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Enzymatic modification of the sugar moieties of natural glycosides

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Abstract

Glycosides of various classes of natural products are widely distributed in nature. Many of these compounds are pharmacologically important molecules or possess other interesting properties and, quite often, their sugar moieties are acylated with aliphatic and aromatic acids.

In recent years the regioselectivity of lipases and proteases has been exploited by different research groups for the synthesis, in organic solvents, of specific esters of natural glycosides. The selectivity of the β-1,4-galactosyl-transferase (GalT) from bovine colostrum, has also been investigated, aiming to produce new glycosyl derivatives of these compounds.

The applications of these two bio-transformations will be discussed in this review.

Keywords: Natural glycosides; Enzymes; Regioselectivity; Glycosyltransferases; Lipases; Proteases

1. Introduction

Glycosides of various classes of natural products are widely distributed in nature, where they are often present acylated with aliphatic and aromatic acids (mainly acetic, malonic, *p*-coumaric, and ferulic acid) at specific OHs of their sugar moieties. Many of these compounds are pharmacological important molecules or possess other interesting properties [1]. Examples are given by *ginsenosides* (e.g. **1**), sweet terpene glucosides (e.g. **2**), haemolytic saponins (e.g. **3**), cardiac glycosides (e.g. **4**). In recent years, we have exploited the regioselectivity of lipases and proteases in organic solvents as well as the selectivity of the β-1,4-galactosyl-transferase (GalT) from bovine colostrum to obtain new glycosyl derivatives of these compounds.

The application of these enzymatic methodologies for the modification of compounds **1**–**4** and of other glycosides will be presented in this account, based on the content of the lecture given at the Biotrans 2001 Conference, but reviewing also the papers published on this topic by different research groups.

2. Regioselective acylation of natural glycosides in organic solvents

Several esters of glycosides can be found in nature. The formation of these esters is the last step in the biosynthetic pathway and it is catalyzed by different acyltransferases, enzymes that usually show relative flexibility towards the acyl moiety but strict selectivity for the substrate to be esterified. Additionally,

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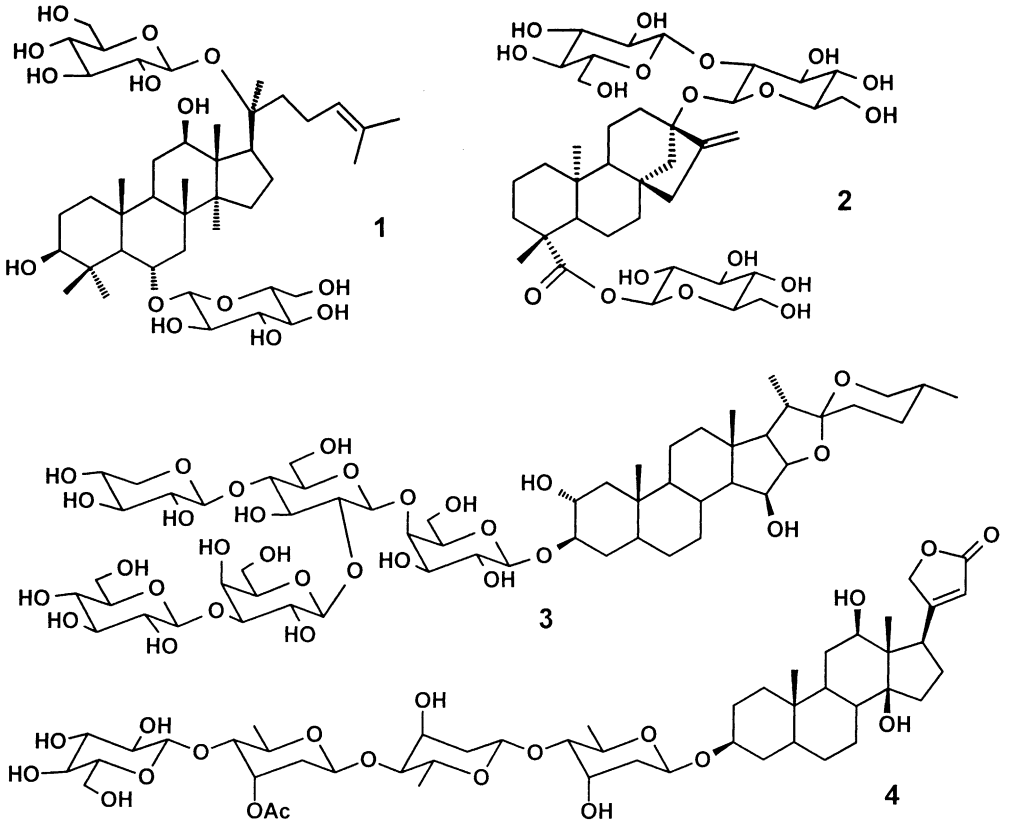


