Novel Mechanism for Oxidative Cleavage of Glycosidic Bonds: Evidence for an Oxygen Dependent Reaction

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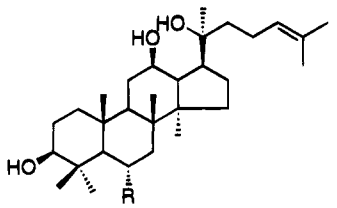
In a previous work from our laboratory, an optimized procedure was worked out for cleavage of the glucosidic bonds in ginsenosides (Cui, J. F.; Garle, M.; Lund, E.; Björkhem, I.; Eneroth, P. *Anal. Biochem.* **1993**, *210*, 411-417). When the reaction was performed in *n*-butanol, alkaline conditions were found to give a considerably better and almost quantitative yield of *intact* aglyconic-specific products than did acidic conditions. This is surprising in view of the current concept that glucosidic bonds are more stable under alkaline than acidic conditions. It is shown here that the alkaline cleavage is *oxygen dependent* and that there is little or no conversion when oxygen or air is replaced with nitrogen. Addition of an anti-oxidant, glucose or water also reduces the degree of cleavage under the conditions employed. Replacement of *n*-butanol for *sec*-, iso- or 2-methyl-2-propanol, decreased the yield of products to about 75% and when *n*- or isopropanol was used as solvent the yield decreased to about 40%. It was shown that the glucose moiety was completely degraded under the conditions employed and that formate and carbonate, in a ratio of 5/1, were the major products. A mechanistic rationale for the oxygen dependent cleavage of the glucosidic bonds is suggested. The possibility that this mechanism may be of protective importance in biological systems under some specific conditions is also discussed.

Introduction

Methods including lead tetraacetate oxidation, anodic oxidation, UV-irradiation, acid- and soil bacterial hydrolysis for cleavage of glycoside linkages in oligoglycosides such as saponins, have been developed during the last decades.[2] The yields are generally poor, and many of these methods often causes secondary changes of the aglycon and results in the formation of artifact aglycons.

In recent work from our laboratory, we reported that treatment of various ginsenosides with alkali in butanol under some specific conditions gives a very high yield of the two sapogenins (S)-20-protopanaxadiol (**1**) and (S)-20-protopanaxatriol (**2**).[1] In contrast, acid hydrolysis



1: R = H
2: R = OH

gives 10 side products in addition to the above genuine sapogenins. The side products were formed from the genuine sapogenins by cyclization, epimerization and hydroxylation of the side chain.

Table 1. Effect of O_2, N_2, and Air on Cleavage of Ginsenosides Rg1, Rb1, Ro, (α)- and (β)-methyl- and 3β-O-Cholesteryl Glucoside[a]

		conversion (%) under		
entry	glucoside	O_2	N_2	air
1	Rg1[b,d] (**3**)	>95	2	82
2	Rb1[b,c] (**4**)	>95	3	82
3	Ro[b,c] (**5**)	>95	20	78
4	α-methyl glucoside	>99	0	85
5	β-methyl glucoside	>99	0	84
6	3β-O-cholesteryl glucoside	>99	0	

[a] Reactions were carried out using general procedure A or B (entries 1–3). [b] Analytical data (GC/MS) of the isolated aglyconic sapogenin is presented in ref 1. [c] Panaxadiol (**6**) is used as internal GC-standard. [d] Panaxatriol (**7**) is used as internal GC-standard.

The efficient cleavage of the glucosides, including ginsenosides, cholesteryl- and alkyl glucosides, under alkaline conditions is surprising in view of the general concept that glycosidic bonds are more sensitive to hydrolysis under acidic conditions than under alkaline conditions. In the present work it is shown that the previously demonstrated alkaline cleavage of ginsenosides in butanol is *oxygen* dependent. Possible mechanisms for this is discussed.

Results

When the ginsenosides Rg1 (**3**), Rb1 (**4**), Ro (**5**), α- or β-methyl glucoside and 3β-O-cholesteryl glucoside were treated with NaOH or NaOMe in *n*-butanol for 15–24 h at 90 °C in a closed system under standard conditions, little or no conversion was observed when the air was replaced with nitrogen (Table 1). When replacing the air with oxygen, the degree of conversion was dramatically increased. We have previously shown that the degree of cleavage of ginsenoside Rg1 (**3**) into (S)-20-protopanaxatriol (**2**) under the above standard conditions in air is about 80%. When replacing the air with oxygen the degree of cleavage further increased to > 95%.

