CROATICA CHEMICA ACTA CCACAA **74** (4) 787–799 (2001)

ISSN-0011-1643
CCA-2767 *Authors' Review*

Ester-linked Glycopeptides as Tools for Studies of Biological Phenomena

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Received May 15, 2001; revised June 27, 2001; accepted June 29, 2001

Modifications of plasma proteins, structural proteins and other macromolecules by glycation (the Maillard reaction) contribute to the development of accelerated atherosclerosis and other complications in diabetes and are also involved in the pathogenesis of aging. Monosaccharide esters, which mimic the reactivity of sugar 6-phosphate esters in nonenzymatic glycations, were prepared from endogenous opioid peptide leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu), smaller peptides of different lengths (Tyr-Pro-Phe-Val, Tyr-Pro-Phe, Tyr-Pro) and from only one amino acid (Tyr) and were used as model compounds for a study of the Maillard reaction *in vitro*. It was found that these compounds readily undergo intramolecular reactions leading to different types of products, such as Amadori compounds, imidazolidinones, glycosylamine, diketopiperazine, pyrrololactone, *etc.* The obtained results demonstrate that the chemical properties of the glycopeptides studied are determined by the structure and length of the peptide chain, suggesting that similar products may be also generated in the early stages of the Maillard reaction *in vivo*.

Key words: glycopeptides, enkephalin, opioid, imidazolidinone, Maillard, glycation.

INTRODUCTION

Undesirable pharmaceutical and biopharmaceutical properties often hinder clinical development of biologically active compounds. One approach that has been used to improve the physicochemical properties is preparation

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of ester prodrugs. Recent investigations have revealed that glycoconjugates possessing an ester bond between the bioactive moiety and one of the sugar hydroxyls are valuable new tools in biomedical research. These monosaccharide esters seem to be able to improve antiviral[1,2] and antibacterial activity,[3] increase the intestinal permeability,[4] alter receptor-selectivity,[5] or they may be used as monomers in polycondensation reactions[6] as well as building blocks for the solid phase combinatorial synthesis of libraries of novel glycopeptides[7] and in peptide templated glycosylation reactions.[8]

In our approach, structurally well-defined monosaccharide esters of peptides have been used for the first time to study the biological phenomena associated with the complex reaction occurring in every living organism, known as the Maillard reaction. In this reaction, reducing sugars, such as glucose, can react nonenzymatically with proteins, glycoproteins, lipids and nucleic acids to produce a variety of carbohydrate adducts known as advanced glycation end products (AGEs).[9,10] It is generally accepted that in the early stage of the Maillard reaction, sugars react with the amino groups of proteins to form keto-sugar derivatives or Amadori rearrangement products *via* Schiff bases. In the advanced stage, many different complex reactions occur leading to AGE products associated with diabetic complications,[11] aging,[12] vascular diseases,[13] chronic renal failure,[14] and Alzheimer's disease.[15] Although the occurrence of the Maillard reaction with a variety of proteins has been demonstrated, the reactivity of individual sugar and peptide components as well as the sequence of reactions that Amadori compounds undergo to generate AGEs are still poorly understood.

In order to gain a better insight into the mechanisms and products of the Maillard reaction, we have used the sugar-peptide esters in which different monosaccharide moieties are linked through their C-6 hydroxy groups to the C-terminal carboxy group of the endogenous opioid pentapeptide leucine-enkephalin as well as to some other small peptides.[5,16] We assumed that such 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 the reducing sugar moiety. They will mimic the reactivity of the sugar 6-phosphate esters in the glycation reactions, and for steric reasons, the attached peptide is more likely to form carbohydrate-peptide adducts than the parent saccharide alone.

We have also examined the reactivity of monosaccharide esters in which glucose is linked to amino acid residues as models for teichoic acid fragment.[17] It occurred to us that the ability of Gram-positive strains to stimulate the production of pro-inflammatory cytokines, which may be important in the pathogenesis of shock caused by these bacteria,[18] reveals some strik-

ing similarities with the effects caused by AGE products, capable of triggering cellular processes linked to accelerated vascular and inflammatory complications.[19] Since diverse physiological functions of Gram-positive bacteria are controlled by the degree of esterification of teichoic acids with D-alanine,[20] one of our goals was to examine on the model compounds whether, in Gram-positive pathogens, reactivity of the free amino group of D-alanine esters in teichoic acid could be responsible for producing specific AGE-like compounds, which would have a direct impact on inflammatory processes.