acyltransferases are not very convenient even on a lab-scale synthesis, as they require as acyl donor the corresponding acyl-coenzyme A, that must be either used in stoichiometric amounts or regenerated in situ. On the other hand, the one-step selective chemical acylation of glycosides is still a distant target because of the present lack of suitable reagents and protocols to discriminate among the primary OHs of the various sugar units present in the same molecule or among the several secondary OHs present in a single carbohydrate moiety.

In recent years, hydrolytic enzymes (more specifically esterases, lipases, and proteases) have become valuable tools in organic synthesis due to their large availability, low cost, wide substrate spectrum and no need of added cofactors [2]. More specifically, it has been found that glycosides and polyhydroxy compounds can be selectively acylated at specific OHs of their molecules by the action of an activated ester in the presence of a suitable hydrolase suspended in organic solvent. This will be the topic of the first part of this account.

At the beginning of 1988, Klibanov and coworkers reported on the use of the protease subtilisin for the modification of primary hydroxyl groups of di- and oligosaccharides as well as of natural glycosides [3]. The compounds utilized in this work (depicted in Fig. 1) can be considered the “natural ancestors” of the numerous applications that have been reported since then. Among them, adenosine (**7**) and uridine (**8**) are nucleosides. As the enzymatic acylation of this class of compounds has been described in a recent review [4], no further examples will be described and commented upon here.

2.1. Enzymatic acylation of flavonoid glycosides

Flavonol glycosides and their esters are an important group of natural compounds widely distributed in the plant kingdom. For instance, the *p*-coumarate of

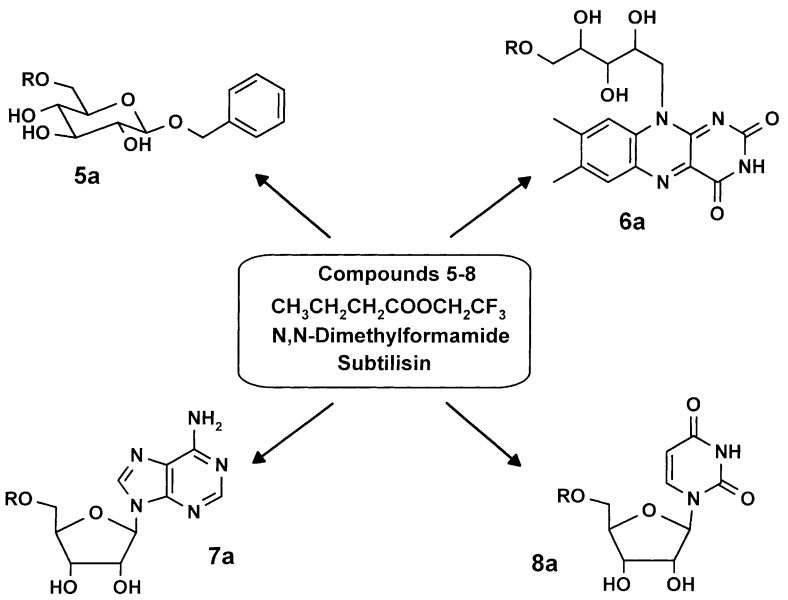


Fig. 1. First enzymatic acylation of natural glycosides. Substrates **5**–**8**, R = H; products **5a-8a**, R = PrCO [3].