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(1) Cui, J. F.; Garle, M.; Lund, E.; Björkhem, I.; Eneroth, P. *Anal. Biochem.* **1993**, *210*, 411–417.
(2) Kiatagawa, I.; Kamigauchi, T.; Ohmori, H. and Yoshikawa, M. *Chem. Pharm. Bull.* **1980**, *28*, 3078–3086, and references cited therein.

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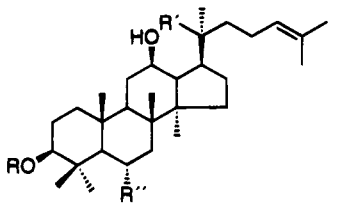
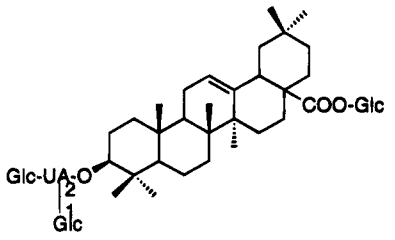


3: R = H; R′ = R″ = β-D-Glc-O;
4: R = D-Glc(β1-2)D-Glc; R′ = D-Glc(β1-6)D-Glc; R″ = H
Glc = glucopyranosyl



5: Glc = β-D-glucopyranosyl; GlcUA=β-D-glucoronic acid

The degree of cleavage of ginsenoside Rg1 (3) at 90 °C and under oxygen atmosphere, is shown as a function of time (Figure 1). The reaction was found to be completed, and all of the ginsenoside consumed, after about 13 h. The corresponding sapogenin (2) was formed quantitatively. When the NaOH was replaced with NaOMe, the reaction was faster and completed already after 5 h. In a separate study it was shown that it was not possible to further speed up the reaction rate by adding more of the sodium- hydroxide or methoxide. In fact 50 mg (approx 1000 equiv with respect to Rg1) of NaOMe gave essentially the same result as 200 mg under the conditions employed (Figure 2). *No conversion for any of the glucosides was found in the absence of alkali.*

When the cleavage of ginsenosides Rg1 (3), Rb1 (4) and Ro (5) was carried out under oxygen, air or nitrogen at 90 °C for 5 h, the results suggest that the effect of NaOMe on the cleavage under the three different gases was the same as that with NaOH (Table 1). The temperature has a significant effect on the conversion. At room temperature and under air atmosphere, the degree of cleavage was only about 50% after 60 days.

Addition of water, 0.7%, to the reaction mixture in the general procedures, decreases the degree of conversion by more than 40%, and when the butanol phase is saturated with water the conversion is only 6%. The effect of different solvents on the conversion of ginsenoside Rg1 (3) is shown in Table 2. Replacement of *n*-butanol for *sec*-, iso- or 2-methyl-2-propanol, decreases the degree of conversion with about 25%. When the reaction is performed in *n*- or isopropanol the conversion is decreased by more than 60%.

Since the reaction is oxygen dependent, addition of an anti-oxidant would be expected to reduce the rate of, or even inhibit the reaction. Addition of 0.9 mg of 4-*tert*-butylphenol to the reaction mixture of Rg1, under general conditions, was found to reduce the conversion by 25%. When 50 mg was added, there was no conversion at all. Likewise, the addition of 5 mg of D-(+)-glucose decreased the rate- and conversion of the reaction by more than 50%. Addition of 10 mg or more of D-(+)-glucose decreased the rate and conversion to about the same extent, probably due to a limited solubility.

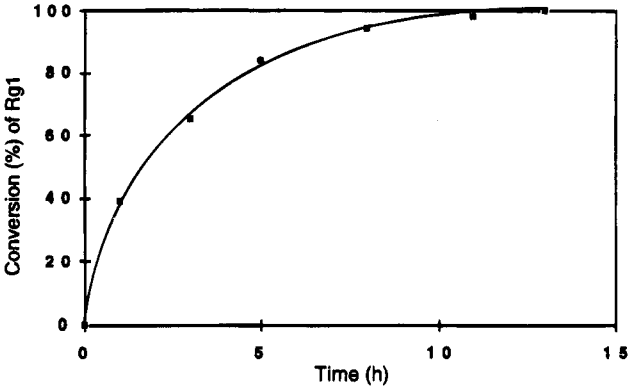


Figure 1. Conversion of ginsenoside Rg1, using standard procedure A under oxygen atmosphere.

