detected in solutions of the Amadori compounds **2**–**4** over the incubation period. Relative concentrations of the fluorophores
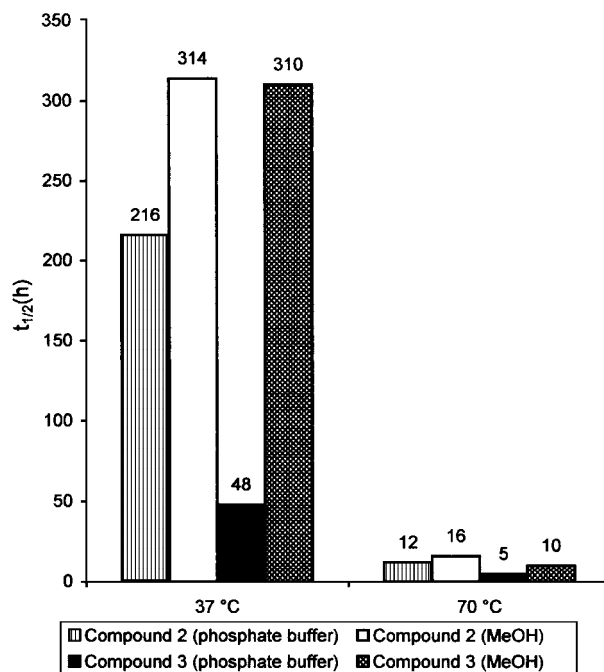

Figure 3 Half-lives of hydrolysis of Leu-enkephalin-related Amadori compounds **2** and **3** in 0.05 M phosphate buffer (pH 7.4) and methanol at 37° and 70 °C.
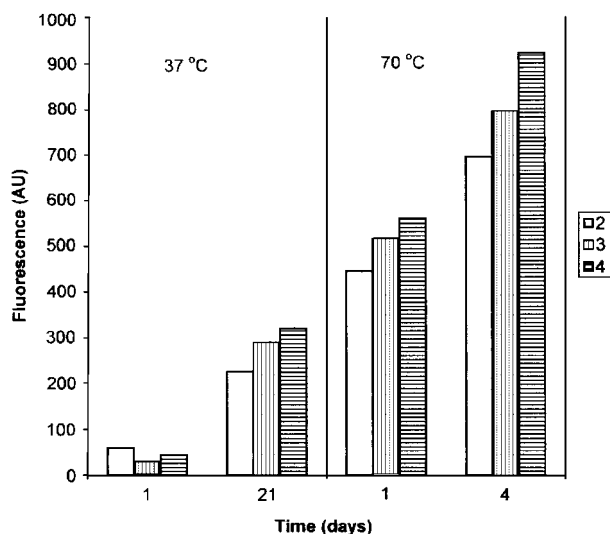
Figure 4 Production of fluorescence at the wavelength specific to AGEs (excitation wavelength 320 nm, emission wavelength 420 nm) in phosphate buffer (pH 7.4) solutions of Amadori compounds **2**–**4** incubated at 37° and 70 °C.

appear to be related to peptide (amino acid) structure and decreased in the order **5** ≫ **4** > **3** > **2**. These results imply that, if the formation of similar glycated opioid peptides occur under physiological conditions, plasma degradation will be significantly reduced compared with degradation of the parent peptide compound and, not less important, accompanied by the slow formation of AGE degradation products.

INTRAMOLECULAR MAILLARD EVENTS IN PEPTIDE/AMINO ACID ESTERS CONTAINING CARBOHYDRATE MOIETIES AT THE *C*-TERMINAL POSITION

In order to gain better insight into the mechanisms and products of the Maillard reaction, the monosaccharide esters of Leu-enkephalin **7**–**9** in which D-glucose (**7**), D-mannose (**8**) or D-galactose (**9**) are linked through their C-6 hydroxy groups to the *C*-terminal carboxy group of the pentapeptide were used as model compounds (Figure 5). Such structurally well-defined carbohydrate esters represent an ideal model system for the study of the Maillard reaction *in vitro* since these molecules contain a free amino group at the *N*-terminus of the peptide moiety as well as a reducing sugar epitope. They also mimic the reactivity of the sugar 6-phosphate

esters in the glycation reaction and, for steric reasons, the attached monosaccharide should be more likely to form carbohydrate-peptide adduct than the parent sugar alone. It was demonstrated that, in pyridine-acetic acid as the solvent, esters **7**–**9** readily undergo intramolecular rearrangement reaction leading to bicyclic Amadori adducts with either D-fructofuranose (**10**; obtained from compounds **7** or **8**) or D-tagatofuranose (**11**; obtained from ester **9**) ketohexose moieties (Figure 5) [68]. Hydrolysis of compounds **10** and **11** resulted in *N*-(1-deoxy-D-fructos-1-yl) (**2**) and *N*-(1-deoxy-D-tagatos-1-yl) (**12**) Amadori products of Leu-enkephalin, indistinguishable from **2** and **12** obtained by different reaction methods.

It appears that the formation of bicyclic products **10** and **11** from carbohydrate esters **7**–**9** is favoured, compared with the formation of glycated products **2** and **12** from their parent sugars (glucose, mannose or galactose) and Leu-enkephalin. Since the formation of Amadori products requires the open-chain form of the reducing sugar, the obtained results indicate that esterification of the primary hydroxy group of the monosaccharide moieties increase the abundance of the acyclic *aldehydo* sugar form in solution. In fact, the reactivity of esters **7**–**9** closely resembled that found for D-glucose 6-phosphate, which reacted considerably faster with amines than did D-glucose itself [69].

