Preliminary Results concerning Chemical Galactofuranosylation of Tocopherol (Vitamin E) And Thiamine (Vitamin B1)

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D,L-α-Tocopherol was galactofuranosylated by boiling it for 7-8 h in dry toluene with tetra-O-benzoyl α-D-galactofuranosyl bromide, cadmium carbonate as promoter and calcium sulfate as water scavenger. Reaction product was submitted to Zemplen saponification, and D,L-α-tocopheryl β-D-galactofuranoside was purified by column chromatography on silica gel and characterized by spectral (ESI-MS), chemical and chromatographical means. Contrary to this method of glycosylation, heating was avoided in the course of galactofuranosylation of thiamine (vitamin B1). In this sense, glycosylation was made by stirring a suspension consisting of thiamine, tetra-O-benzoyl α-D-galactofuranosyl bromide, calcium sulfate, cadmium carbonate and silver salicylate in dry toluene for 72 h at room temperature. Of reaction mixture, thiamine β-D-(2,3,5,6-tetra-O-benzoyl) galactofuranoside was separated. Protecting groups were removed by Zemplen saponification and salinity was avoided by neutralization with Dowex 50W X8 (H^+). Thiamine β-D-galactofuranoside was characterized physico-chemically and chromatographically, and a small portion peracetylated and ESI-MS spectra registered.

Key words: D,L-α-tocopheryl β-D-galactofuranoside, thiamine β-D- galactofuranoside, mass spectrometry, Koenigs-Knorr synthesis

Many vitamins, both hydrophilic and lipohilic, are found in living matter as glycosides, sugar moiety being most frequently D-glucose and L-rhamnose [1, 2]. The glycosidic moiety can play a crucial role for the activity and improving biological properties. Glycosylated vitamins (B_1, B_2, B_6, C) have advantages over the original aglycones in their solubility in water, stability against ultraviolet light, heating, and air oxidation, reduction of a bitter taste, a stimulative tongue-pricking taste, and longer remanence in the tissues [2, 3]. At the same time, glycosylation strongly alters the transport through physiological barriers, e. g., brain barrier, placental barrier. Remarcably, some glucosides can be actively carried into the brain tissue by using the glucose-transport system. Contrary, many glucuronides are blocked by placental barrier, thus preventing intoxication of foetal tissue by conjugates of xenobiotics [2]. Another aspect is the interaction between some glycosidic fragment and the respective complementary zone of receptors or lectins. A good example is the high affinity of β-galactosides to hepatocytes due to galectin-C occurring in high concentrations on cell surface [4]. Many vitamin glycosides present spectacular and unique biochemical, physiological and therapeutical properties. The action of ascorbic acid glucoside to heal melasma, being applied either manually or by sonophoresis, proved its efficiency when the illustrating indices – erythema/melanin were measured by a mexameter or dermoscopically [5]. In a tentative to improve biological properties of vitamin C, a stable form of this compound have been invented, 2-O-α-D-glucopyranosyl-L-ascorbic acid. This stable hydrophilic derivative, successfully passed all biological and medical tests and has been accepted as a skin care main ingredient by the Japanese Government. Subsequently, its transdermal activity has been improved by attaching a hydrophobic tail on C-6 of aglycone. The availability of vitamin C of this compound was proved by the fact that a series of glycosides of the type 2-O-α-D-glucopyranosyl-6-O-acyl-L-ascorbic acid, the acyl group being successively C_4, C_6, C_8, C_{10}, C_{12}, C_{16}, were alternatively cleaved by tissues enzymes of guinea pig and rat, either at glycosidic or at ester bond or at both [6]. The pyridoxine glucosides are either deconjugated by a mucosal glucosidase or they are absorbed intact and hydrolyzed in various tissues, especially liver and serum [7, 8]. The beneficial action of vitamin glycosides have been demonstrated in a combined strategy: tumor sensitization to cancer radiotherapy, as produced by using nitrotriazole (N-(2'-methoxyethyl)-2-(3"-nitro-1"-triazolyl) acetamide) as radiosensitizer, and normal tissue protection by two glycosides, ascorbic acid glucoside and α-tocopherol glucoside [9].