In this paper we will attempt to give an overview of our recent results and provide some new insights into the Maillard reaction mechanisms, obtained by using monosaccharide esters of peptides and amino acids as investigation tools. All compounds mentioned in this article were purified using RP HPLC and fully characterized structurally by elemental analysis, NMR and MS spectroscopy.

INTRAMOLECULAR REACTIONS OF SUGAR-PEPTIDE ESTERS RELATED TO LEUCINE-ENKEPHALIN

In our previous studies we have demonstrated[5] that introduction of saccharides into leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu), belonging to the class of important endogenous neuropeptides,[21] has a modifying influence on the biological activity profile of the parent peptide compound. Thus, evaluation of the opioid activities in the guinea pig ileum and mouse vas deferens assays of the D-*gluco*- (**1**), D-*manno*- (**2**) and D-*galacto*-ester (**3**) of leucine-enkephalin, illustrated in Figure 1, revealed that introducing different monosaccharide moieties at the C-terminal position of the pentapeptide changes the opioid activity profile and results in an enhanced interaction with the μ-opioid receptor.[5] Monosaccharide esters **1**–**3** were used as model compounds for the study of the Maillard reaction.

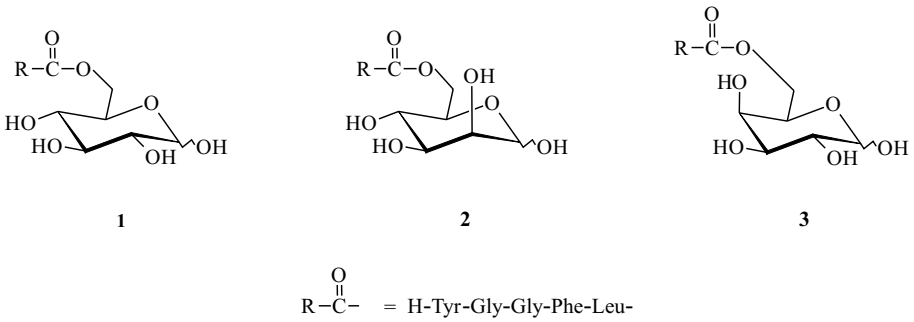
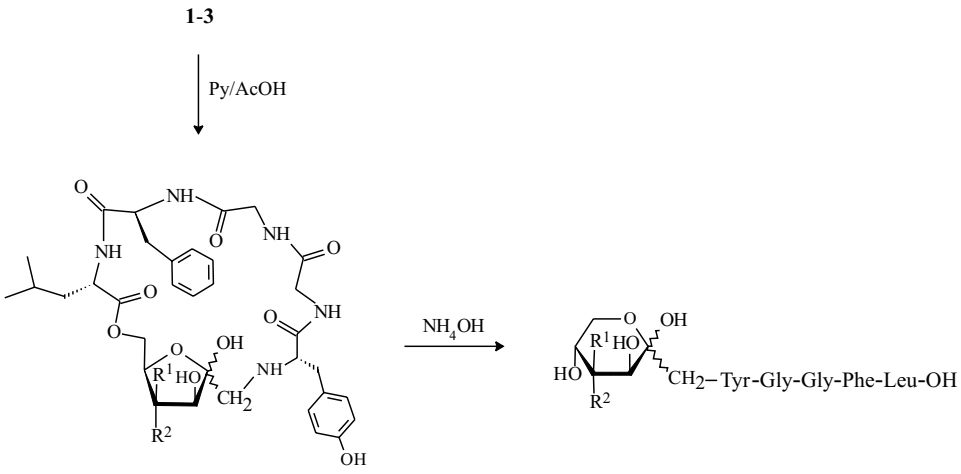


Figure 1. Structure of the monosaccharide esters of leucine-enkephalin **1**–**3**.