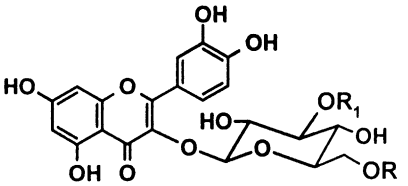
some kaempherol and quercetin disaccharide monoglycosides are active components (together with ginkolides) of the very popular medicinal extract of the leaves of *Ginko biloba*, which is used to increase peripheral and cerebral blood flow. Due to the presence of several reactive groups even on their aglycon moieties, these compounds are particularly challenging substrates for enzymatic esterification. The behavior of several hydrolases towards the acylation of flavonol monoglycosides (i.e. isoquercitrin, **9**, and quercitrin, **10**) and of more complex flavonol disaccharide monoglycosides (i.e. rutin, **11**) with the activated ester trifluoroethyl butanoate was examined first. The protease subtilisin gave interesting results in terms of selectivity with compounds **9** and **11** and the corresponding esters (**9a**, **9b**, and **11a**) were isolated in good yields, while the rhamnopyranosyl derivative **10** was recovered unaffected [5,6].

Then, a chemo-enzymatic approach to some 6″-*O*-(3-arylprop-2-enoyl) derivatives (cinnamate, *p*-coumarate, feruloate) of isoquercitrin **9** was explored, to overcome the inability to directly introduce these acyl moieties by the subtilisin-catalyzed reaction of **9** with the corresponding activated ester. This approach was based on the enzymatic regioselective introduction of a methyl malonate residue at the CH_2OH of the sugar moiety to form the mixed diester **9c**. The subsequent selective chemical hydrolysis of the methoxycarbonyl function of **9c** was unsuccessful and therefore this compound was subjected to an enzymatic chemo-selective hydrolysis. The malonic monoester **9d** was finally reacted in a Knoevenagel-type condensation with the appropriate aromatic aldehydes to afford the esters **9e**–**g** [7].

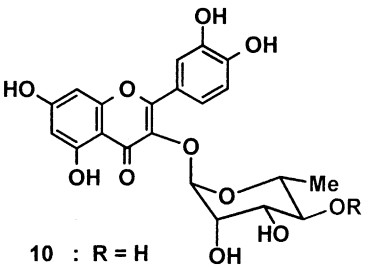
In a more recent report [8], it was shown that the malonic monoester **9d** could be more efficiently prepared by another two-steps chemo-enzymatic approach. Specifically, it was found that dibenzylmalonate is a good acyl donor substrate for the lipase B from *Candida antarctica* (at that time marketed under the trade name of Novozym 435), even in an acetone solution containing 10% pyridine to dissolve **9**. Under these conditions, isoquercitrin was transformed into the mixed diester isoquercitrin 6″-*O*-benzyl malonate (**9h**) with high regioselectivity and good isolated yields (74%). Catalytic hydrogenation of **9h** on Pd/C in THF solution afforded pure **9d** in quantitative yields, without need of further purification steps.

Later on, the selectivity of Novozym 435 towards sugar derivatives was systematically investigated [9] and it was found that this enzyme was also able to acylate quercitrin to give the corresponding 4″-*O*-acetyl derivatives (**10a**), while rutin gave the expected diacetate **11b**.