In this paper, two new derivatives of β-tocopherol and thiamine have been synthesized, separated and characterized, i. e., D,L-α-tocopheryl-β-D-galactofuranoside and thiamine β-D-galactofuranoside. They could be new metabolites of α-tocopherol and thiamine or serve as new drug delivery systems of the two vitamins in gastrointestinal tract.

Experimental part

The mixture of 1,2,3,5,6-penta-O-benzoyl-αβ-D-galactofuranoses was prepared as indicated [10], by heating D-galactose in pyridine and adding benzoyl chloride while the sugar solution is still hot. Alternatively, this compound was prepared *via* the following sequence of reactions [11, 12]: D-galactose→1,2-5,6-di-O-izopropylidene-α-D-galactofuranoside→1,2-O-izopropylidene-α-D-galactofuranoside→1,2-O-izopropylidene-3,5,6-tri-O-benzoyl-α-D-galactofuranoside → 3,5,6-tri-O-benzoyl-αβ-D-galactofuranoses → 1,2,3,5,6-penta-O-benzoyl-αβ-D-galactofuranoses. Penta-O-benzoyl derivative was converted to glycosylation donor, tetra-O-benzoyl-α-D-galactofuranosyl bromide, by reaction with HBr in glacial

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acetic acid [10, 13]. D,L-α-Tocopherol was galactofuranosylated by boiling it for 7-8 hr in dry toluene with tetra-O-benzoyl α-D-galactofuranosyl bromide, cadmium carbonate as promoter and calcium sulfate as water scavenger [10, 13, 14]. Reaction product was submitted to Zemplen saponification, and D,L-α-tocopheryl β-D-galactofuranoside was purified by column chromatography on silica gel and characterized by spectral (ESI-MS), chemical and chromatographical means. Contrary to this method of glycosylation, heating was avoided in the course of galactofuranosylation of thiamine (vitamin B1), taking into account the fact that this vitamin is thermolabile. In this sense, glycosylation was made by stirring a suspension consisting of thiamine, tetra-O-benzoyl α-D-galactofuranosyl bromide, calcium sulfate, cadmium carbonate and silver salicylate in dry toluene for 72 h at room temperature [15]. Of reaction mixture, thiamine β-D-(2,3,5,6-tetra-O-benzoyl) galactofuranoside was separated. Protecting groups were removed by Zemplen saponification and salinity was avoided by neutralization with Dowex 50W X8 (H^+). Thiamine β-D-galactofuranoside was studied *per se* except a small portion that was peracetylated in view of ESI-MS analysis.

ESI MS spectra of all intermediates, including aglycones and galactofuranosylated vitamins, were registered in peracetylated form at a GC MS-MS Varian Saturn 3 equipment.

Thin-layer chromatography (TLC) was performed on ready-to-use glass plates covered with silica gel 60 (E. Merck). The following solvent mixtures were used: solvent system (SS) 1 (toluene-methanol, 7/1, v/v), SS 2 (chloroform-methanol-water, 50/10/1, v/v), SS 3 (toluene-ethanol, 5/1), SS 4 (chloroform-methanol-water-concentrated ammonia, 70/30/4/1, v/v), SS 5 (acetone-n-butanol-water, 5:4:1, v/v).

Two types of acidic hydrolysis were taken into account: a mild hydrolysis, currently used for galactofuranosides, accomplished by boiling the sample in 0.3 N H_2SO_4 in a mixture of water-ethanol (1:1, v/v) [16] and an energetic hydrolysis, destined to stronger glycosidic bonds, made by refluxing the compounds in 3 N H_2SO_4 [17]. Chemical constituents of D,L-α-tocopheryl-β-D-galactofuranoside, preceded by acidic hydrolysis and partition between chloroform and water, were determined by characteristic methods: D-galactose by anthrone [18] and D,L-α-tocopherol by Emmerie-Engel reaction [19]. In case of thiamine galactoside, the sugar was determined by the same method and thiamine by a fluorimetric method after oxidation to thiochrome [20].

Column chromatography was made on silica gel, acylated compounds being eluted with a gradient of ethyl acetate in n-hexane, and the relatively polar compounds by a gradient of methanol in chloroform.