As the Amadori rearrangement is known to be a complex acid-base catalyzed reaction in which the balance of the acidity and basicity in the reaction system controls the simultaneous and consecutive reactions,[22] pyridine-acetic acid was used as an ideal solvent for the study of the Amadori product formation. When dissolved in this solvent and incubated at ambient temperature for 24 h, esters **1** and **2** were readily transformed to the D-fructofuranose-related bicyclic Amadori product **4** in 58 and 50% yield, respectively, whereas D-galactose ester **3** incubated at 4 °C for 24 h afforded D-tagatofuranose-related compound **5** (Figure 2) in 20% yield.[23] Hydrolysis of compounds **4** and **5** resulted in *N*-(1-deoxy-D-fructos-1-yl) (**6**) and *N*-(1-deoxy-D-tagatos-1-yl) (**7**) Amadori products of leucine-enkephalin, indistinguishable from **6** and **7** obtained by different reaction methods.



	R^1	R^2
4	H	OH
5	OH	H

	R^1	R^2
6	H	OH
7	OH	H

Figure 2. Amadori rearrangement products of monosaccharide esters **1**–**3**.

It appears that the formation of bicyclic products **4** and **5** from monosaccharide esters **1**–**3** is favoured compared to the Amadori product **6** and **7** formation from their parent sugars (glucose, mannose, galactose) and leucine-enkephalin. The esterified carboxy group of the pentapeptide does not account *per se* for the increased reactivity of compounds **1**–**3** since leucine-enkephalin methyl ester, incubated with D-glucose under identical conditions, showed similar reactivity as the carboxy unprotected peptide. In fact,

the behaviour of esters **1–3** closely resembled that found for D-glucose 6-phosphate, which reacted considerably faster with amines than did D-glucose itself.[24] Since formation of the Amadori compounds requires the open-chain form of the reducing sugar, it can be assumed that, similarly to D-glucose 6-phosphate, the acyclic forms of compounds **1–3** are either more abundant in solution or more efficiently trapped, whereafter the equilibria producing the acyclic forms are rapidly restored.

In contrast to this study, we provided evidence that intramolecular cyclization of leucine-enkephalin monosaccharide esters **1–3**, in methanol as the solvent, resulted in the formation of novel glycation products in the Maillard reaction having imidazolidinone ring in the molecule (compounds **8–10**) (Figure 3).[25–27] Considering the mechanism of the formation of imidazolidi-