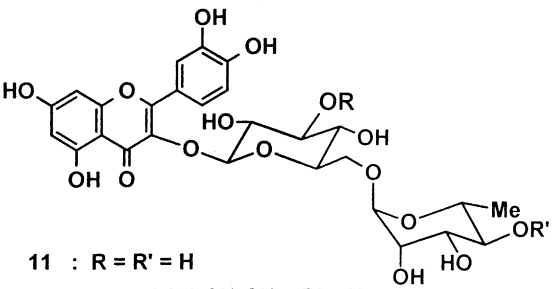
More recently, it was shown that it is also possible to use this versatile biocatalyst to directly introduce phenylpropenoic moieties on flavonoid glycosides [10–13]. The approach suggested by Vulfson and coworkers [13] is of particular interest as the target derivatives (i.e. **9e–g**) were obtained from the aglycone using a two-steps enzymatic protocol (glycosydases-catalyzed glycosylation in supersatured solutions, followed by Novozym 435-catalyzed acylation in organic solvents).



9 : R = R_1 = H
9a : R = $COCH_2CH_2CH_3$; R_1 = H
9b : R = R_1 = $COCH_2CH_2CH_3$
9c : R = $COCH_2COOCH_3$; R_1 = H
9d : R = $COCH_2COOH$; R_1 = H
9e : R = $COCH{=}CHC_6H_5$; R_1 = H
9f : R = $COCH{=}CHC_6H_4$(*p*)OH; R_1 = H
9g : R = $COCH{=}CHC_6H_3$(*p*)OH(*m*)OCH_3 ; R_1 = H
9h : R = $COCH_2COOCH_2C_6H_5$; R_1 = H



10 : R = H
10a : R = $COCH_3$



11 : R = R' = H
11a : R = $COCH_2CH_2CH_3$; R' = H
11b : R = R' = $COCH_3$

2.2. Enzymatic acylation of terpene glycosides

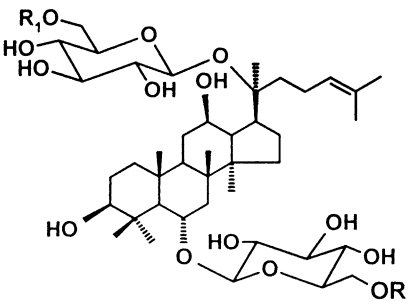
Ginsenosides are an important class of dammarane-type triterpene oligoglycosides which are isolated from the water soluble portion of the dried roots and leaves of Panax ginseng C.A. Meyer, a plant widely used in the traditional Chinese and Korean medicine. Recently, a careful examination of white Ginseng extracts has revealed that some ginsenosides are present as monoesters of malonic acid, the acylation site occurring invariably at one of the primary OHs of the sugar moiety. These carboxyacetyl ginsenosides behave as acidic saponins, and besides being more soluble in water than the ordinary glycosides, they also cause a remarkable increase of the solubility of the other ginsenosides and, as a consequence, they might deeply influence the absorption of these drugs in humans.

In spite of these interesting properties, no information was available on the synthesis of malonyl ginsenosides or, more generally, on the preparation of specific esters of ginsenosides with aliphatic carboxylic acid. Therefore, the behavior of some of these glycosides towards enzymatic acylation was investigated. Using the ginsenoside Rg_1 (**1**) as a substrate, the best results were again obtained with Novozym 435, in this case suspended in *t*-amyl alcohol in the presence of the acyl donor vinyl acetate. A complete conversion

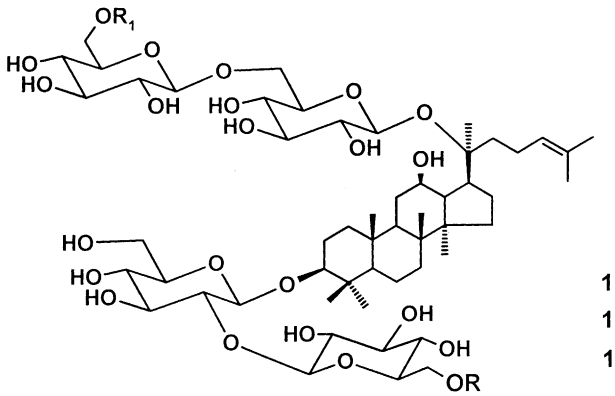
to only two products in a 22:1 ratio took place and the two products were identified, on the basis of an extensive analysis of their ^{1}H-NMR spectra, as 6′-*O*-acetyl- and 6′,6″-*O*-diacetyl-ginsenoside Rg_1 (**1a**) and (**1b**), respectively [14].