Results and discussion

By a carefully led TLC analysis, the product(s) of galactofuranosylation could be detected for both aglycones, D,L-α-tocopherol and thiamine. In case of glycosylation of D,L-α-tocopherol (fig. 1) at least two products could be identified: one migrating faster than aglycon and one slower.

Separation of thiamine β-D-(2,3,5,6-tetra-O-benzoyl) galactofuranoside, produced in the reaction, from unreacted thiamine, is presented in figure 2.

An important test in glycosylation reactions is the identification of products migrating slower than the aglycon, chromatographic analysis being preceded by Zemplen saponification. In case of both aglycons used in this paper, such products could be identified, as illustrated

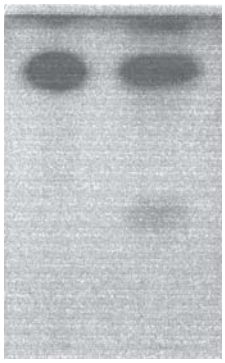

Fig. 1. Evidencing of synthesis product(s) in comparison with aglycon. Lane 1, D,L-α-tocopherol; lane 2, mixture of D-galactofuranosylation products. Migration, SS 1 (toluene-methanol, 7/1, v/v). Visualisation, mostain

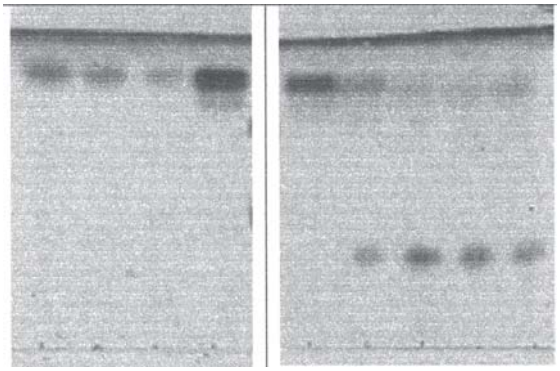

Fig. 2. Separation of thiamine D-(2,3,5,6-tetra-O-benzoyl) galactofuranoside (faster moving compound) of unreacted thiamine. Migration, SS 5 (acetone-n-butanol-water, 5:4:1, v/v); Visualisation, mostain

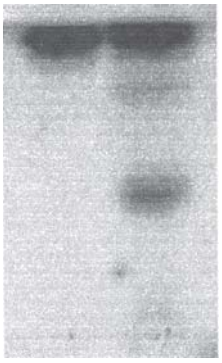

Fig. 3. Evidencing of a reaction product migrating faster than D-galactose (in start) and slower than D,L-α-tocopherol, i. e., in the region of glycolipids. Lane 1, crude reaction products before Zemplen saponification; lane 2, reaction products after Zemplen saponification. Migration, SS 2, chloroform-methanol-water, 50/10/1, v/v. Visualization, mostain

for D,L-α-tocopherol (fig. 3). However, in case of thiamine, relatively polar elution solvents had to be used [21] and it was impossible in this situation to present both glycosylations on the same plate.

Column chromatography produced fractions containing pure compounds, for both thiamine β-D-(2,3,5,6-tetra-O-benzoyl) galactofuranoside (fig. 2) and D,L-α-tocopheryl D-galactofuranoside (fig. 4).

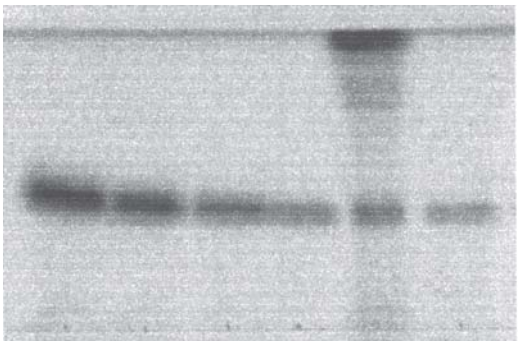

Fig. 4. TLC Analysis of fractions obtained by column chromatography of galactofuranosylation product of D,L-α-tocopherol after Zemplen saponification. Lane 5, total glycosylation mixture; lanes 1, 2, 3, 4, 6, successive fractions. Migration, SS 2, chloroform-methanol-water, 50/10/1, v/v. Visualization, mostain