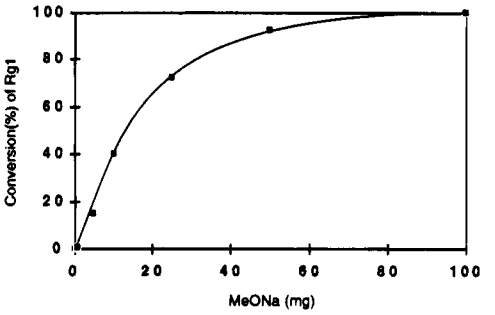


Figure 2. Effect of amount of added MeONa on cleavage of ginsenoside Rg1, using general procedure B under oxygen atmosphere.

Table 2. Effect of Different Solvents on Cleavage of Rg1[a]

solvent	conversion (%)
n-BuOH	100
s-BuOH	73
i-BuOH	74
t-BuOH	73
n-PrOH	38
i-PrOH	35

[a] All reactions were carried out using general procedure B.

It was observed that the oxidative cleavage of the ginsenosides always was associated with development of a yellow color in the butanol phase that did not occur under nitrogen atmosphere. A similar but more pale yellow color appeared when D-glucose or the methyl- or cholesteryl glucosides were subjected to the same treatment as the ginsenosides in the presence of oxygen. Isolation of the oxidized products of the glucose in the crude butanol phase after the oxidative cleavage of the ginsenosides were performed. Only *traces* of glucose and other hexoses could be identified in the reaction mixture after the cleavage, but in quantities *considerably* less than expected if unchanged glucose had been the only cleavage product. However, in all reactions, formate was found in large quantity together with carbonate. When α- or β-methyl glucoside or 3β-O-cholesteryl glucoside were subjected to the oxidation procedures, generally no glucose could be recovered. Again, formate (5 equiv) was formed together with carbonate (1 equiv), as the only remaining components of the carbohydrate, indicating an extensive oxidation. It was observed that when the amount of added base (NaOH or NaOMe) was < 6 equiv with respect to the glucoside, the *initial* rate of consumption of oxygen was essentially the same as that when a

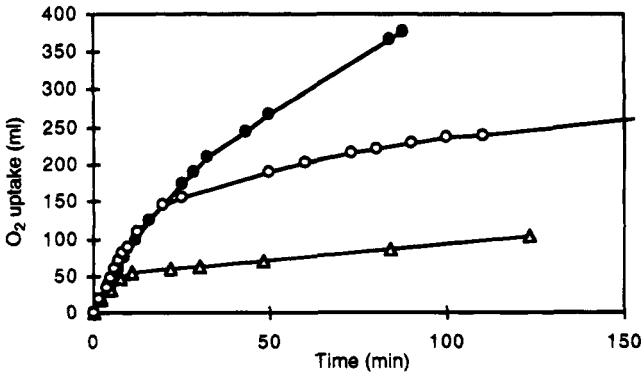


Figure 3. Effect of 4 equiv (△), 5 equiv (○), and 21 equiv (●) of added base (NaOH) on oxygen uptake for methyl glucoside.

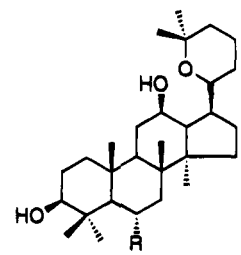
large amount (>20 equiv) of base was used. When 4 and 5 equiv of NaOH was used, the consumption of oxygen ceased after 20 and 50 min respectively (Figure 3). After 24 h, formate and carbonate were the only components which were isolated from the reaction mixture together with unreacted glucoside, indicating an incomplete oxidative cleavage of the glucoside. When the amount of base was increased to 20 equiv or more, the rate of consumption of oxygen was further increased, and the uptake of oxygen was now continued until all of the glucoside was cleaved and the carbohydrate quantitatively converted to formate and carbonate.

Minor amounts of a side product in the butanol phase after the oxidative cleavage of the glucosides was identified by mass spectrometry as *n*-butanal and condensation products of this compound. Such oxidized products could not be formed, however, when the reaction was performed in 2-methyl-2-propanol.