For the synthesis of malonyl-Rg_1, ginsenoside **1** was enzymatically reacted with dimethyl-malonate

Interesting results were also obtained with the more complex ginsenoside Rb_1 (**12**), carrying a gentiobiose unit at C-20 and a sophorose unit at C-3. The monoacetate at the primary OH of the external glucose of the disaccharide moiety at C-20 (**12a**) was mainly obtained, accompanied by the diacetate **12b** [15].



1 R = R' = H
1a R = COMe ; R' = H
1b R = R' = COMe
1c R = $COCH_2COOMe$; R' = H
1d R = $COCH_2COOH$; R' = H
1e R = $COCH_2COOCH_2CCl_3$; R' = H



12 R = R' = H
12a R = H ; R' = COMe
12b R = R' = COMe

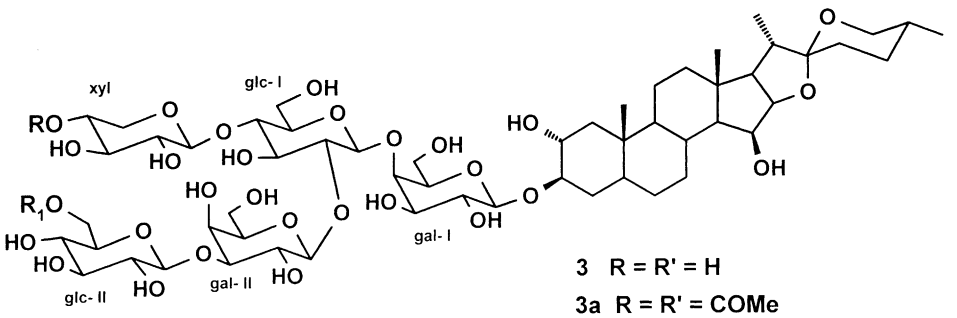
under the usual conditions to afford the mixed diester **1c**, which, in turn, was selectively hydrolysed by pig liver esterase to the expected malonate **1d**. However, this acidic saponin proved to be very unstable during the necessary chromatographyc purification (due to its high propensity to undergo either complete hydrolysis to the parent **1** or decarboxylation to the acetate **1a**) and was isolated in low yield. The synthesis of **1d** was improved by exploiting an alternative chemo-enzymatic approach based on the lipase-catalyzed enzymatic formation of the 6″-*O*-(2,2,2-trichloroethyl)-malonate **1e**, followed by the chemical removal of 2,2,2-trichloroethanol by action of Zn/AcOH [14].

Digitonin (**3**) is a complex monodesmosidic steroidal saponine isolated from the seeds of *Digitalis purpurea*, which possesses the distinctive characteristic to cause the rupture of the membrane of the red blood cells. This haemolytic activity is shared by other natural saponins and it is greatly influenced by the structure of the aglycone and by the number and type of sugars as well as by their glycosidic linkages. It is assumed that haemolysis depends on the ability to form a complex with cholesterol, a central component of the erythrocyte membrane. For the elucidation of the structural requirements for haemolysis, a 1:1 complex of digitonin and cholesterol has been prepared and extensive NMR studies have been

undertaken to clarify the sites of interaction between the two molecules [16]. To support this work, specific derivatives were needed in order to evaluate their aptitude to form complexes with cholesterol. Digitonin contains a branched pentasaccharide moiety and therefore it represents a challenging target for selective modification. Out of the 4 primary and 13 secondary hydroxyl groups present in this molecule, only the C-4 OH of the xylopyranosyl moiety and the C-6 OH of the external glucopyranosye (glc-II) were recognized by Novozym 435 suspended in a *t*-amyl alcohol solution. In the presence of vinyl acetate and vinyl laurate this enzyme catalyzed the exclusive formation of the diacetate **3a** and of the monoacetates **3b** and **3c**, and of the monolaurate **3d**, respectively [17]. The hemolytic activity of these compounds proved to be lower than that of the parent **3**.

