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Synthesis, Enzymatic Cleavage and Chemical Sulfation of Cholesteryl-ß-D-glucopyranoside

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Abstract

D-Glucose was peracetylated by means of reaction with a mixture of acetic anhydride and pyridine. Penta-O-acetyl-ß-D-glucopyranose was brominated with hydrobromic acid in glacial acetic acid. 1-Bromo-1-deoxy-tetra-O-acetyl-α-D-glucopyranoside produced in this way was used as glycosylating donor for cholesterol in a Koenigs-Knorr reaction, chemical condensing agent being either cadmium carbonate or basic zinc carbonate. The reaction mixture, containing as the main product tetra-O-acetyl-ß-D-glucopyranosyl-cholesterol, was submitted to Zemplen saponification and cholesteryl-ß-D-glucopyranoside was purified by column chromatography on silica gel and characterized by chemical means. Enzymatic cleavage of the synthetic glycoside was accomplished by incubating with a crude extract of Helix pomatia and taurocholic acid. Alternatively, chemical sulfation was made by using chlorosulfonic acid in pyridine, the main products being a mono- and a bis-sulfate of cholesteryl-ß-D-glucopyranoside.

Keywords: cholesteryl ß-D-glucopyranoside, sulfate ester, bis-sulfate ester, ß-glucosidase, acetobromoglucose

Introduction

Cholesteryl ß-D-glucopyranoside is a natural substrate of steryl-ß-glucosidase (EC 3.2.1.104); besides, it acts on glucoside of sitosterol but not on some related sterols such as coprostanol [1].

Cholesteryl ß-D-glucopyranoside was probably amongs the first steroid glycoside prepared by organic synthesis [2] [3]. However, its biological functions were disclosed 80 years later [4] [5] [6] [7]. Steryl glucoside and its 6'-*O*-acyl derivatives are common constituents of higher plants, fungi and bacteria. An esterified form of steryl glucoside was found in potato tuber lipids and soybean phosphatides. It contains sterol, glucose and fatty acid in the molar ratio 1 : 1 : 1. Four types of sterols were identified, among them ß-sitosterol and stigmasterol, while the fatty acids present are palmitic, stearic, oleic, linoleic and linolenic acids, the latter compounds being located on C-6 of the monosaccharide [8]. Cholesteryl-ß-D-glucopyranoside is one of the two major glycolipids of *Mycoplasma gallinarum* strain J [9] [10]. Biosynthesis of steryl glucoside has been studied in immature soybeans seeds [11] [12] The sequential transfer of glucose and rhamnose to quercetin, forming the flavanol glycoside rutin, has been demonstrated in leaves of Phaseolus [13]. The biosynthesis of cholesteryl glucoside by *Mycoplasma gallinarum* strain J takes place by the transfer of glucose from uridine-5'-diphosphoglucose to membrane-bound sterol [14]. It was demonstrated by feeding D-[^{14}C]glucose at *Borrelia hermsi*, that labeled glucose was incorporated into cholesteryl

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glucoside and acylated cholesteryl glucoside [15]. A mixture containg predominantly the ß-anomer of diosgenin glucoside and a small amount of yamogenin glucoside was synthesized and its effects on cholesterol homeostasis in monkeys (*Macaca fascicularis*) were tested. The respective mixture, containing mainly diosgenin glucoside, reduced cholesterolemia, decreased intestinal absorption of exogenous cholesterol and increased secretion of endogenous cholesterol. It was demonstrated that the glucoside was well tolerated when long-term studies were undertaken, no toxic signs being observed. An interesting conclusion has been drawn: diosgenin glucoside and other synthetic glycosides with similar activities could be used in the management of hypercholesterolemia and atherosclerosis [16]. Three kinds of glycolipids, accounting for about 25% of the total lipid, were identified in a lipidic extract from *Helicobacter pylori*, their structures being cholesteryl-α-D-glucopyranoside, cholesteryl-6-*O*-tetradecanoyl-α-D-glucopyranoside and cholesteryl-6-O-phosphatidyl-α-D-glucopyranoside [17]. Beta-glucosidase is an important microbial taxonomic marker, an excellent substrate being cyclohexenoesculetin-ß-D-glucoside [18]. Two glycosides, sitosteryl-D-glucoside and stigmasteryl-D-glucoside, were isolated from the fresh fruit of *Annona glabra* and characterized by physical and spectral evidence [19].