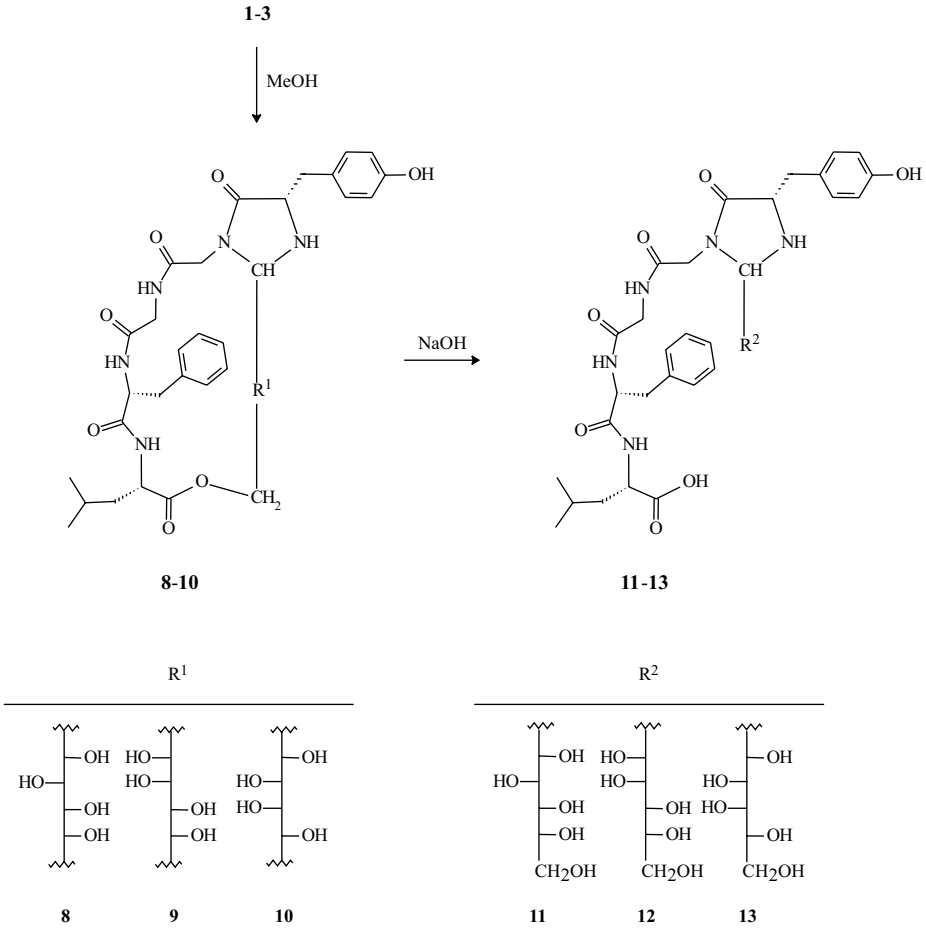


Figure 3. Formation of imidazolidinones from esters **1–3**.

nones **8**–**10**, in the first step, similarly to the formation of Amadori products **4** and **5** from esters **1**–**3**, the aldehyde group of the open-chain form of the carbohydrate moiety is attacked by the free amino terminus of the peptide moiety and the cyclic Schiff base **14** is formed as an intermediate (Figure 4). In the subsequent step, the Schiff base, instead of Amadori rearrangement to the corresponding keto-sugars **4** and **5**, undergoes nucleophilic attack by the Gly^2 nitrogen to yield imidazolidinones **8**–**10** 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. Transformation of mannose ester **2** to imidazolidinone **9** took place completely stereospecifically,[26] while the conversion of glucose ester **1** and galactose ester **3** to the corresponding derivatives **8** and **10**, respectively, resulted in the formation of imidazolidinone diastereoisomers having *cis* or *trans* relative geometry of the substituents at the imidazolidinone ring moiety.[27]

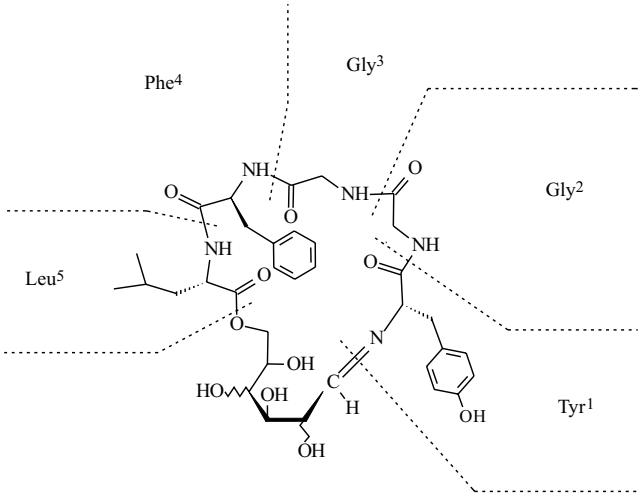


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Figure 4. Structure of the Schiff base **14**.

Cleavage of ester bonds in both the major and minor isomers of compounds **8**–**10** led to the corresponding D-*gluco*- (**11**), D-*manno*- (**12**) and D-*galacto*-related (**13**) imidazolidinones (Figure 3) in 77–96% yields.

It is important that this study demonstrated for the first time, by *in vitro* experiments, that in addition to Amadori rearrangement, an alternative pathway for carbohydrate-induced modification is possible. The experimental fact that monosaccharide esters **1**–**3**, the behaviour of which closely re-

sembles the reactivity of hexose 6-phosphates, yield either the corresponding Amadori products or imidazolidinones, points to the possibility that, depending on the physiological environment, adducts similar to imidazolidinones **11**–**13** may be also generated *in vivo* by the reaction of hexose sugars with the available amino groups on peptides and proteins.

EFFECTS OF THE PEPTIDE OR AMINO ACID MOIETY ON THE PATHWAYS OF INTRAMOLECULAR REACTIONS IN SUGAR ESTERS

The finding that enkephalin-related monosaccharide esters **1**–**3** in pyridine-acetic acid readily undergo intramolecular reaction to Amadori compounds raised the question whether the observed rearrangements are general phenomena or depend upon the length and the amino acid sequence in the peptide moiety of the sugar ester.