At least theoretically, both aglycons used in this paper could led to Friedel-Crafts products instead of Koenigs-Knorr synthesis products (i. e., carbon-carbon bonds instead of carbon-oxigen ones). Although such products are natural products [22] and they could be valuable compounds in affinity chromatography and in modulating enzymatic reactions, by mimicking the structure of natural ligands or substrates, they did not constitute the aim of this work. A valuable chemical or enzymatic test evidencing the presence of glycosides *versus* Friedel-Crafts products is total hydrolysis. Comparative acidic hydrolysis of the two glycosides undoubtfully indicated furanosic ring for the sugar [13, 14, 16], due to their susceptibility to acidic hydrolysis: the use of 0.3 N sulfuric acid solution produced complete cleavage in less than half an hour (fig. 5), making superfluous the use of 3 N sulfuric acid and longer times of reaction. Determination of chemical constituents indicated a ratio 1:1 between sugar and aglycon in both galactosides, of D,L-α-tocopherol and thiamine.

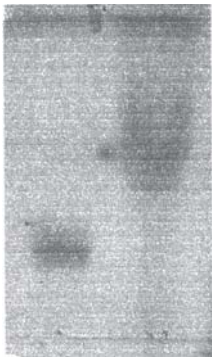

Fig. 5. By acidic hydrolysis, D,L-α-tocopheryl D-galactofuranoside (lane 1) produced D,L-α-tocopherol (lane 2) (D-galactose is not seen in the lane, as expected, because hydrolysis mixture was partitioned between chloroform and water, before chromatography). Migration, SS 3 (toluene-ethanol, 5/1); visualization, mostain

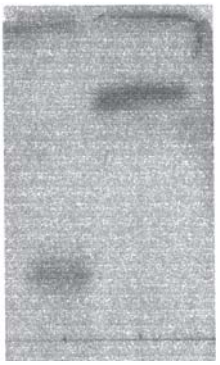

Fig. 6. An appreciable increase between R_F value of D,L-α-tocopheryl D-galactofuranoside (lane 1) and peracetylated D,L-α-tocopheryl D-galactofuranoside SS 3 (toluene-ethanol, 5/1)

A chemical chromatographical test, comparable with quantitative IR spectroscopy (determination of the ratio between the number of hydroxy groups and methylene groups) [23], was elaborated. It was noted that compounds posessing one hydroxy group per molecule (cetyl alcohol, cholesterol, D,L-α-tocopherol) present a relatively low increase in R_F value by acetylation. In case of D,L-α-tocopherol, the two values for free and acetylated compound are 0.89 and 0.77, respectively. On the other hand, compounds containing 4-6 hydroxy group per molecule (cerebrosides, cholesteryl hexosides, diacyl glycerol hexosides) present a significant increase in R_F value by acetylation. This test applied to the synthesized D,L-α-tocopheryl galactoside (fig. 6) disclosed the following R_F values for free and peracetylated galactosides, respectively: 0.75 and 0.21. In this case, if we consider the lower R_F value as a unit, the following values are obtained: 1.15 for D,L-α-tocopheryl acetate in comparison with D,L-α-tocopherol and 3.57 for D,Lα-tocopheryl D-(2,3,5,6-tetra-O-acetyl)galactofuranoside relatively to D,L-α-tocopheryl D-galactofuranoside.