The cardioactive glycoside content of *Digitalis purpurea* leaf is 0.15–0.4% (w/w), consisting of about 30 different structures. The glycosides comprise two series of compounds, carrying either a tetrasaccharide unit (glucose-(digitoxose)$_3$–, i.e. lanatoside C, **4**) or a trisaccharide unit ((digitoxose)$_3$–, i.e. digitoxin, **13**, or digoxin, **14**), the second group being obtained by action of β-glucosidases which remove the terminal glucose. Digoxin (**14**, trade names: Digacin, Lanicor, Lanoxin) is regarded as the drug of choice for the treatment of congestive heart failure. Acetylation or methylation of the terminal digitoxose residue produces derivatives with improved oral absorption. Examples are α-acetyldigoxin (**14a**, trade names: Dioxanin, Lanatilin, Sandolanid) and β-acetyldigoxin (**14b**, trade name: Novodigal).

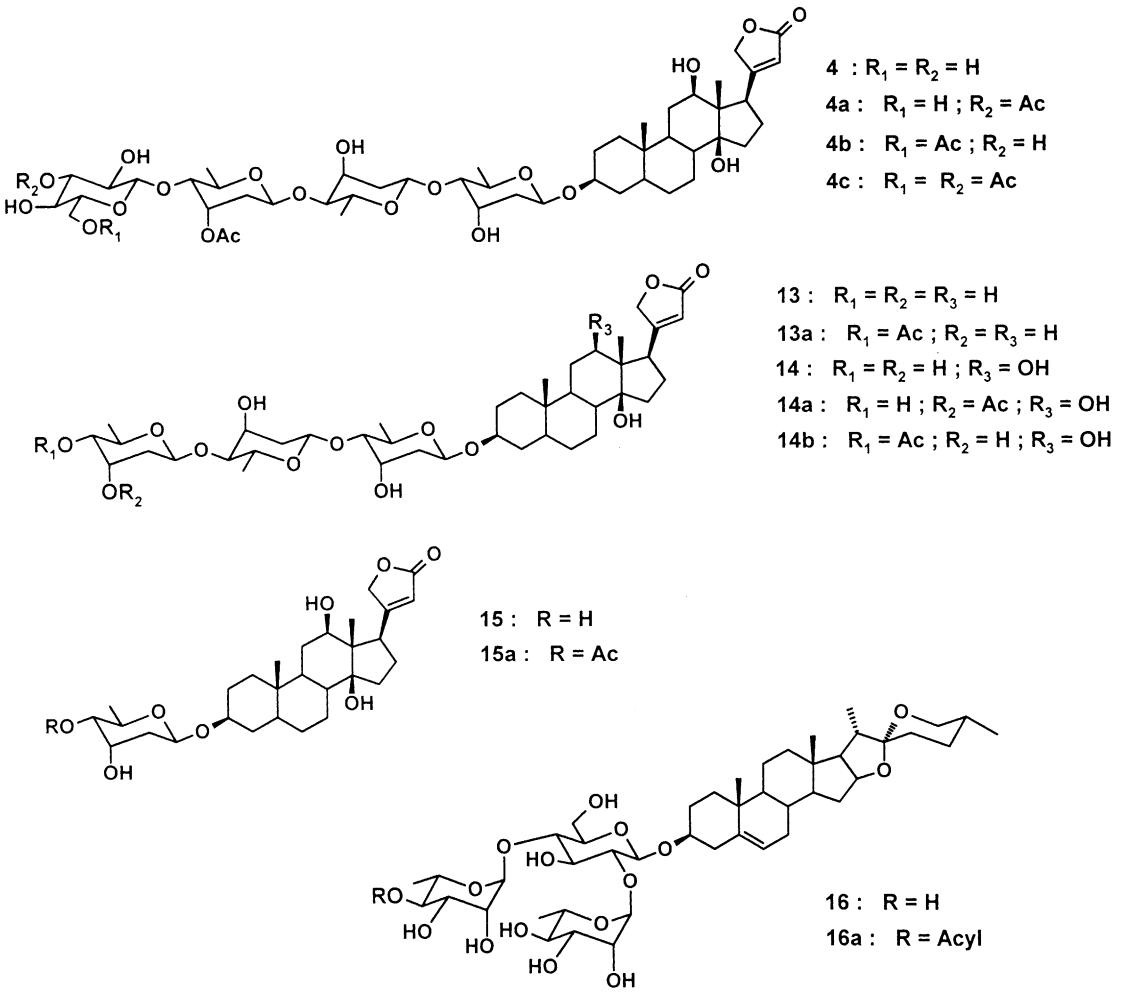
Regioselective acylation of the terminal glucopyranosyl unit of **4** was successfully accomplished with