With the aim to examine the chemical reactivity of peptide and amino acid monosaccharide esters, we have prepared conjugates **15**–**18** (Figure 5) in which D-glucose is linked to Tyr-Pro-Phe-Val (**15**), Tyr-Pro-Phe (**16**), Tyr-Pro (**17**), and to only one amino acid Tyr (**18**) by an ester bond involving hydroxy group at C-6 of the D-glucopyranose moiety.[16,17] The relation between the structure and reactivity of glycoconjugates **15**–**18** has been studied in pyridine-acetic acid solvent system.

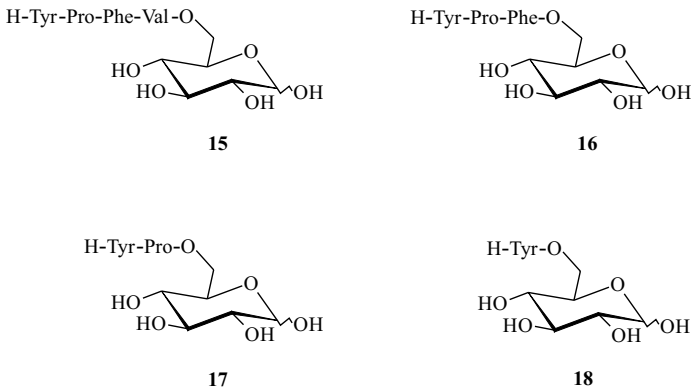

Figure 5. Structure of monosaccharide esters **15**–**18**.

Incubation of tetrapeptide ester **15**, whose amino acid sequence corresponds to the 51–54 fragment of human β-casein (found to possess weak opioid activity),[28] for 24 h at room temperature resulted in bicyclic keto-su-

gar derivative **19** (Figure 6), an Amadori compound, in the β-furanose form.[16] In fact, the product of the intramolecular transformation of tetrapeptide ester **15** was analogous to compound **4**, obtained under identical reaction conditions from monosaccharide ester **1**.

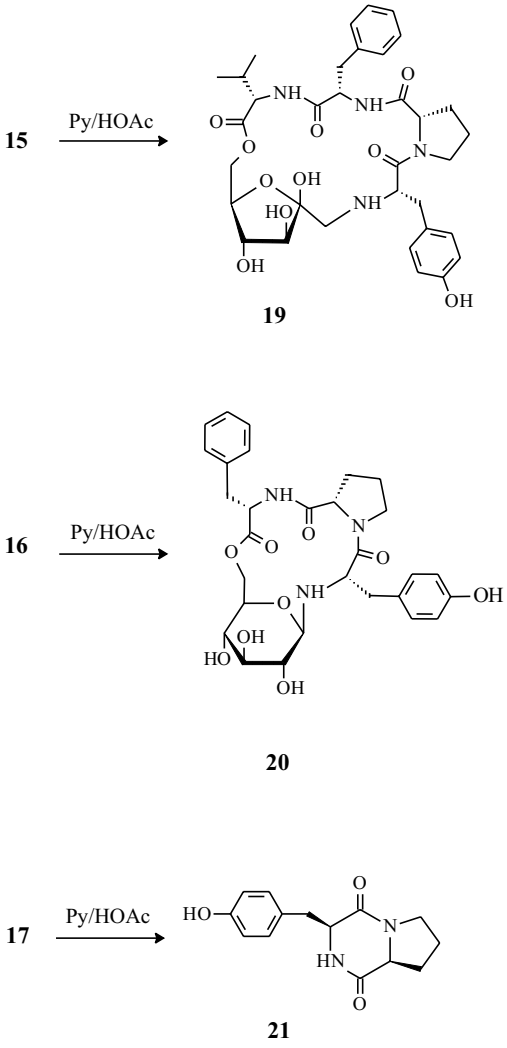

Figure 6. Products of intramolecular reactions of compounds **15**–**17**.