In the present paper, cholesteryl-ß-D-glucopyranoside has been synthesized by ,eans of a Koenigs-Knorr reaction of 1-bromo-1-deoxy-tetra-O-acetyl-glucopyranoside in the presence of either cadmium carbonate or basic zinc carbonate, as promoters, followed by mild alkaline hydrolysis and column chromatography. Orthoester formation was ruled out by enzymatic cleavage of reaction product by a crude ß-galactosidase from snail *Helix pomatia*. Alternatively, cholesteryl-ß-D-glucopyranoside has been sulfated with chlorosulfonic acid in pyridine, a monosulfate and a bis-sulfate being obtained.

Materials and Methods

Materials. Cholesterol, glucose, acetic anhydride, pyridine, chlorosulfonic acid, silicagel for column chromatography, precoated thin layer plates, florisil, were either from Merck or from Fluka.

Methods. 1. **Penta-O-acetyl-ß-D-glucopyranoside** was synthesized as indicated LEMIEUX [20]. In a 0.25-L, 3-necked flask equipped with a magnetic stirrer and a thermometer, 40 mL of acetic anhydride was cooled in an ice and water mixture, and 0,24 mL of 70 % perchloric acid was added dropwise. The solution was then warmed to room temperature, and 10 g of anhydrous D-glucose was added to the stirred mixture at such a rate to keep the reaction temperature between 30 and 40 ^{0}C. At the end of the reaction, ice and chloroform were added in the reaction mixture, organic layer was separated and washed three times successively with small volumes of saturated sodium bicarbonate solution and then with water. Organic layer was then dried on magnesium sulfate, filtered and concentrated to dryness by rotavapor. The reaction product, penta-O-acetyl-ß-D-glucopyranoside, was purified by crystallysation from ethanol.

2. **1-Bromo-1-deoxi-2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside** was synthesized from 2.9 g penta-O-acetyl-ß-D-glucopyranoside and hydrobromic acid according to LEMIEUX [20] : a sample of 32-33 % solution of hydrobromic acid in dry acetic acid was placed in a flask protected against humidity and cooled on ice. Then penta-O-acetyl-ß-D-glucopyranoside and 1,2-dichloroethane were added and the reaction was run on ice and subsequently at room temperature. Bromoderivative was extracted with cold chloroform, washed with sodium bicarbonate solution and water and then dried on magnesium sulfate. The drying agent was removed by filtration and the solution was concentrated to dryness and thoroughly dried in vacuum on phosphorous pentoxide.

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3. **Glycosylation of cholesterol** with 1-bromo-1-deoxi-2,3,4,6-tetra-O-acetyl-α-D-gluco-pyranoside was made in dry toluene in the presence of calcium sulfate and a promoter that was either cadmium carbonate or basic zinc carbonate. The reaction went on for at least 7 hrs by refluxing the mixture.

4. **Cholesteryl-ß-D-glucopyranoside**. Reaction mixture was diluted with chloroform, stirred and filtered. The solution was evaporated to dryness and the residue was resumed in methanol and mixed with sodium methoxide (Zemplen reaction or mild alkaline hydrolysis). Reaction was monitored by thin layer chromatography; it was usually complete in about 8 hrs at room temperature, so it was let to proceed overnight by stirring.

5. **Column chromatography of cholesteryl-ß-D-glucopyranoside**. When mild alkaline hydrolysis was complete, neutralization with methanolic hydrochloric acid followed, the salts were removed by partition and the organic layer was concentrated and the residue submitted to column chromatography on silica gel in a gradient of methanol in chloroform. The advance of separation was followed by thin layer chromatography and the adequate fractions were mixed and the solvent removed.