Discussion

Alkaline cleavage of ginsenosides has been reported in three previous publications,[1,3,4] but the mechanism of the reaction has not previously been studied. Ogihara et al.[3] used sodium or potassium in alcohols containing a small amount of water and assumed that a reduction step was involved in the reaction (cleavage of Saikosaponin C). We have here clearly shown that the alkaline cleavage of the glucosidic bond requires oxygen. Thus there was little or no reaction when the reaction was performed under nitrogen atmosphere. Furthermore, addition of an anti-oxidant or D-glucose decreased the rate and the conversion of the reaction. The amount of anti-oxidant required to completely inhibit the reaction of **3**, **4**, and **5**, was such that it was likely to consume most of the oxygen in the closed system used for the procedures A and B.

Our observation that alkaline conditions and elevated temperature are essential for the oxidative cleavage suggests that the mechanism may be a base catalysed or activated autoxidation. It is well known that when a strong enough base is added to a hydrocarbon with active hydrogens the carbanion formed will be autoxized by oxygen or air.[5] It is tempting to assume that the anomeric carbon C1 of the carbohydrate would be autoxidized in the same manner. This seems unlikely however since the pKa for the C1- proton must be much higher than that of the hydroxyls of the glucoside. Under oxygen atmosphere at 90 °C in n-BuOH and without added base, the glucosides were recovered uncleaved even after 24 h. However, as soon as NaOH or NaOMe was added to the reaction mixture, oxygen was consumed and a white precipitate formed already after a few minutes. The observation that MeO^-, with a slightly higher pK_a, performs a faster reaction than does HO^-, suggests that the reaction is initiated by the formation of an alkoxy anion at C-6 of **8**, Scheme 1. Oxygen then abstracts an α-proton to form an intermediate aldehyde which is further oxidized to the carboxylate **9**. Decarboxylation of **9** gives CO_2, which is trapped under the alkaline conditions as carbonate, and the intermediate anion from **9**, reacts with oxygen to give the peroxy anion **10**. The peroxide decomposes to the dialdehyde **11** and the aglyconic ester **12**, which is hydrolyzed to the free aglycon and formate. Under the conditions, **11** is further degraded by HOO^-, either sequentially via **14**, or directly to **15**, which results in the formation of another two equivalents of formate. Finally, **16** rearranges in a Dakin or Bayer-Villiger type oxidation to **17** which in turn will form another two equivalents of formate. Thus, a total of five equivalents of formate are formed.



6: R = H
7: R = OH

An alternative but thermodynamically less favorable route is the reaction of an anion of the solvent with oxygen to form an alkoxy radical (BuO•) and a superoxide anion radical (-O-O•). The butoxy radical then abstracts the hydrogen at C1, which in turn reacts with oxygen in a radical chain reaction. A similar mechanism for α-tocopherol has been suggested by Sawyer.[6]

The oxidative degradation of the glucose moiety of the glucosides was extensive under the conditions employed. Oxidation of an equimolar amount of glucose or methyl glucoside under the standard procedures resulted in an almost complete conversion to formate and carbonate (5/1). We found that the oxidative cleavage was inhibited by water. In contrast, Isbell isolated lower homologes of the parent carbohydrate from the degradation of carbohydrates by oxygen in alkaline *aqueous* solutions.[7] Under the conditions employed in our investigation, C-3 to C-6 compounds would have been detected by the analytical system used.

Since the *n*-butanol used as solvent in the general procedures was oxidized itself to yield *n*-butanal, the possibility was considered that the oxidation of the glucosides was mediated by an oxidized product such as a peracid of the solvent. 2-Methyl-2-propanol cannot be oxidized to an aldehyde, however, and since 2-methyl-2-