In contrast to the products formed from monosaccharide esters in pyridine-acetic acid, intramolecular cyclization of compounds **7**–**9**, in methanol as the solvent, resulted in the formation of novel glycation products in the Maillard reaction containing an imidazolidinone ring in the molecule with *trans* and *cis* geometry with respect to the arrangement of the ring substituents (compounds **13**–**15**; Figure 6) [70–72]. The conversion of D-*manno*-related ester **8** to the corresponding imidazolidinone **14** took place completely stereospecifically, and the relative configuration of the *C*-substituents at the imidazolidinone ring in compound **14** was determined to be *trans* [71]. On the contrary, **7** → **13** and **9** → **15** transformations exhibited a low degree of stereocontrol resulting in 2.7 : 1 and 1.3 : 1 diastereoisomeric mixture of imidazolidinones, respectively [72]. X-ray analysis of the minor isomer of D-galactose-related compound **15** uniquely defined the absolute configuration of the new chiral centre at the imidazolidinone moiety as C2(*S*), indicating *cis* arrangement of the C2 and C5 substituents at the 5-membered heterocyclic ring moiety [73].
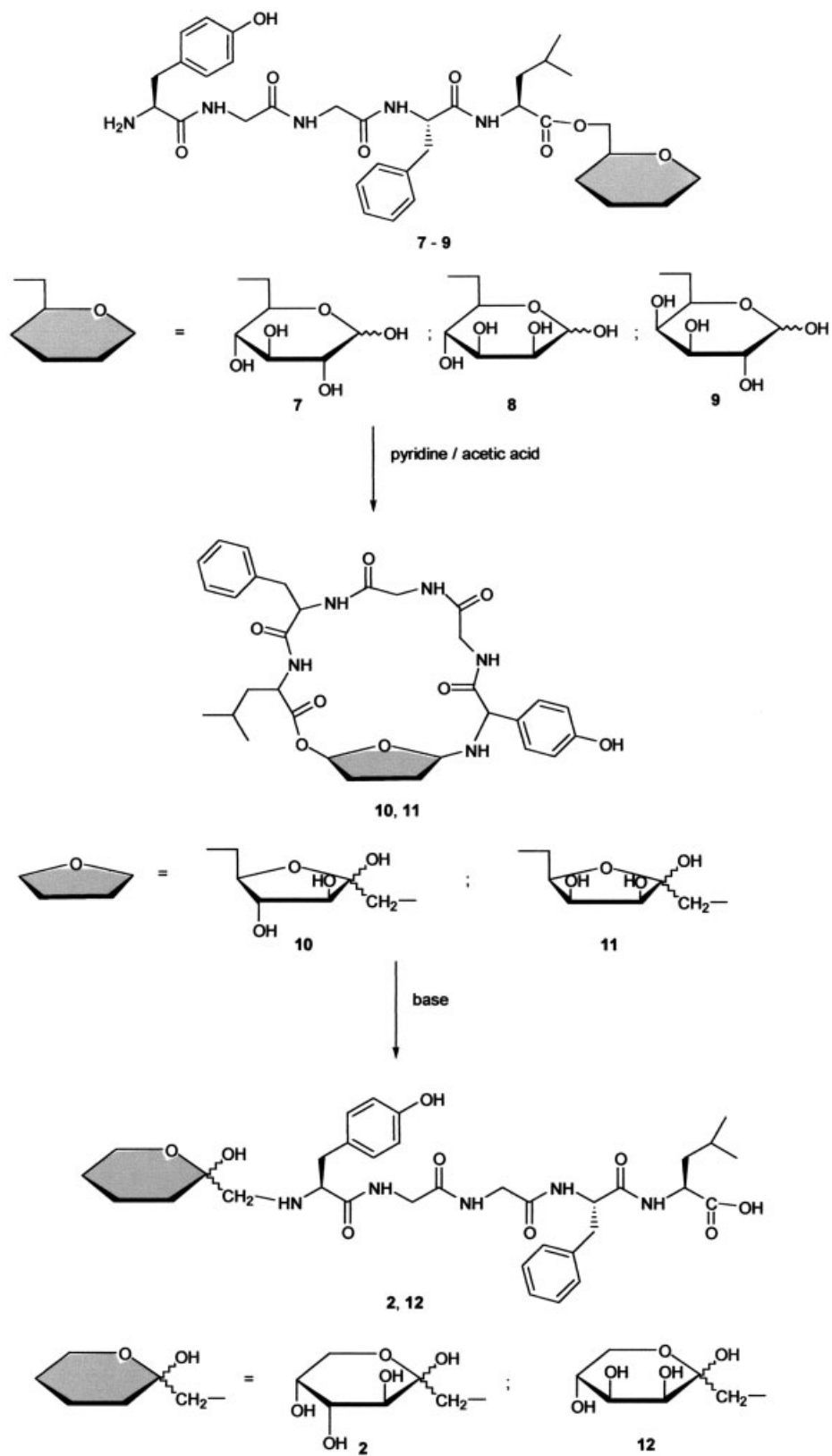
Figure 5 Intramolecular rearrangements of the monosaccharide esters of Leu-enkephalin **7**–**9** in pyridine-acetic acid.
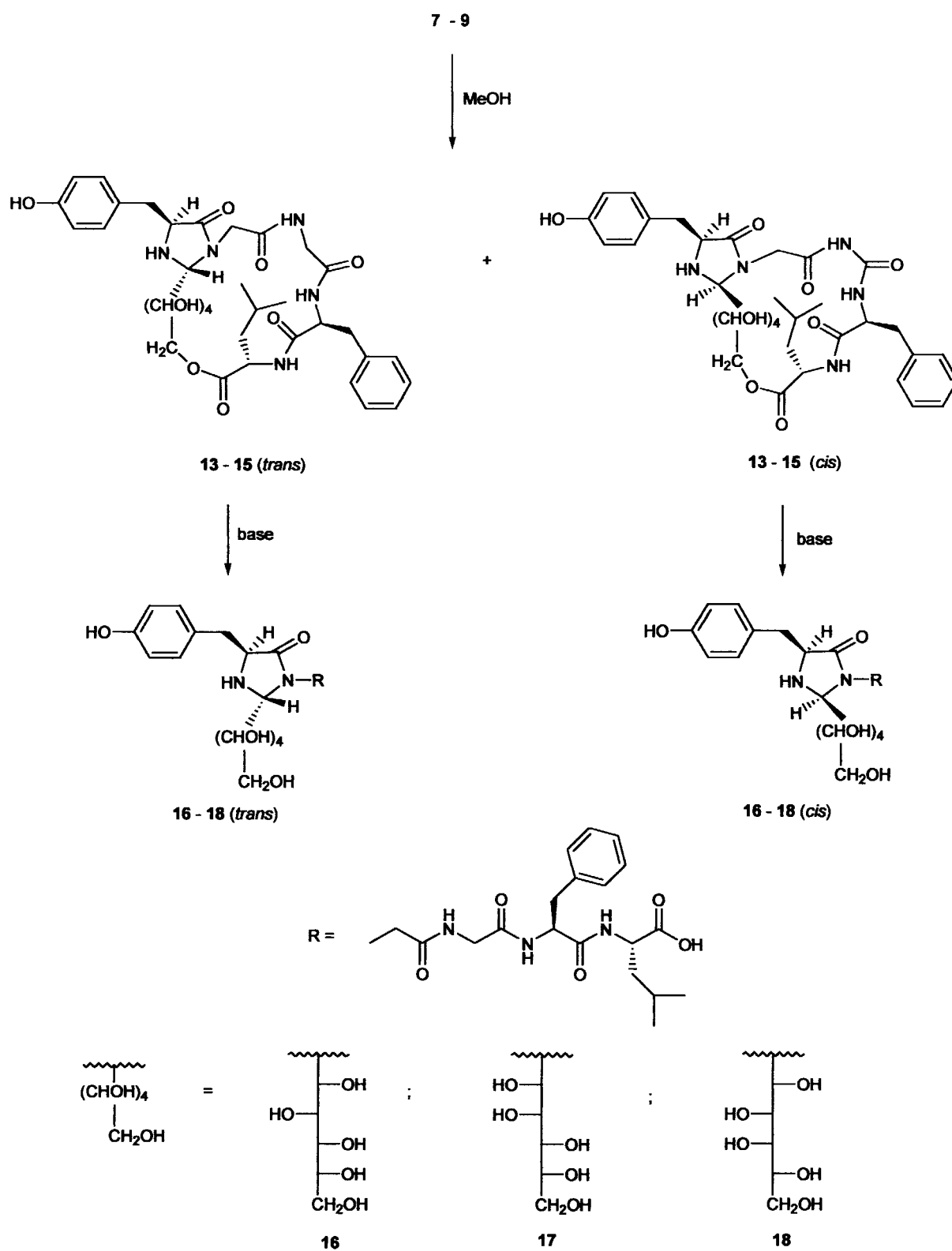
Figure 6   Formation of imidazolidinone products from esters **7**–**9** in methanol.