6. **Enzymic hydrolysis of cholesteryl-ß-D-glucopyranoside**. A uniform emulsion of cholesteryl-ß-D-glucopyranoside was prepared as follows: the substrate was solved in chloroform-methanol and added to a solution consisting of buffer and sodium taurocholate [21]. Organic solvents were removed by heating on a boiling water bath for 5 min, the uniform emulsion was then cooled to room temperature and mixed with a crude extract of snail (*Helix pomatia*). The progression of the reaction was followed by thin layer chromatography.

7. **Chemical sulfation of cholesteryl-ß-D-glucopyranoside**. Cholesteryl-ß-D-glucopyranoside was solved in pyridine and a stoichiometric amount of chlorosulfonic acid was added [22]. The progression of the reaction was followed by thin layer chromatography.

Results and Discussion

In general, it is difficult to follow the advance of glycosylation reaction chromatographically, glycosylation mixtures being extremely complex. It is much easier instead to detect glycosylation products after mild alkaline hydrolysis. This reaction determines chemical transformations of the reagents and products, so that it is easy to distinguish chromatographically between them: unreacted 1-bromo-derivative is transformed in D-glucose that is easily removed by partition, and the reaction product becomes cholesteryl-ß-D-glucopyranoside, the latter product being separated from unreacted cholesterol. The progression of Zemplen reaction is presented in Figure 1; formation of the main product is quite evident. A comparison between the qualities as promoter of cadmium carbonate and basic zinc carbonate is presented in Figure 2: reaction product in the case of cadmium carbonate is more homogenous.

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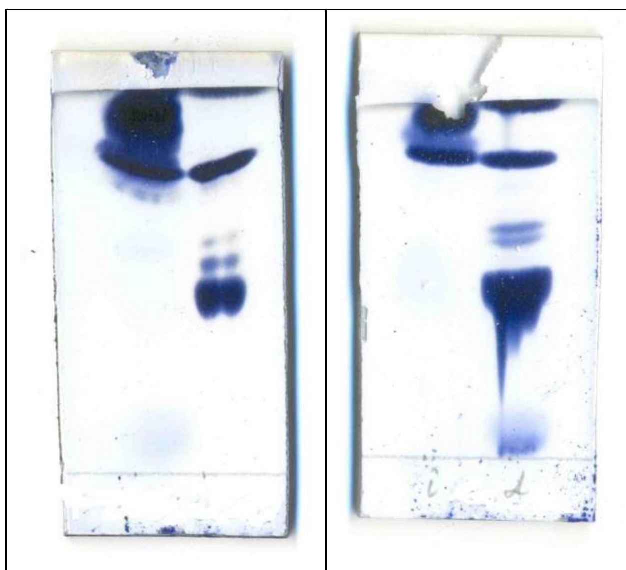

Figure 1. The advance of mild alkaline hydrolysis of glycosylation product. The compound having R_F 0.45 is cholesteryl-ß-D-glucopyranoside. On both plates: left, before alkaline hydrolysis; right, at 5 min (plate one) or 15 min (plate two) after starting of alkaline hydrolysis. In both cases, promoter was basic zinc carbonate. Migration, chloroform-metanol-water, 10/5/1; visualysation, mostain.

Purification of cholesteryl-ß-D-glucopyranoside by silica gel column chromatography allowed the removal of impurities and obtaining of a pure compound (Figure 3). Glycosylation of cholesterol by using 1-bromo-1-deoxi-2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside can lead to a real glycoside or to an orthoester of acetate (Fig 4). The two products can be distinguished at least by two means: by NMR spectra or enzymatically. Incubation of our product with a crude enzymic extract from snail (*Helix pomatia*) in the presence of taurocholic acid indicated its cleavage. In this way, the presence of acetate orthoester was ruled out. Sulfation of cholesteryl-ß-D-glucopyranoside with chlorosulfonic acid in pyridine produced two compounds: a minor one migrating very near to sulfo-ß-D-galactopyranoside-ceramide (sulfatide) and a major one having lower R_F.

