Hemolytic Properties of Synthetic Glycosides

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Abstract □ Cholesteryl α-L-rhamnopyranoside, tigogenyl α-L-rhamnopyranoside, tigogenyl β-D-fucopyranoside, smilagenyl β-D-fucopyranoside, cholesteryl β-maltoside, tigogenyl β-maltoside, and smilagenyl β-maltoside were synthesized and characterized. The rhamnosides and fucosides, as well as some other steroid monoglycosides, proved to be extremely insoluble in water. The concentration giving 50% hemolysis, H_{50}, was of the same order of magnitude for all synthetic glycosides. Ghost cells collected from blood hemolyzed by smilagenyl maltoside and tigogenyl maltoside had appreciable amounts of absorbed aglycones. All results are in accordance with previous investigations on the mechanism of saponin and sapogenin hemolysis.

Keyphrases □ Glycosides, various—synthesized, hemolytic activity evaluated □ Hemolytic activity—various glycosides evaluated

Saponins have a wide range of biological activity, some of which might be of medical value. Most saponins possess antibiotic properties (1), especially against fungi (2–4). Escin (5), the active principle of *Aesculus hippocastanum*, has antiexudative and anti-inflammatory activities. Various saponins are antiviral agents (6, 7), and tumor inhibitory saponins were isolated from *Acer negundo* (8, 9) and *Entada phaseolides* (10). However, therapeutic application of saponins is very limited. When taken orally, they are poorly absorbed from the intestine; when administered by injection, especially intravenously, they are very toxic and induce marked hemolysis.

BACKGROUND

Saponins in their original form are generally nonhemolytic (11, 12) and nonfungicidic (13), but these properties are attained after the glycosidic bonds are hydrolyzed by appropriate membrane glycosidase enzymes to liberate the active aglycone[1]. The presence of several glycosidases in extracts of human erythrocytes was described (14). The mechanism by which saponins induce hemolysis is the basis for the biological activity of various glycosides, *i.e.*, anthraglycosides (15) and phloridzin (16, 17). It can be assumed that biological activities other than hemolysis are also based on the same mechanism.

Since the nature of the glycosidases varies from one type of cell to another, it should be possible to synthesize nonhemolytic saponins whose glycosidic bonds will be hydrolyzed only by some target cell and thereby set free the active aglycones. The synthesis of such glycosides should obviously be guided by the glycosidases active in whole erythrocytes. However, slight hemolysis was obtained when erythrocytes were incubated with nitrophenyl glycosides, so the nature of whole erythrocyte glycosidases could not be determined.

The purposes of the present investigation were to synthesize various new glycosides of hemolytically active aglycones and to test their hemolytic activity.

RESULTS AND DISCUSSION

The glycosides were synthesized according to the Koenigs–Knorr method (18–20) by condensing the acetyl bromoglycosides with the sapogenins in the presence of silver oxide as described by Wulff *et al.* (21). Optimal yields were obtained when the final hydrolytic step, usually performed according to Zemplen and Pacsu (22), was modified. Instead of methanolysis with catalytic amounts of sodium methoxide under reflux, hydrolysis was performed with 0.25 *M* sodium methoxide at room temperature.

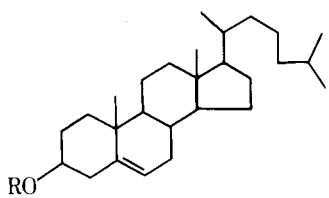
The following new glycosides were synthesized: cholesteryl α-L-rhamnopyranoside (I), tigogenyl α-L-rhamnopyranoside (II), tigogenyl β-D-fucopyranoside (III), smilagenyl β-D-fucopyranoside (IV), cholesteryl β-maltoside (V), tigogenyl β-maltoside (VI), and smilagenyl β-maltoside (VII). The constitution of the glycosides was assured by elemental analysis and TLC of the products obtained after acid hydrolysis. The stereochemical assignments of the glycosidic bonds were deduced from the synthetic methods applied (23).