ESI MS spectra of the two galactosides were made comparatively on peracetylated compounds – aglycons and glycosides. In case of D,L-α-tocopheryl D-(2,3,5,6-tetra-O-acetyl)galactofuranoside, $C_{43}H_{68}O_{11}$, two molecular ions were registered: 784 ($M + Na^+$) and 779 ($M + NH_4^+$). A more complicated discussion has to be made in case of thiamine and its galactoside: Zemplen saponification exposed the compound to alkaline medium and, at the same time, peracetylated glycoside was heated in order to remove reagents (pyridine, acetic anhydride, acetic acid). A suite of transformations take place in the molecule of thiamine as a function of physico-chemical agents acting on it [24, 25] (fig. 7). The three structures were taken into consideration as a consequence of ion species identified by ESI MS spectra. The following values were found: for structure (a), $C_{28}H_{36}N_4SO_{11}$, the value 674 ($M - H + K^+$); for structure (b), $C_{28}H_{37}N_4SNaO_{12}$, the value 694 ($M + NH_4^+$); for structure (c), $C_{28}H_{36}N_4O_{12}$, the value 637 ($M - H + NH_4^+$). One raises the question if chemical glycosylation is adequated enough for thiamine glycosylation and the answer is yes providing that protecting groups are removed enzymatically by an esterase [26] and rotavapor concentration is replaced by freeze-drying.

Concerning the configuration of the two galactofuranosides synthesized in this paper, the following arguments can be invoked at this stage of the work. It is well known that the configuration of synthesized glycoside can be influenced by the promotor of the reaction. In this sense, the use of zink chloride leads to glycosides having α configuration while p-toluenesulfonic acid determines the formation of β-configuration [27, 28]. Synthesis of the two galactofuranosides by using p-toluenesulfonic acid as promoter led to products chromatographically indistinguishable from glycosides described above (D. P. Iga, unpublished results). Of this reason, it can be envisioned that the galactofuranosides of D,L-α-tocopherol and thiamine synthesized in this paper both have β configuration.

On the other hand, a number of hydroxysteroids have been galactofuranosylated, by using cadmium carbonate as promotor, as being representative in terms of type of hybridization of carbon bearing hydroxyl group, position on cyclopentenoperhydrophenantrene nucleus and, implicitly, the degree of shielding: cholesterol, estrone, androstanolone, 11-α-hydroxyprogesterone, prednisolone

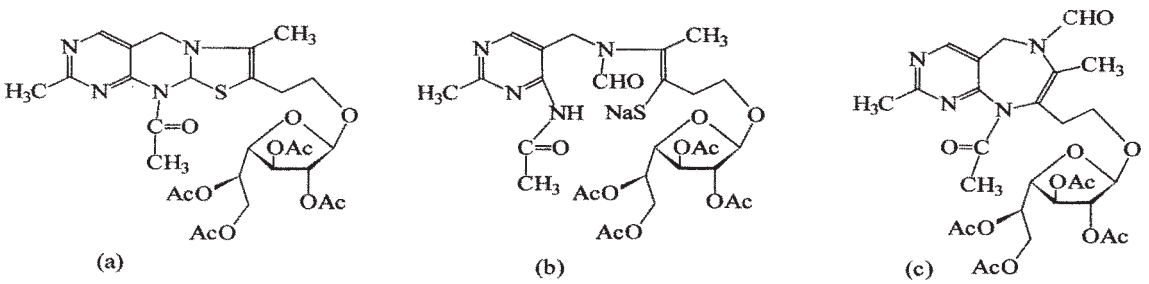

Fig. 7. Degradation products obtained by exposing thiamine to alkaline environment and/or heating

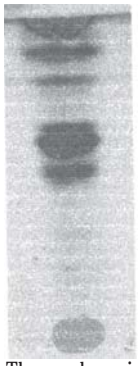

Fig. 8. Three glycosides were produced, separated and characterized by cholesterol glycosylation [29]. Migration, SS 2, chloroform-methanol-water, 50/10/1, v/v. Visualization, mostain