Considering the mechanism of formation of imidazolidinone compounds **13–15**, in the first step, similarly to the formation of Amadori products **10** and **11** from esters **7–9**, the open-chain form of the carbohydrate moiety is attacked by the free amino terminus of the peptide moiety and a cyclic Schiff base is formed as an intermediate (Figure 7). Since the Amadori rearrangement is a complex acid–base catalysed reaction, pyridine-acetic acid appears to be an ideal solvent for the further transformation of the Schiff base catalysing all steps up to keto-sugar derivatives **10** and **11**. In methanol as the solvent, the Schiff base, instead of Amadori rearrangement, undergoes nucleophilic attack by the Gly^2 nitrogen to yield the *cis* and *trans* isomers of imidazolidinone compounds **13–15** in which C-1 of the sugar moiety forms a bridge

between the amino group of the *N*-terminal tyrosine residue and the amide nitrogen of the Tyr^1-Gly^2 peptide bond. Experiments carried out in methanol as the solvent, suggest the participation of methanol in the imidazolidinone ring-closing reaction by formation of a proton-transferring (both donation and abstraction) transition state. According to structural analysis performed on the D-*manno*-**14** and D-*galacto*-**15** imidazolidinone products it appears that the relative configuration of the substituents in the major product formed during ring closure is *trans*, meaning that the pentitolyl and hydroxybenzyl groups, point in opposite directions with respect to the plane defined by the heterocyclic ring moiety.