Generally, β-glycosides are obtained from sugars belonging to the D-series, while L-sugars give rise to α-glycosides. The configuration was further confirmed from molecular rotation differences according to Klyne (24) who showed that the rotation contribution of the carbohydrate component, ΔC, in steroid glycosides is almost independent of the steroid component and is approximately equal to the molecular rotation, $[M]_D$, of the corresponding α- or β-methyl glycosides:

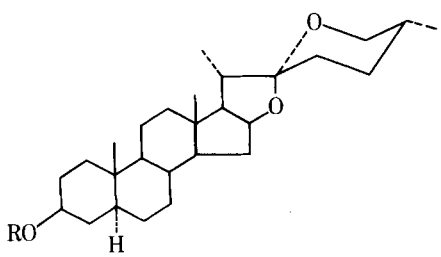
$$[M]_D = [\alpha]_D \times \text{mol. wt.}/100 \qquad \text{(Eq. 1)}$$

The value of ΔC is determined from:

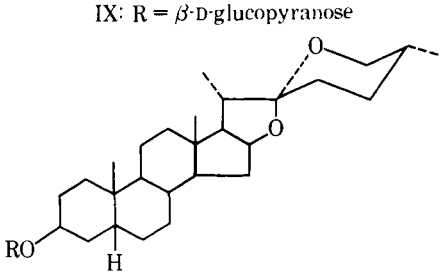
$$\Delta C = [M]_D \text{ of steroid glycoside} - [M]_D \text{ of free steroid} \qquad \text{(Eq. 2)}$$



I: R = α-L-rhamnopyranose
V: R = β-maltose
XII: R = β-D-glucopyranose



II: R = α-L-rhamnopyranose
III: R = β-D-fucopyranose
VI: R = β-maltose
VIII: R = H
IX: R = β-D-glucopyranose



IV: R = β-D-fucopyranose
VII: R = β-maltose
X: R = H
XI: R = β-D-glucopyranose

[1] Saponins possessing very high surface activities (*e.g.*, digitonin) may induce hemolysis by a parallel mechanism, probably similar to that of other surfactants.

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Table I—$[M]_D$ Values of the Synthesized Glycosides and the Corresponding Aglycones and Calculated ΔC Values and Literature $[M]$ Values for the α- and β-Methyl Glycosides

Compound	Proposed Configuration	$[M]_D$ Glycoside	$[M]_D$ Aglycone	ΔC	$[M]_D$ Methyl Glycopyranosides[a] α	β
I	α	−300	−153	−147	−111 (24)	+170 (24)
II	α	−568	−258	−310	−111 (24)	+170 (24)
III	β	−340	−258	−82	+332 (25)	—
IV	β	−310	−287	−23	+332 (25)	—
V	β	+120	−153	+273	—	+273 (26)
VI	β	−100	−258	+158	—	+273 (26)
VII	β	−40	−287	+247	—	+273 (26)

[a] Literature reference in parentheses.

Table I summarizes the ΔC values obtained for the new synthetic glycosides and the available $[M]_D$ values for the methyl glycosides. Considerable differences between some observed and literature data exist. This discrepancy may be attributed to the difference in the solvents used (24); the methyl glycosides were measured in water whereas the steroid glycosides were measured in chloroform or methanol. However, the ΔC values are all of the same sign and order of magnitude as the corresponding methyl glycosides.

The configurations of the glycosidic bonds could not be confirmed by either one of the two most reliable methods, NMR spectroscopy or degradation with specific glycosidases, because of the extremely low solubility of the glycosides in all organic solvents and in water.

The hemolytic activities of the synthetic glycosides, tested on bovine erythrocytes, are expressed by the H_{50} values, the concentrations inducing 50% hemolysis. These values were determined by plotting percentage hemolysis *versus* hemolysin concentration (27). Some sapogenin monoglycosides also were tested. All data are summarized in Table II.