In contrast, by heating tripeptide ester **16** in the same solvent system for 24 h at 37 °C and at 50 °C, two different products were obtained. At 37 °C, by intramolecular cyclization of **16**, the major product isolated in a 52%

yield was bicyclic β-D-glucosylamine derivative **20** (Figure 6), whereas incubation of **16** at a higher temperature (50 °C) gave *cyclo*-(Tyr-Pro) (**21**) (68%).[16] Formation of glucosylamine tripeptide derivative **20** was explained by formation of a Schiff base as intermediate, which through intramolecular reaction of the sugar C-5 hydroxy group with C-1 then closes to give hemiacetal **20**. The fact that the incubation mixture of monosaccharide ester **16** at 37 °C contained, in addition to **20**, only traces of diketopiperazine **21**, while incubation at 50 °C gave exclusively **21**, suggested the bicyclic compound **20** as the precursor of *cyclo*-(Tyr-Pro) **21**. We speculated that at higher temperatures, for steric reasons, the proline carbonyl atom in **20** is more vulnerable to nucleophilic attack by the secondary NH group of the tyrosine residue, resulting in diketopiperazine **21** formation after cyclization and cleavage of the easily hydrolyzed *N*-glucosyl-amino acid bond.[29]

It is generally accepted that the Amadori rearrangement, transformation of the Schiff bases, or their cyclic forms glycosylamines, into 1-amino-1-deoxy-2-keto-sugar derivatives, readily occurs with *N*-glycosylamino acids.[29] Surprisingly, attempts to rearrange glucosylamine tripeptide derivative **20** into the corresponding keto-sugar derivative were unsuccessful. However, heating of D-glucose with tripeptide Tyr-Pro-Phe at 37 °C for 2 days in pyridine-acetic acid afforded the corresponding Amadori product *N*-(1-deoxy-D-fructos-1-yl)-Tyr-Pro-Phe as the major product (26%) whereas no glucosylamine derivative of the tripeptide was detected in the reaction mixture.[16] It is rational to presume that the inability of **20** to rearrange into the keto-sugar derivative is due to conformational reasons, however, further NMR and molecular modeling analyses were initiated to elucidate this assumption.

When dipeptide ester **17** was dissolved in pyridine-acetic acid and kept at 4 °C for 2 days, diketopiperazine **21** was the only product generated in 48% yield. 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 because of good leaving groups (alcohol molecules),[30] it is interesting that neither the corresponding glucosylamine derivative nor the Amadori product were detected in the reaction mixture as a consequence of the intramolecular reaction between the sugar and dipeptide moiety in ester **17**.

These results have evidenced that monosaccharide esters **15**–**17** easily undergo intramolecular chemical transformations, subsequent to the attack of the free *N*-terminal amino group on the peptide backbone or on the anomeric position of the D-glucose moiety. The data also indicate that the structure and the length of peptides direct the specific chemical reactions and the products formed in the early stage of the Maillard reaction.

Surprisingly, and in contrast to the results obtained with monosaccharide esters of peptides, we have demonstrated[17] that the tyrosine-related ester **18**, a model of teichoic acid fragment, due to the enhanced reactivity, readily undergoes intramolecular reactions leading to a heterogeneous mixture of compounds. As evidenced by RP HPLC, after 24 h of incubation in pyridine-acetic acid, the abundance of degradation products exceeded 90% in ester **18**. From the numerous reaction products formed from **18** (Figure 7), it was possible to isolate pure pyrrololactone **22** (5%) as the major rearrangement product. Considering the formation of lactone **22** from monosaccharide ester **18**, we assume that in contrast to the intramolecular reactions of esters **1**–**3** yielding stable bicyclic Amadori products **4** and **5** under identical reaction conditions, the unstable keto-sugar derivative formed from **18** rearranges by enolization and β-elimination further to the reactive deoxyhexosone amino acid ester. For steric reasons, the nucleophilic attack of the