Cleavage of the ester bonds in *trans*-**14** as well as in both the *cis* and *trans* isomers of compounds
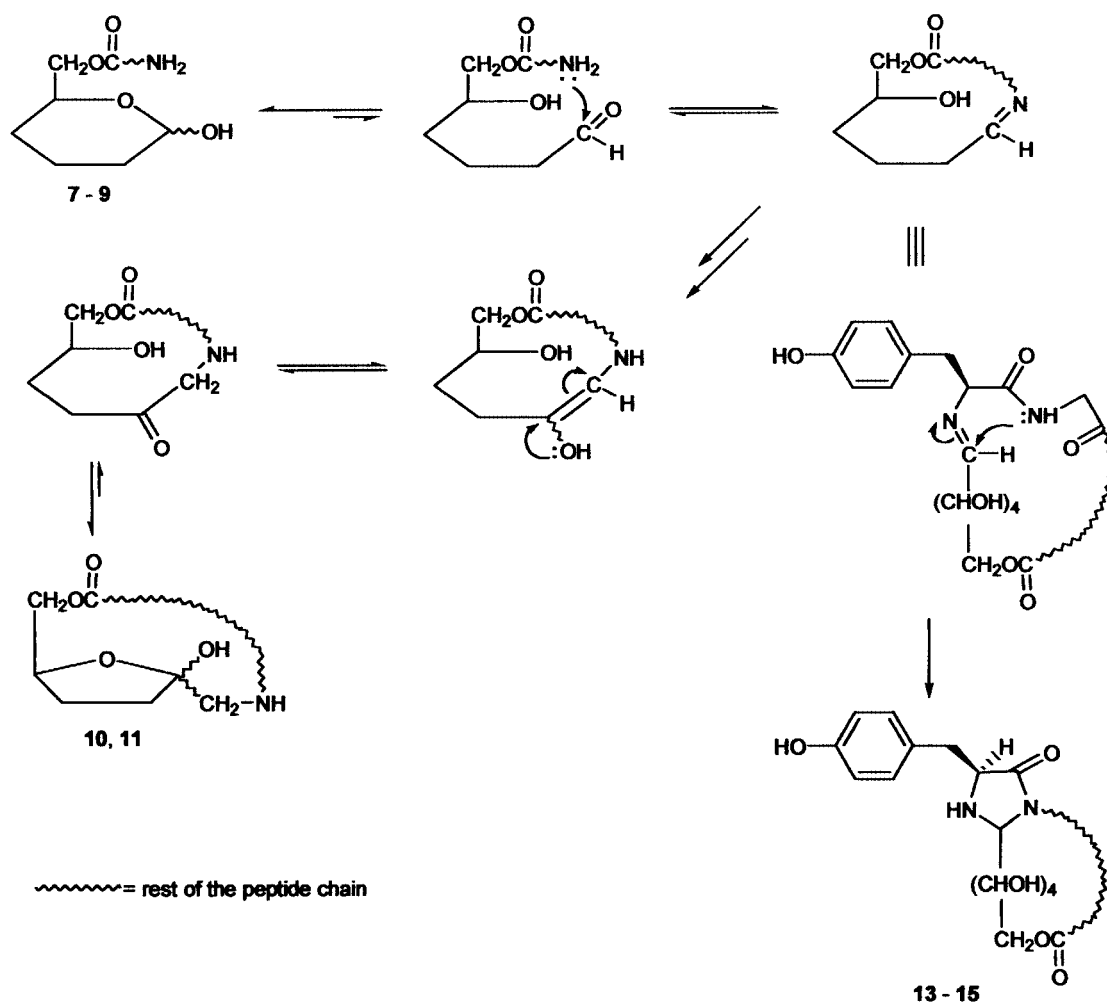

Figure 7 Proposed mechanism of formation of Amadori compounds **10** and **11** and imidazolidinones **13–15** from monosaccharide esters **7–9**.

13 and **15** led to the corresponding D-*gluco*- (**16**), D-*manno*- (**17**) and D-*galacto*-related (**18**) imidazolidinones (Figure 6) in very good yields. The fact that from the same sugar-peptide esters, by changing the reaction conditions, either Amadori rearrangement products or imidazolidinones could be obtained, points to the possibility that, depending on the physiological environment, adducts similar to compounds **16**–**18** may also be generated *in vivo* by the reaction of hexoses with the available amino groups at the *N*-terminus of peptides and proteins.

The results obtained with the Leu-enkephalin-related monosaccharide esters **7**–**9** raised the question whether the observed rearrangements are general phenomena or depend upon the length and the amino acid sequence in the peptide part of the sugar ester. In order to better understand the effects imposed by esterification of the amino acid/peptide with a carbohydrate moiety on the reactivity of the amino group and pathways of intramolecular reactions, the chemical properties of sugar esters **19**–**22** (Figure 8) in which Tyr (**19**), Tyr-Pro (**20**), Tyr-Pro-Phe (**21**) or Tyr-Pro-Phe-Val (**22**) is linked to

the C-6 hydroxy group of D-glucose were examined in pyridine-acetic acid solvent system.

In contrast to peptide esters **7**–**9**, for the tyrosine-related ester **19**, the formation of numerous products generated by intramolecular and intermolecular glycation reactions was observed, indicating a high reactivity of the amino acid ester studied [74]. From the heterogeneous mixture of degradation products formed from **19**, compounds **23**–**28** (Figure 9) were isolated and their structures confirmed by NMR, chemical synthesis and mass spectrometry. Considering the formation of pyrrolo-lactone **24**, it was assumed that, in contrast to the intramolecular reactions of esters **7**–**9** yielding stable bicyclic Amadori products **10** and **11** under identical reaction conditions, the unstable keto-sugar derivative formed from **19** rearranges by enolization and β-elimination to the reactive deoxyhexosone amino acid ester from which, by intramolecular reaction and dehydration, lactone **24** is formed. The Amadori compound **23**, 4-hydroxybenzaldehyde (**25**), 4-hydroxybenzoic acid (**26**), 4-hydroxyphenylacetic acid (**27**) and *N*-acetyl-tyrosine (**28**), are assumed to arise from
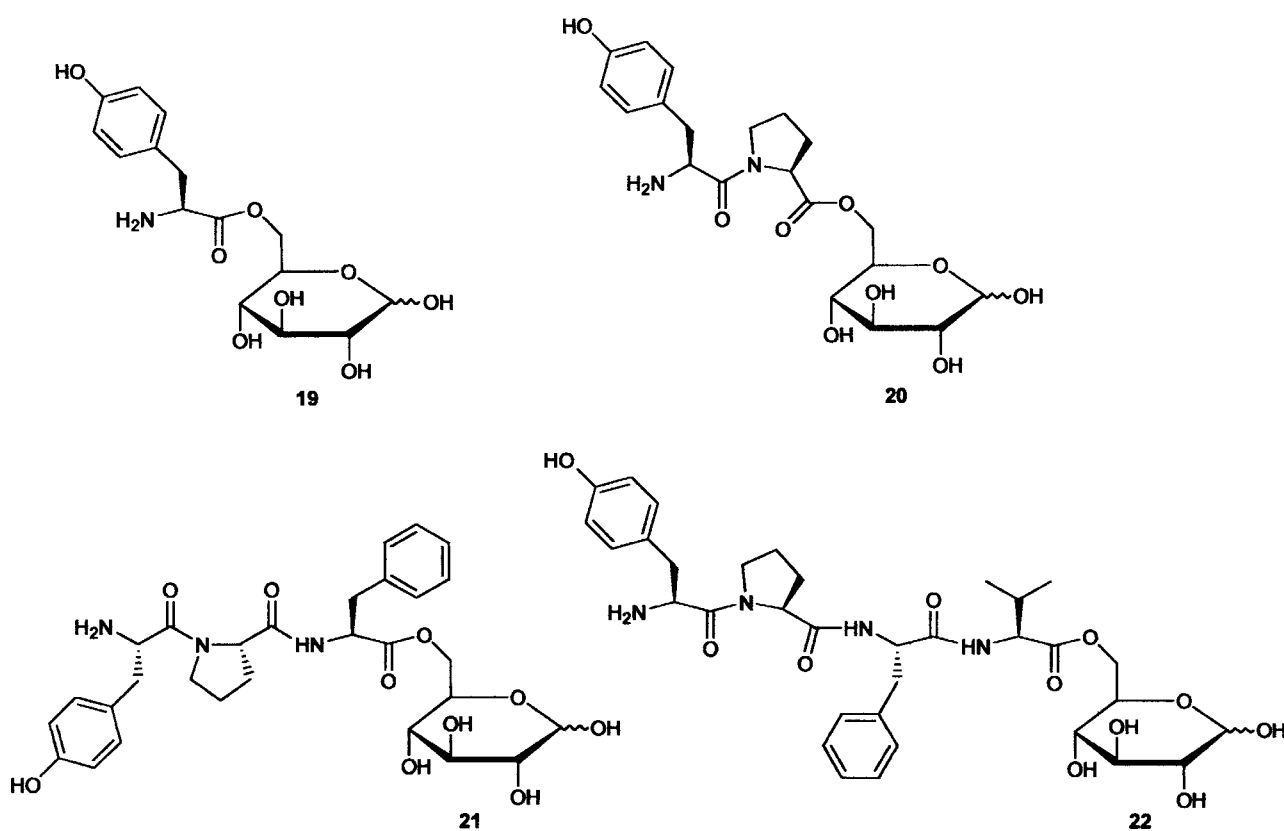