Most tested monoglycosides proved to be extremely water insoluble. In previous investigations (27) on the hemolytic activity of various sapogenins and steroids, dimethyl sulfoxide–water was an excellent system for dissolving these compounds and for performing the hemolysis tests. However, contrary to expectation, this system was completely inadequate for dissolving monoglycosides. Higher solubility was attained with isopropyl alcohol, which was nonhemolytic[2] at a final concentration of 10%. This solvent system was adequate for dissolving smilagenyl glucoside and, to some extent, tigogenyl glucoside for which the activity was tested on the saturated solution without determining its concentration. However, neither dimethyl sulfoxide nor isopropyl alcohol could be used for dissolving the glycosides of the desoxy sugars, *i.e.*, the rhamnosides and the fucosides. Although these compounds are by themselves soluble in both solvents, they precipitate from the solution on the addition of water.

Awareness of the fact that the monoglycosides are extremely insoluble, often even less soluble than the corresponding aglycones, is important in preparing water-soluble derivatives of lipophilic compounds.

In contrast to the monoglycosides, the maltosides (which are diglucosides) proved to be fairly soluble in dimethyl sulfoxide and isopropyl alcohol and could be diluted with water without precipitation. All three maltosides tested were highly hemolytic (Table II). All maltosides gave 50% hemolysis at the same concentration. The same concentration also gave 50% hemolysis in the only soluble glucoside, smilagenyl glucoside, and was determined previously (28) for the aglycone itself.

Ghost cells were collected from blood hemolyzed with either tigogenyl or smilagenyl maltoside and were extracted with dichloromethane. The extracts, when tested by TLC, were found to contain significant quantities of absorbed free aglycones, although the original maltoside solutions were devoid of any aglycone. These findings agree with previous conclusions that hydrolysis of the glycosidic bonds by membrane glycosidases, which free the active aglycones, precedes the hemolytic phase (11). Such a mechanism also rationalizes the identical H_{50} values of smilagenin, smilagenyl glucoside, and smilagenyl maltoside, as well as the identical H_{50} values of the three maltosides, since all monohydroxy steroids and sapogenins possess identical hemolytic activity (28).

Saponin hemolysis generally is ascribed to the fact that saponins form stable insoluble complexes with cholesterol (29). Since cholesterol is an erythrocyte membrane constituent, it is assumed that hemolysis is induced by the formation of an insoluble complex between the saponin (or sapogenin) and this constituent (30). The finding that cholesteryl maltoside is exactly as active as smilagenyl and tigogenyl maltosides is of special relevance to this theory. In tigogenyl and smilagenyl maltosides, the aglycones were detected in extracts of the ghost cells.

Because of the presence of cholesterol in every erythrocyte membrane extract, the aglycone could not be demonstrated in cholesteryl maltoside but may be assumed. (The use of isotopically labeled cholesteryl maltoside was not feasible because of the very low yields in which the maltosides were obtained.) Consequently, the generally accepted conception that a stable insoluble complex between the hemolysin and the membrane cholesterol is the factor inducing hemolysis must be reconsidered. The possibility that the sapogenins, including cholesterol, combine with

[2] The identity of the H_{50} values of tigogenin maltoside in dimethyl sulfoxide and isopropyl alcohol (Table III) indicates that the latter has no enhancing or inhibiting effect on hemolysis.