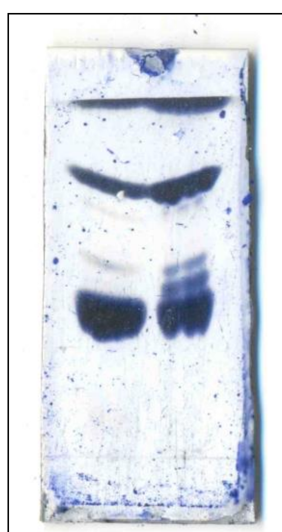

Figure 2. Comparison between reaction products of the two promoters: cadmium carbonate (left) and basic zinc carbonate (right) (see also Figure 1).

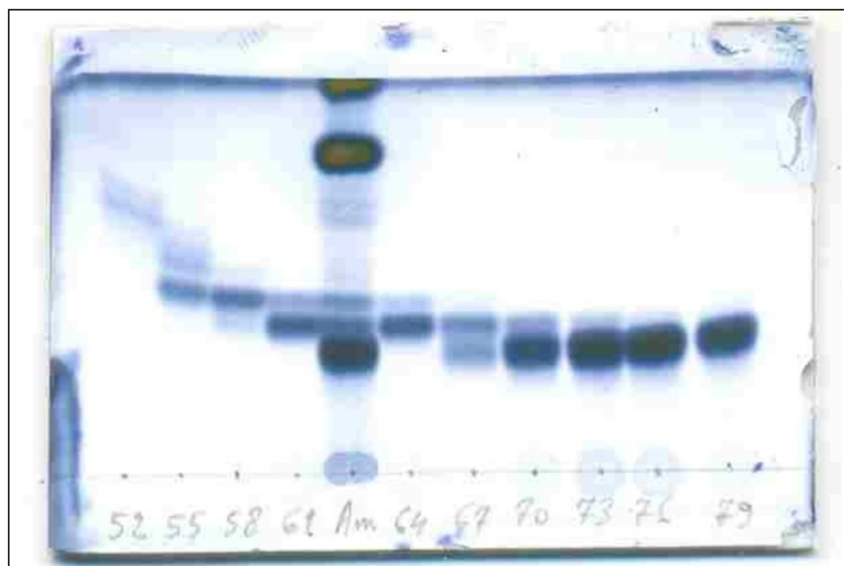

Figure 3. Purification of cholesteryl-ß-D-glucopyranoside by column chromatography (see also Figure 1).

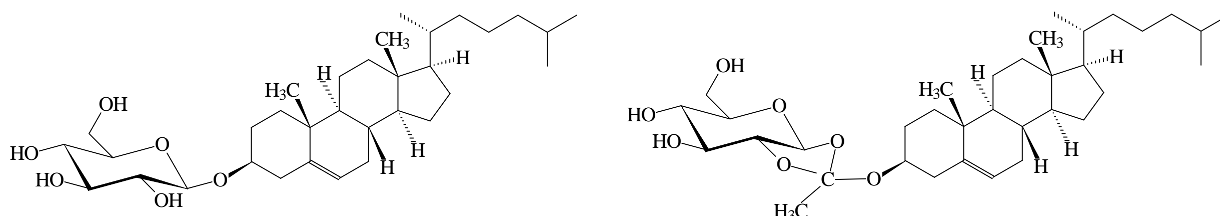

Figure 4. Cholesteryl-ß-D-glucopyranoside (left) and its orthoester (right) as possible products of Koenigs-Knorr reaction.