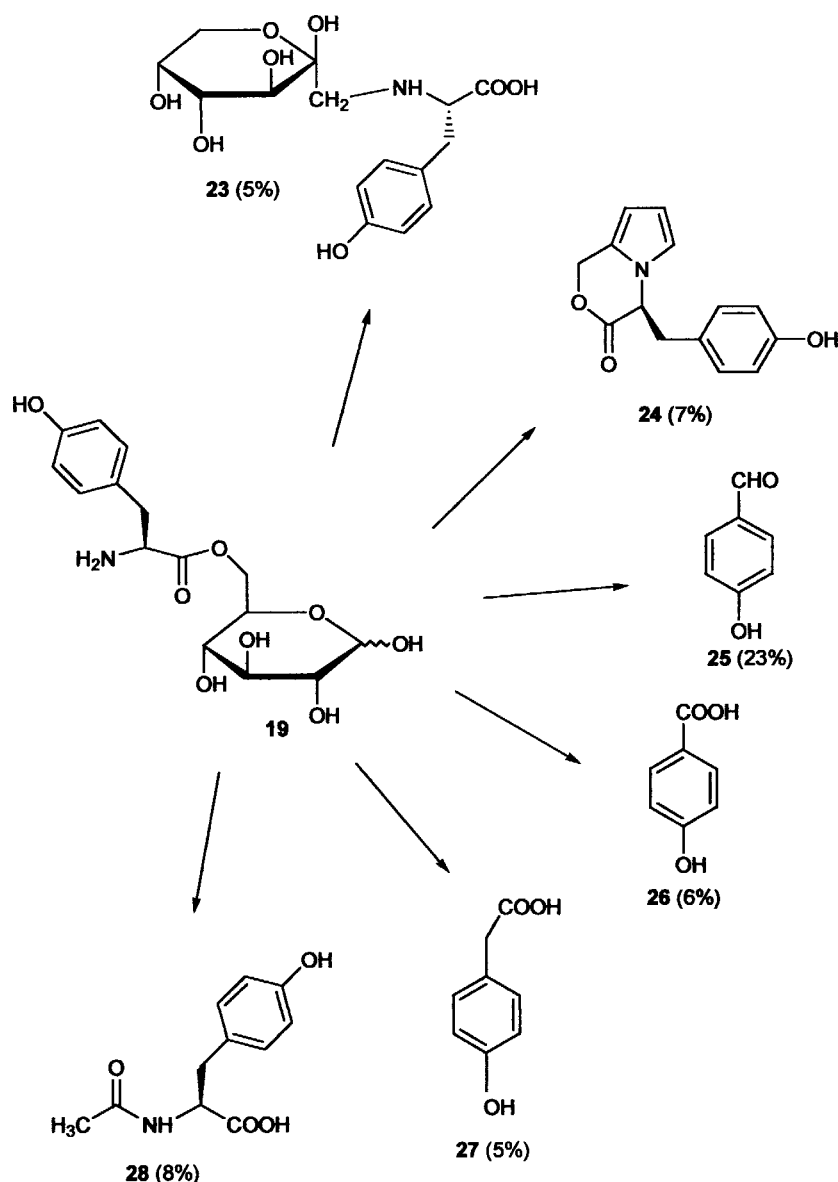Figure 8 Structure of monosaccharide esters **19**–**22**.

Figure 9   Products isolated from the incubation mixture of monosaccharide ester of tyrosine **19** in pyridine-acetic acid.

rearrangements and/or Strecker degradation of the carbohydrate-amino acid adducts. Since ester **19** could be considered a model for the teichoic acid fragment, a component of the cell wall of Gram-positive bacteria containing D-alanine residues esterified with carbohydrate moieties [75], these findings raise the question whether, in Gram-positive pathogens, the free amino group of the esterified D-alanine residue could form specific AGE-like products which could participate in the molecular recognition processes of bacteria by producing bioactive chemical messengers capable of altering the properties of

the host cells, or may have direct impact on colonization processes.