Table II—H_{50} Values of Glycosides and Aglycones

	Compound	H_{50}, M[a]	Solvent
VIII	Tigogenin	2.5×10^{-5} (25)	Dimethyl sulfoxide
II	Tigogenyl α-L-rhamnopyranoside	Insoluble	Dimethyl sulfoxide and isopropyl alcohol
III	Tigogenyl β-D-fucopyranoside	Insoluble	Dimethyl sulfoxide and isopropyl alcohol
VI	Tigogenyl β-maltoside	1×10^{-5}	Dimethyl sulfoxide and isopropyl alcohol
IX	Tigogenyl β-D-glucopyranoside[b]	5[c]	Isopropyl alcohol
X	Smilagenin	3.9×10^{-5} (25)	Dimethyl sulfoxide
IV	Smilagenyl β-D-fucopyranoside	Insoluble	Dimethyl sulfoxide and isopropyl alcohol
XI	Smilagenyl β-D-glucopyranoside[b]	1.2×10^{-5}	Isopropyl alcohol
VII	Smilagenyl β-maltoside	1.1×10^{-5}	Dimethyl sulfoxide
I	Cholesteryl α-L-rhamnopyranoside	Insoluble	Dimethyl sulfoxide and isopropyl alcohol
XII	Cholesteryl β-D-glucopyranoside	Insoluble	Dimethyl sulfoxide and isopropyl alcohol
V	Cholesteryl β-maltoside	1.5×10^{-5}	Dimethyl sulfoxide

[a] Literature reference in parentheses. [b] Obtained from Professor G. Wulff, University of Bonn, Federal Republic of Germany. [c] Percent hemolysis of saturated solution.

Table III—TLC Data for Acetylated Steroid Glycosides and Their Corresponding Aglycones

Acetylated Glycoside	R_f	Aglycone	R_f	Solvent System
Cholesteryltriacetyl α-L-rhamnoside	0.85	Cholesterol	0.78	Acetone–petroleum ether (bp 60–80°) (2:3)
Tigogenyltriacetyl α-L-rhamnoside	0.83	Tigogenin	0.77	Acetone–petroleum ether (bp 60–80°) (2:3)
Tigogenyltriacetyl β-D-fucoside	0.89	Tigogenin	0.83	Acetone–petroleum ether (bp 60–80°) (1:1)
Smilagenyltriacetyl β-D-fucoside	0.80	Smilagenin	0.78	Acetone–petroleum ether (bp 60–80°) (2:3)
Cholesterylheptaacetyl β-maltoside	0.73	Cholesterol	0.78	Acetone–petroleum ether (bp 60–80°) (2:3)
Tigogenylheptaacetyl β-maltoside	0.73	Tigogenin	0.78	Acetone–petroleum ether (bp 60–80°) (2:3)
Smilagenylheptaacetyl β-maltoside	0.73	Smilagenin	0.78	Acetone–petroleum ether (bp 60–80°) (2:3)

Table IV—Solvent Systems and R_f Values for TLC of Glycosides

Compound	Solvent System	R_f
I	Chloroform–methanol (9:1)	0.36
II	Chloroform–methanol (9:1)	0.46
III	Acetone–petroleum ether (bp 60–80°) (2:1)	0.42
IV	Chloroform–methanol (9:1)	0.43
V	Chloroform–methanol (9:1)	0.72
VI	Chloroform–methanol (9:1)	0.70
VII	Chloroform–methanol (9:1)	0.73

Table V—Analytical Data of Synthetic Glycosides

Compound	Solvent Used for Column Chromatography[a]	Solvent for Crystallization	Melting Point	Yield, %	$[\alpha]_D$[b] (Solvent Concentration)	Formula	Analysis, %	Calc.	Found
I	A	Methanol	213–214°	42	−58° (0.38 chloroform)	$C_{33}H_{56}O_5$	C	74.40	74.71
							H	10.52	10.67
II	A	Methanol	229–231°	50	−101° (0.20 chloroform)	$C_{33}H_{54}O_7 \cdot CH_3OH$	C	67.0	67.38
							H	9.76	9.18
III	B	Methanol	216–217°	45	−60.4 (0.3 chloroform)	$C_{33}H_{54}O_7 \cdot H_2O$	C	68.02	67.96
							H	9.6	9.5
IV	A	Methanol	196–198°	54	−55.4° (0.20 chloroform)	$C_{33}H_{54}O_7 \cdot \frac{1}{2}CH_3OH$	C	69.4	69.76
							H	9.63	9.86
V	B	Chloroform–methanol (4:1)	213°	21	+16.9° (0.28 methanol)	$C_{39}H_{66}O_{11} \cdot 2H_2O$	C	62.6	62.97
							H	9.38	9.12
VI	C	Chloroform–methanol (4:1)	157°	28	−14.7° (0.3 methanol)	$C_{39}H_{64}O_{13} \cdot H_2O$	C	61.74	61.57
							H	8.71	8.85
VII	C	Chloroform–methanol (4:1)	298–300°	25	−5.4°	$C_{39}H_{64}O_{17} \cdot H_2O$	C	61.74	61.27
							H	8.71	8.75