Elaboration by Koenigs and Knorr [23] of the synthesis christened by their name consisted in integration of chemical knowledge, appeared before them, indicated by the following reactions: (a) substitution of H atoms of all OH groups of a monosaccharide by acyl groups - peracylation - leads to the closure of the molecule as a pyranosic or furanosic ring, in case of aldohexoses, aldopentoses, 2-ketohexoses, and exclusively as furanosic ring in case of aldotetroses and 2-ketopentoses; (b) linkage of the substituted sugar to an aglicone - formation of glycosidic bond - needs the activation of C-1 atom. Koenigs and Knorr accomplished this by substitution of acylate group by Br or Cl; (c) formation of glycosidic bond produces a strong acid, HBr or HCl, that must be instantaneously consumed, because glycosidic bond is vulnerable to acidic medium. Koenigs and Knorr solved this aspect by adding a chemical promoter, Ag carbonate; in this way insoluble Ag salts of Br or Cl are formed; (d) water formed in reaction is also instantaneously eliminated by adding a dehydrant, anhydrous Ca sulfate. In fact, in all steps of the reaction, a dry medium is accomplished by using anhydrous organic solvents: glacial acetic acid, chloroform, 1,2-dicloretan, diclormethane, etc. In an important reaction, Koenigs and Knorr anticipated a strategy that would accomplish valuable things in Organic Chemistry, syntheses in aprotic solvents: N,N-dimethylformamide, dimethylsulfoxide, tetrahydrofuran, etc. In the next one hundred years that followed to the Koenigs-Knorr synthesis, tens of variants of this reaction were elaborated and the steps indicated by the two authors would become real principles. Moreover, almost all of the respective variants were christened Koenigs-Knorr reaction in the honour of the two chemists [24] [25] [26]. Preparation of 1-bromo-tetraacyl-α-D-glucopyranoside was constantly improved [20] [27]. Alkylglucopyranosides, in the range C_6-

C_{12}, were synthesized from the corresponding alcohol and bromoacetoglucopyranoside [28]. Apigenin 7,4'-di-O-ß-D-glucopyranoside, a component of blue pigment protodelphin, was synthesized from naringenin by two successive Koenigs-Knorr glycosylation with 1-halogeno-tetraacetylglucopyranoside [29]. Preparation of cyclohexenoesculetin-ß-glucoside was accomplished by a modified Koenigs-Knorr reaction: 3,4-cyclohexenoesculetin was dissolved in potassium hydroxide and to this was added an equimolar proportion of α-acetobromoglucose dissolved in acetone [18]. C_7–C_{16}-alkyl D-glucopyranosides, α and ß, were prepared by reaction of the respective alkanol and penta-acetylglucopyranoside by using tin chloride(IV) as promoter [30]. Cadmium carbonate proved to be a useful promoter in the Koenigs-Knorr synthesis of 2-(4-methoxybenzyl)cyclohexyl-β-D-glycopyranosides [31]. Naturally occurring glucosides of benzyl alcohol, (±)-menthol, (+)-borneol, thymol, carvacrol and eugenol were synthesized by the Koenigs-Knorr-Zemplén method [32]. Recently [33] two ß-glucopyranosides were synthesized, brasoside and littoralisone, as well as some steryl-galactosides [34]. Numerous natural biologically-active compounds, especially vitamins, were found in nature as glucopyranosides [35]. 1-O-Cholesteryl-ß-D-glucopyranoside promises to be a protection against gastric ulcer due to its capacities to activate heat shock factor and to induce heat shock proteins, the latter possessing cytoprotective effect [7].

Conclusions

1.Glycosylation of cholesterol with 1-bromo-1-deoxy-2,3,4,6-tetra-O-acetyl-D-glucopyranose produced, after mild alkaline hydrolysis, cholesteryl-ß-D-glucopyranoside.
2.Cholesteryl-ß-D-glucopyranoside was cleaved by a ß-galactosidase from snail (*Helix pomatia*).
3.Chemical sulfation of cholesteryl-ß-D-glucopyranoside produced two sulfate esters, a monosulfate and a bis-sulfate.

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