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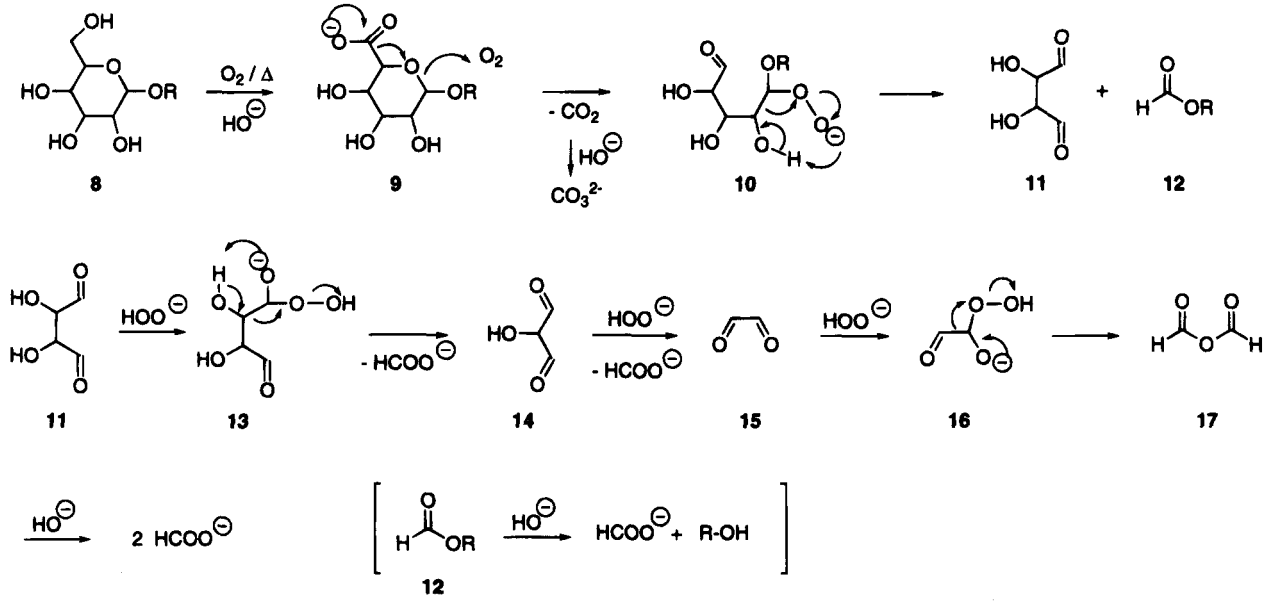
(7) Isbell, H. S. *Carbohydrate Research*, **1976**, *49*, C1-C4.

Scheme 1



propanol also can be used as a solvent in the oxygen dependent cleavage of the glucoside, it can be concluded that oxidation of the solvent can not be an obligatory step in the reaction. Furthermore, butyric acid or remaining peroxides were not found in any of the reactions. The fact that *n*- and isopropanol is so much worse a solvent than *n*-butanol (Table 2), may be explained by a difference in solubility of intermediates formed in the reaction.

Despite the omnipresence of one-electron processes in nature, free-radical damage presents a serious constant threat to living organisms. One available source of radicals in the body is the superoxide anion radical, which is formed in a large number of reactions of biological importance in both enzymatic and non enzymatic processes.[8] It is evident that the type of oxidative cleavage of glucosidic bonds studied here can not be of importance in solutions in biological systems. In biomembranes, however, the situation may be different. It can thus not be excluded that glucosides may act as anti-oxidants and protect against oxidative attacks under certain conditions. It was recently reported that ginsenosides have anti-oxidant properties in some specific systems.[9]

The present type of oxidative method appears potentially useful for the cleavage of glucosidic compounds when the recovery of an intact agluconic moiety is essential. This concept is being studied further in our laboratories.

Experimental Section

General. GC was performed on a HP 5890A gas chromatograph, equipped with a FID and a DB-5 column (length 15 m). Column (He) flow rate was 2.0 mL/min. The peak areas were measured with an HP 3396A integrator. GC/MS was performed on a Hewlett-Packard 5995C GC/MS. ^{13}C NMR spectra were obtained on a Bruker AM-400 instrument at 100.6 MHz. They were recorded in D_2O containing MeOH as internal standard, and are reported in δ-units.

Materials. *n*-Butanol, *sec*-butanol, isobutanol, 2-methyl-2-propanol, *n*-propanol, isopropanol, 4-*tert*-butylphenol, D-(+)-glucose, NaOH and NaOMe were of analytical reagent grade (Merck, Darmstadt, Germany). Ginsenoside Rg1 (**3**), Ro (**5**), panaxadiol (**6**), panaxatriol (**7**) and oleanolic acid were obtained from the Chinese National Institute for the Control of Pharmacological and Biological Products (Beijing, China). Ginsenoside Rb1 (**4**) was obtained from Sigma (St. Louis, MO), N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) from Pierce (Rockford, IL), trimethylchlorosilane (TMCS) from Fluka (Buchs, Switzerland) and trimethylsilylimidazol (TMSI) from Machery-Nagel (Duren, Germany). 3β-*O*-cholesteryl glucoside was prepared according to Elyakov.[10]