[a] Solvent system A, chloroform–methanol (36:1); B, petroleum ether (bp 60–80°)–acetone (6:1); and C, chloroform–methanol (6:1). [b] Optical rotations were determined on a Perkin-Elmer polarimeter-241.

membrane cholesterol in some micellar structure resulting in hemolysis is, of course, not precluded.

The role of membrane cholesterol in the hemolyzing mechanism is now being investigated.

EXPERIMENTAL

The following materials were obtained commercially: L-rhamnose[3], D-fucose[3], maltose[4], tigogenin[4], smilagenin[4], cholesterol[4], crystalline silver nitrate[5], and hydrogen bromide solution in acetic acid[6].

TLC was performed on silica gel G coated plates.

Synthesis of Peracetylated Sugars (31–33)—To a solution of the sugar (30 m*M*) in pyridine (20–40 ml) at 4°, an equal volume of acetic anhydride was added. The reaction solution was stirred for several hours at 4° and monitored by TLC [acetone–petroleum ether (bp 60–80°) (2:3)]. The R_f of the sugar component was 0; the R_f of peracetylated sugars was 0.4–0.7. When the components with low R_f values had disappeared, ether and ice water were added.

The organic phase was collected, washed with cold aqueous sulfuric acid (5%), sodium bicarbonate (5%), and ice water, and dried (magnesium sulfate). Then the solvent was evaporated. The monosugars yielded yellow viscous products, and maltose yielded a white solid. The acetates were stored over phosphorus pentoxide and used without further purification.

Synthesis of Acetyl Sugar 1-Bromides (34–36)—To a solution of the peracetylated sugar in acetic acid (3 ml for monosaccharides and 30 ml for maltose), excess hydrogen bromide–acetic acid was added (7 ml for monosaccharides and 23 ml for maltose). The reaction was monitored by TLC [acetone–petroleum ether (bp 60–80°) (2:3)]. The bromoacetates generally had slightly higher R_f values than the peracetylated sugars.

After 2–3 hr, the reaction was complete (*i.e.*, all peracetylated sugars disappeared). Chloroform and then ice water were added. The organic phase was collected, washed with cold sodium bicarbonate (5%) and ice water, and dried (magnesium sulfate); then the solvent was evaporated. The bromoacetates were used for glycoside synthesis without purification immediately after their preparation.

Glycoside Syntheses—The general method was a modification of the Koenigs–Knorr reaction (18–20). A solution of the aglycone (1.5 moles, about 600 mg) in freshly prepared (37) dry ether (30–50 ml), finely powdered silver oxide (11 mmoles, 2.6 g), and several grains of calcium sulfate[7] were stirred in the dark in a two-necked bottle equipped with a calcium chloride tube and a separator. A solution of the bromoacetyl glycoside in dry ether (10 ml) containing a few grains of calcium sulfate was added slowly (~30 min).

The reaction mixture was stirred vigorously until no more aglycone could be detected by TLC. The chromatographic data are summarized in Table III. When the reaction was completed after 2–4 hr, the mixture was filtered, and the solvent was evaporated. The residue, a white powder, was stored over phosphorus pentoxide until alcoholysis was performed.