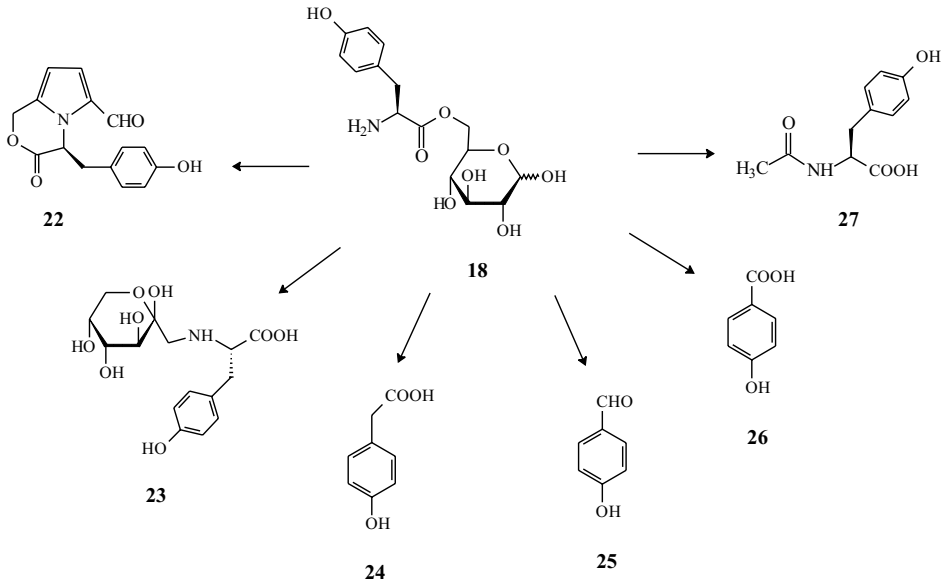

Figure 7. Isolated products of intramolecular reactions of the monosaccharide ester **18**.

free amino group on the reactive hexosone carbonyl group is facilitated, resulting, after dehydration, in lactone **22**.[17]

In addition to lactone **22**, from the reaction mixture of the starting compound **18** it was possible to isolate, in less than 1% yield each, Amadori compound **23**, 4-hydroxyphenylacetic acid (**24**), 4-hydroxybenzaldehyde (**25**),

4-hydroxybenzoic acid (**26**) and *N*-acetyl-tyrosine (**27**) (Figure 7), assumed to arise from rearrangements and/or Strecker degradation of the initially formed intramolecular carbohydrate-amino acid adducts.

In order to gain better understanding of the effect imposed by esterification of the amino acid with a carbohydrate moiety on the reactivity of the amino group, ester **18** as well as tyrosine and glucose were incubated separately in pyridine-acetic acid at 50 °C for 24 h. The relative amounts of lactone **22** and compounds **24**–**27** determined in both reaction mixtures clearly showed that the formation of these products from ester **18** is favoured in comparison with the formation of the same compounds from D-glucose and tyrosine.[17]

Taken together, we have demonstrated an activating effect of the sugar substituent on the amino acid residue, resulting in a complex mixture of products. Our findings also suggest that the carbohydrate esters of D-alanine present in teichoic acids of Gram-positive bacteria could be considered participants in molecular recognition processes by producing bioactive chemical messengers capable of altering the properties of the host cells.

Acknowledgement. – This work was supported by the Ministry of Science and Technology of Croatia grant No. 00980704.

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SAŽETAK

Esterski povezani glikopeptidi kao modeli za proučavanje bioloških pojava

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Glikacijom uzrokovane promjene plazmatskih proteina, strukturnih proteina i ostalih makromolekula (Maillardova reakcija) pridonose razvoju ubrzane ateroskleroze i ostalih komplikacija kod dijabetesa, a također su uključene i u patogenezu sta-

renja. Esteri monosaharida, koji po reaktivnosti sliče 6-fosfatnim esterima šećera, pripravljeni su iz endogenog opioidnog peptida leucin-enkefalina (Tyr-Gly-Gly-Phe-Leu), manjih peptida različite duljine (Tyr-Pro-Phe-Val, Tyr-Pro-Phe, Tyr-Pro) i iz samo jedne aminokiseline (Tyr) te su uporabljeni kao modelni spojevi za proučavanje Maillardove reakcije *in vitro.* Pronađeno je da ti spojevi lako podliježu intramolekulskim reakcijama, pri čemu nastaju različiti tipovi produkata, npr. Amadorijevi spojevi, imidazolidinoni, glikozilamin, diketopiperazin, pirol-lakton itd. Dobiveni rezultati jasno pokazuju da kemijska svojstva proučavanih spojeva ovise o strukturi i duljini peptidnog lanca i upućuju na moguće nastajanje sličnih produkata u ranom stupnju Maillardove reakcije *in vivo.*