Increase in the length of the peptide part of the carbohydrate esters **20–22** gave rise to specific chemical reactions [76]. As presented in Figure 10, when dipeptide ester **20** was kept in pyridine-acetic acid, the only product formed was diketopiperazine **29**. Although it is known that diketopiperazine formation by intramolecular nucleophilic addition of the terminal amino group to the carbonyl carbon of the second amino acid residue occurs easily in dipeptide esters [77], it is interesting that no other product resulting from the intramolecular reaction
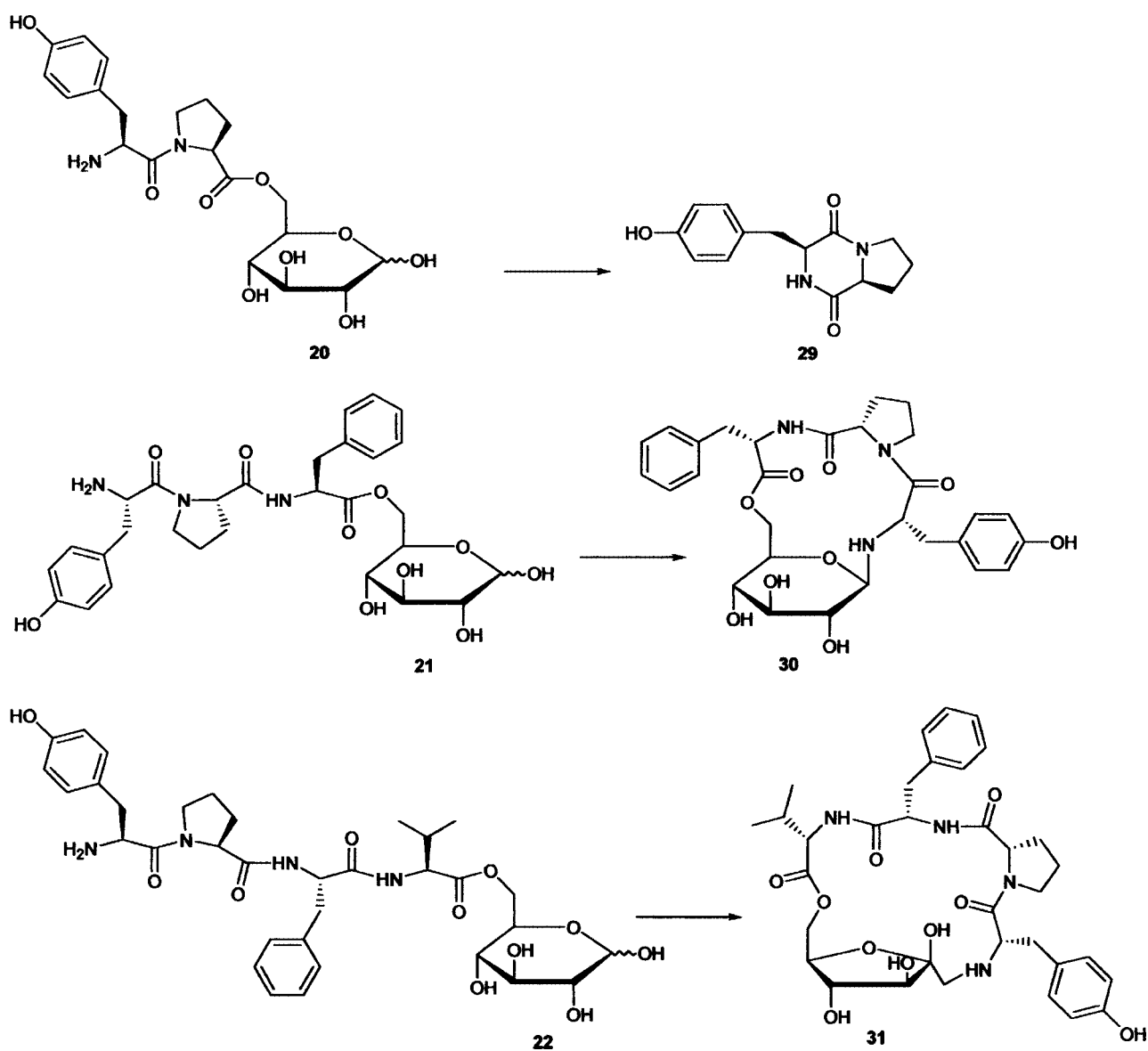
Figure 10   Products of intramolecular reactions of esters **20–22**.

between the sugar and dipeptide moieties in ester **20** was detected, even at low reaction temperatures. In contrast, by heating Tyr-Pro-Phe ester **21** in the same solvent system for 24 h at 37 °C and at 50 °C, two different products were obtained. At the lower temperature, bicyclic glucosylamine derivative **30** was obtained as the major product, whereas incubation of **21** at 50 °C gave *cyclo*-(Tyr-Pro) (diketopiperazine **29**) as the only product. Amadori rearrangements, transformation of the Schiff bases, or their cyclic forms glycosylamines, into 1-deoxy-2-keto-sugar derivatives, readily occurs via *N*-glycosylamino acids [78]. Surprisingly, attempts to

rearrange glucosylamine tripeptide derivative **30** into the corresponding keto-sugar derivative, were unsuccessful. A combined use of NMR spectroscopy and molecular modelling revealed that the main reason for this is a rigid conformation of the central 14-membered ring in compound **30** with the hydrophobic tyrosine and the phenylalanine side-chains stacking against the proline ring and the hydrophilic glucopyranose moiety occupying the opposite side of the molecule [79]. Incubation of tetrapeptide ester **22**, whose amino acid sequence corresponds to the 51–54 fragment of human β-casein, resulted in bicyclic Amadori compound **31**.

In contrast to glycosylamine **30**, the conformational analysis of the Tyr-Pro-Phe-Val-related cyclic Amadori compound **31**, with an 18-membered glycopeptide ring, has shown more flexibility in the peptide backbone and amino acid side-chains. However, mutarotation of the carbohydrate part is hindered, resulting in only β-anomeric configuration of the 1-deoxy-D-fructofuranose moiety in solution.