Basic Alcoholysis of Acetyl Glycoside—The general method was a modification of the Zemplen and Pacsu method (22). Sodium methoxide was added to a solution of the acetyl glycoside in absolute methanol (dried over molecular sieve 3) to give a final concentration of 0.25 *M* with a total volume of ~50 ml. The reaction mixture was stirred at room temperature and monitored by TLC (solvent systems and R_f values are summarized in Table IV).

When alcoholysis was complete, the solvent was evaporated. Then the residue was washed several times with cold water and chromatographed on a column of silica gel. Crystallization yielded the pure glycosides. All analytical data concerning the individual glycosides are summarized in Table V.

Acid Hydrolysis of Synthetic Glycosides—A solution of the glycoside (3 mg) dissolved in methanol (10–20 ml), with concentrated hydrochloric acid added to give a final concentration of 1 *N*, was heated under reflux for 3 hr. It was then neutralized with concentrated ammonia, and most of the solvent was evaporated. The aglycones were extracted from the aqueous methanolic residue; both fractions were tested on TLC, authentic aglycones and sugars being used as references. The TLC data for the aglycones are given in Table III. For TLC of sugars, chloroform–methanol (7:6) was used as the developing solvent; the R_f values were: glucose, 0.47; rhamnose, 0.57; and fucose, 0.52.

Hemolysis Test—*Hemolysin Solutions*—The glycosides were dissolved in dimethyl sulfoxide or isopropyl alcohol. The dimethyl sulfoxide solutions were diluted with water to give a final concentration of 30%, and the isopropyl alcohol solutions were diluted to give a final concentration of 10%. All solutions were checked carefully to determine whether the solute had precipitated on addition of water.

Blood—Citrated bovine blood was used in all experiments.

Determination of Hemolytic Activity—The method employed was described previously (28). Ghost cells were collected, extracted, and tested by TLC by the method described recently (38).

[3] Sigma.
[4] Merck, A. G. Darmstadt.
[5] J.T. Baker Chemical Co., New York, N.Y.
[6] B.D.H.
[7] Drierite.

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Nonisothermal Aqueous Calorimetry: Computation of Process-Dependent Temperature Change and Aspects of Calorimeter Design

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Abstract □ A general method for determining the process-dependent (intrinsic) temperature change in a nonisothermal calorimeter is presented. The nonisothermal approach to calorimetric investigations requires an estimate of the magnitude of the process independent (extrinsic) temperature change during the reaction period. The proposed method can be applied to any calorimeter whose output is a discrete or continuous temperature–time profile. It is based on a first-derivative transformation of the temperature–time profile and the partitioning of the observed temperature variation into two components: pure extrinsic variation, which occurs outside the reaction period, and the combined extrinsic and intrinsic effects during the reaction period. Close examination of the pure extrinsic variation was considered essential, since it provided the basis for identifying the form of a descriptive mathematical function consistent with the observed extrinsic behavior. Once a suitable function was selected, parameters for the equation were determined through a linear regression procedure. The resulting equation was used to predict the extrinsic variation within the reaction period. Subtraction of predicted extrinsic variation from the observed total variation and integration over the time course of the experiment provide an estimate of the process-dependent temperature change. The differential approach was examined for processes performed in a calorimeter of simple design. Aspects of calorimeter design and advantages of the proposed method of data analysis are discussed.

Keyphrases □ Calorimetry, nonisothermal—general method for determining process-dependent temperature change, aspects of calorimeter design □ Instrument design—nonisothermal calorimeter, general method for determining process-dependent temperature change

Immersional calorimetry is a remarkably versatile technique for investigation of the wetting of hydrophilic and hydrophobic solids (1). Some applications of this analytical method were reported (2–7); yet, in view of its utility, these applications are few and limited.

The information available from calorimetric investigations could be of value in the formulation and evaluation of pharmaceutical products. Implementation of this general experimental technique has proceeded slowly, primarily because of the apparent sophistication necessary in the equipment.

Aspects of calorimeter design and data analysis were included in a general review of calorimetry (8). This paper presents a general method of data analysis and demonstrates its rational development for a calorimeter of simple design.

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