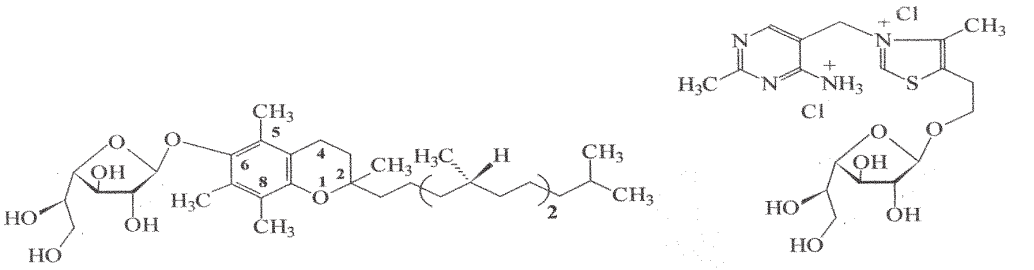

Fig. 9. Structure of synthesized galactosides of D,L-α-tocopherol and thiamine

[10]. Glycosylation of cholesterol produced cholesteryl β-D-galactofuranoside and -α-D-galactofuranoside in the ratio 10:1 [10, 13, 29]. The major product of galactofuranosylation of the other hydroxysteroids was also the corresponding β-D-galactofuranoside [10]. Two furanosylated glycosphingolipids – β-D-galactofuranosyl-6- and β-D-galactofuranosyl-3-β-D-galactopyranosyl-1'ceramide – have been synthesized by using galactocerebroside and sulfatide, respectively, as precursors. In all these reactions, both anomers were produced, the β anomer being the major one. Taking into consideration that both silicagel used for column chromatography and TLC can separate the diastereoisomers [29] and the fact that glycosylation products of both thiamine (fig. 2) and D,L-α-tocopherol (fig. 4) are homogenous in comparison with galactofuranosylation products of cholesterol (fig. 8), one can suppose that the reaction products are D,L-α-tocopheryl- and thiamine β-D-galactofuranoside, respectively (fig. 9).

A simple survey run on vitamins glycosides discloses the fact that chemical glycosylation of vitamins constitutes a challenging task. Majority of papers dealing with vitamin glycosides preparation used enzymes, especially the transferasic activity of hydrolases.

2-O-α-D-Glucopyranosyl-L-ascorbic acid was synthesized by the action of the cyclomaltodextrin glucanotransferase from *Bacillus stearothermophilus* [30]. Although the exact location of vitamin E-metabolism has not been determined yet, it is known at molecular level: the lipid-soluble vitamin E is degraded to water-soluble carboxyethyl hydroxychroman metabolites by side-chain degradation without modification of the chromanol head. Carboxyethyl hydroxychroman metabolites are conjugated with glucuronic acid to increase their solubility and excreted in the urine [31]. A novel derivative of vitamin E, 2-(α-D-glucopyranosyl) methyl-2,5,7,8-tetramethyl-chroman-6-ol, was synthesized from 2-hydroxymethyl-2,5,7,8-tetramethylchroman-6-ol and maltose by transglycosylation with α-glucosidase from *Saccharomyces species*. The glycosylated product has relatively high solubility in water and its radical scavenging activity, measured with 1,1-diphenyl-2-picrylhydrazyl, was found to be nearly the same as those of α-tocopherol, 2-carboxy-2,5,7,8-tetramethylchroman-6-ol and ascorbic acid [32]. The following vitamins have been found as glycosides in natural materials: pyridoxine, vitamin D, niacin, pantothenate, and riboflavin. Glycosylated forms of pyridoxine varies between 5% and 75% of the total vitamin B_6 content in fruits, vegetables and grains. The main glycosylated form of pyridoxine in most plant-derived foods is pyridoxine 5'-β-D-glucoside [8]. Ascorbic acid glucoside was prepared by incubating the aglycone with maltose or oligosaccharides and an enzyme produced by genera *Aspergillus* or *Penicillium* [33]. Riboflavin glucoside was produced by cultivating a microorganism belonging to the genus *Bacillus* in a media containig the vitamin and starch [34]. Three glycosides of thiamin were prepared by using transferasic activities of the corresponding glycosidase: O-β-galactoside, O- α-glucoside, O-β-*N*-acetylglucosaminide [2, 35].

Conclusions

Galactofuranosylation of D,L-α-tocopherol by using cadmium carbonate as promotor produced almost exclussively D,L-α-tocopheryl-β-D-galactofuranoside; the corresponding diastereoismer -α-D-galactofuranoside appeared at most in traces.

Galactofuranosylation of thiamine in the presence of cadmium carbonate and silver salicylate at room temperature led to thiamine-β-D-galactofuranoside. However, the product was altered by exposing to alkaline environment and heat.

Combination of chemical and chromatographical methods proved a valuable alternative for separation and characterization of the synthesized furanosides.

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Manuscript received 19.09.2009