These results clearly imply that carbohydrate-peptide esters easily undergo intramolecular chemical transformations and the data presented demonstrate that the structure and the length of the peptide residues direct the pathways and the products formed in the early stage of the Maillard reaction.

MASS SPECTROMETRIC ANALYSIS OF SUGAR-PEPTIDE ADDUCTS

Mass spectrometry (MS) has been successfully employed as a fast and highly sensitive method for the detection of early and advanced glycation products of the Maillard reaction, both in food and in biological systems, leading to new tools for monitoring diabetes, and, on the other hand, to important information on the mechanism(s) of this reaction [80–82]. Soft-ionization methods, such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF), were used to determine the mass spectrometric properties of isolated Amadori products derived from glucose and amino acids as well as of underivatized glycated proteins [80,81] and to distinguish between the Amadori products of different sugars, due to the characteristic mass increases of the glycated peptides [82].

To clarify the fragmentation pathways and to find biomarkers of specific sugar-peptide structures, various types of carbohydrate units and sugar-peptide bonds were chosen to find differences in the fragmentation pattern and to establish characteristic fragments suitable for the identification in complex systems [83–85].

Amadori compounds related to the exogenous milk-derived opioid tetrapeptide morphiceptin (Tyr-Pro-Phe-Pro-NH$_2$) (**6**) and to the 51–54 fragment of human β-casein (Tyr-Pro-Phe-Val) as well as to the structurally smaller fragment Tyr-Pro-Phe have been investigated by using high-energy collision-induced dissociation (CID) [83] and ESI-MS/MS techniques [85]. The most intense ions present in the MS spectra

of the $[M + H]^+$ ions of the studied Amadori compounds arise from losses of water molecules, resulting in the simultaneous formation of a pyrylium, furylium and immonium ions, considered as M + 108 and M + 78 modifications of the parent peptides (Figure 11). These ions have been also detected in the majority of the Amadori products studied [80].

The positive-ion FAB and ESI mass spectra of compound **16**, a novel imidazolidinone-type Maillard product derived from the endogenous opioid pentapeptide Leu-enkephalin and D-glucose, display an abundant $[M + H]^+$ peak, confirming the molecular mass value [84]. The CID spectra of the m/z 718 $[M + H]^+$ ions of *cis*- and *trans*-**16** were completely identical including the same fragmentation pattern as well as the intensity of all ions formed. MS analysis showed that fragmentation of the both isomers of **16** generates two ions at m/z 566 and m/z 598 which were identified as the M + 10 and M + 42 modifications of the *N*-terminus of the parent opioid pentapeptide (Figure 12). These ions with imidazolidinone-type modifications at the *N*-terminus of peptides or proteins could be useful as biomarkers for rapid MS-based detection and identification of such early glycation products in complex mixtures.

The fragmentation under ESI-MS conditions of the monosaccharide esters of Tyr-Pro-Phe (**21**) and Tyr-Pro-Phe-Val (**22**), differ from those of the corresponding Amadori compounds, in spite of having identical molecular mass values. While the presence of a M + 162 fragment indicates a hexose-peptide adduct, fragmentation of the sugar ring produced the corresponding well stabilized fragments formed by elimination of two HOCH=CHOH molecules (Figure 13) [85]. Additionally, it was found that, during ionization, esters **21** and **22** undergo intramolecular nucleophilic reactions affording cyclic Schiff base structures. Fragmentation of these cyclic sugar-peptide structures generates M + 42 modified *N*-terminal peptide fragment ions.

Looking at the fragmentation pathways of sugar-peptide adducts, it can be concluded that the position of the carbohydrate modification at the *N*- or *C*-terminus of a peptide can be deduced from the mass gain on the peptide fragment ions, clearly distinguishing between Amadori, imidazolidinone or ester structure of glycated peptides.

CONCLUSION

The presented results demonstrate that the structure of the peptide moiety and the length of the

Amadori compound (M + 162)

oxonium ion (M + 144)

immonium ion (M + 78)

pyrylium ion (M + 108)

furylium ion (M + 78)