Alkaline Cleavage of Ginsenosides 3, 4, and 5 under Oxygen, Nitrogen or Air. General Procedure A. To 0.2 g of NaOH dissolved in 3 mL of *n*-butanol in a 10 mL test tube was added ginsenoside, 100 μg, and panaxadiol (**6**) or panaxatriol (**7**), 20 μg, as internal standard. Oxygen was bubbled through the solution for 5 min and the tube was sealed with a PTFE-lined cap and warmed at 90 °C for 15 h. The reaction mixture was then transferred to a 5 mL centrifuge tube, washed with water (1.2 mL and 2 × 0.7 mL), vortexed and centrifuged for 2 min. A 100 μL aliquot of the organic phase was then evaporated to dryness at 70 °C under a stream of nitrogen and submitted to GC/FID or GC/MS analysis after TMS-derivatization. Analytical data, see reference 1.

Alkaline Cleavage of Ginsenosides 3, 4, and 5 under Oxygen, Nitrogen or Air. General Procedure B. The procedure is identical to that described in procedure A, except that the NaOH was replaced with NaOMe (100 mg) and a reaction time of only 5 h, since this was found to give the same degree of conversion.

Derivatization of the Cleavage Products. Trimethyl silylation was performed by adding 100 μL of a reagent mixture containing BSTFA:TMSI:TMSCl (3/3/2, v/v/v) to a tube containing the cleavage products. The tube was sealed with a PTFE-lined cap and warmed at 70 °C for 20 min. The solution was then analyzed by GC or GC/MS.

Alkaline Cleavage of Methyl Glucoside. To a stirred solution of 445 mg of NaOH (11.12 mmol, 21 equiv) in 10 mL of *n*-butanol under nitrogen at 90 °C in a 25 mL round flask, was added 102 mg of α- or β-methyl glucoside (0.5258 mmol). No reaction was observed as long as the reaction mixture was kept under nitrogen. The nitrogen was replaced by oxygen, and the reaction mixture was stirred at 90 °C for 18 h. (When the nitrogen was replaced by oxygen, a white precipitate was formed already after a few minutes). The mixture was then allowed to cool to room temperature and centrifuged. The white precipitate was washed with cold *n*-butanol (2 × 5 mL), centrifuged and dried i vacuum to yield 235 mg of a white

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powder. ^{13}C-NMR (100.6 MHz, D2O): δ 171.1 (HCO2Na) and 168.2 (Na2CO3); The white precipitate was confirmed (13C-NMR) to be a mixture of sodium formate and sodium carbonate by comparing to an authentic sample of the two compounds. The formate was further verified by GC/MS. Further, HCl (20 mL, 2M) was added dropwise to 230 mg of the white powder resulting in the formation of CO2 gas (confirmed by IR and authentic sample of CO2 gas) (11.5 mL, 0.52 mmol, corresponding to 1 equiv). The combined butanol phases contained only traces of sodium formate with all of the methyl glucoside consumed (1H-NMR, 13C-NMR, and GC/MS).

Alkaline Cleavage of 3β-O-Cholesteryl Glucoside. The procedure is similar to that of the alkaline cleavage of the methyl glucoside except for the following changes: To a stirred solution of 540 mg of NaOH (13.5 mmol, 25.4 equiv) in 10 mL of *n*-butanol under nitrogen at 90 °C in a 25 mL round flask, was added 330 mg of 3β-O-Cholesteryl glucoside (0.6022 mmol). The reaction mixture was stirred at 90 °C for 24 h. The white precipitate which was formed in the reaction, was washed with cold *n*-butanol (2 × 5 mL), diethyl ether (5 × 5 mL), centrifuged and dried in vacuum to yield 268 mg of a white powder. ^{13}C-NMR (100.6 MHz, D_2O) : δ 171.1 (HCO_2-Na) and 168.2 (Na_2CO_3) which also was confirmed by comparing to an authentic sample of the two compounds. HCl (20 mL, 2M) was then added dropwise to 268 mg of the white powder resulting in the formation of CO_2 gas (13.5 mL, 0.6 mmol, corresponding to 1 equiv) (CO_2 was confirmed by IR). Evaporation of the combined organic phases gave a pale yellow solid which was extracted with diethyl ether (5 × 5 mL). Evaporation of the combined ether extracts gave the intact aglycon 3β-Cholesterol (227 mg, 0.58 mmol, 98%) as an off-white powder (^{1}H-NMR, ^{13}C-NMR, compared to an authentic sample of 3β-Cholesterol).

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