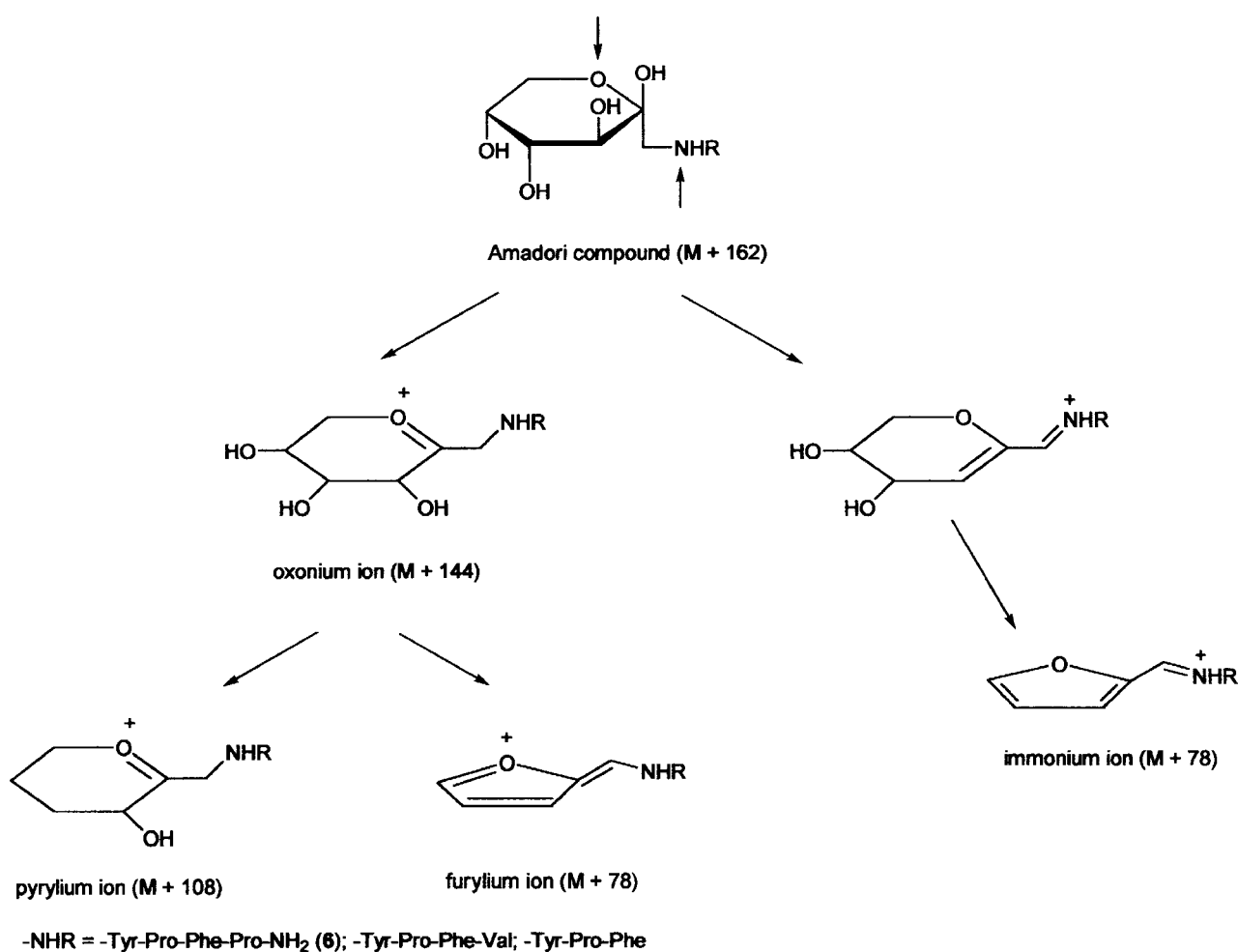-NHR = -Tyr-Pro-Phe-Pro-NH₂ (6); -Tyr-Pro-Phe-Val; -Tyr-Pro-Phe

Figure 11   Diagnostic ions observed in the mass spectra of Amadori compounds related to morphiceptin, 51–54 fragment of human β-casein and Tyr-Pro-Phe.

imidazolidinone compound **16** (M + 162)

~~~~~~COOH = rest of the peptide chain
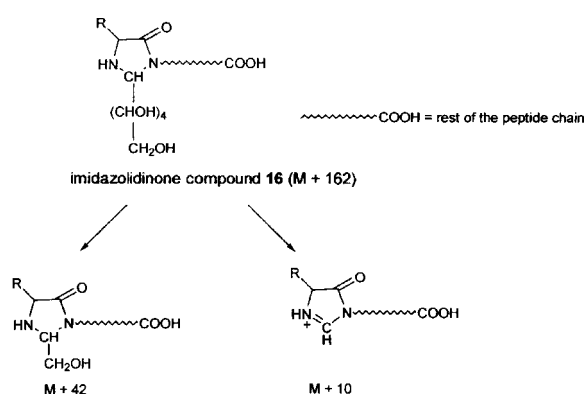
M + 42

M + 10

Figure 12 Fragmentation pattern of imidazolidinone compound **16**.

peptide chain are the main factors that control the degradation of model Amadori compounds as well as their ability to develop Maillard fluorescence. The obtained data indicate that the shorter the peptide chain the more degradation products are formed. It is also to be emphasized that the results obtained with the Leu-enkephalin-related monosaccharide esters demonstrate for the first time that, from the same substrate, in addition to Amadori rearrangement, an alternative pathway for the Maillard reaction is possible yielding imidazolidinone products of the parent peptide from the initially formed Schiff bases.
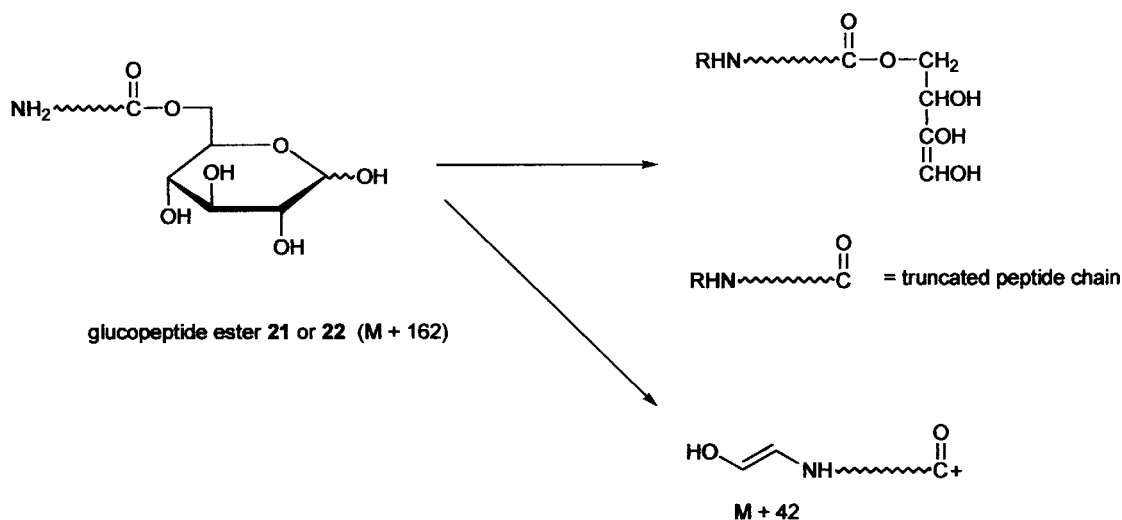
Acknowledgement

Figure 13   Structures of fragment ions obtained from monosaccharide esters **21** and **22**.

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