Many of the plants known to contain cardiac and cardiotonic glycosides have long been used as arrow poisons (e.g. *Strophanthus*) or as heart drugs (*Digitalis*). On this respect, they are used to strengthen a weakened heart and allow it to function more efficiently, though the dosage must be controlled very carefully since the therapeutic dose is so close to the toxic dose. The therapeutic action properties of cardioactive glycosides obviously depend on the structure of the aglycone and on the type and number of sugar units attached. Cardiac glycosides are very similar in their pharmacodynamic actions but vary considerably in their pharmacokinetic properties according to their different lipophilicities.

several hydrolases, and the corresponding mono- and diacylated derivatives **4a–c** were isolated in good yields. On the other hand, lipase PS was the only hydrolase able to acylate the terminal digitoxopyranosyl unit of digitoxin (**13**), digoxin (**14**), and of the monosaccharide derivative digoxygen-monodigitoxid (**15**). The corresponding monoacetyl derivatives **13a**, **14b**, and **15a** were isolated in low yields (20–25%) [18].

Finally, more recently a series of diosgenyl saponins (i.e. dioscin, **16**) were regioselectively acylated by Novozym 435 in THF containing vinyl esters as acylating agents, to afford the corresponding mono- or diacyl diosgenyl saponins (i.e. **16a**) [19].

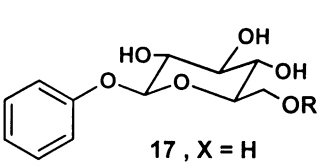


2.3. *Miscellanea*

Few additional reports have been published on the modification of other natural glycosides. In some cases, the substrates were simple monoglycosides, like arbutin (**17** [10]), the rhamnopyranosyl derivative **18** [20], the alkaloids colchicoside (**19**), and thiocolchicoside (**20**) [21]. The corresponding monoacyl derivatives **17a–20a** of these compounds were obtained in good yields.

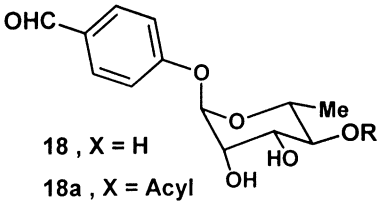
More recently, the enzyme-mediated regioselective acylation of sophorolipids, a class of microbial extracellular surface-active glycolipids, has been reported [22]. Novozym 435 has been shown to be once again an efficient catalyst for this kind of bio-transformations: acylation of sophorolipid methyl ester **21** in dry THF was highly regioselective, and exclusive esterification of the hydroxyl groups on C-6′ and C-6″ took place. Products **21a**, **22**, and **22a** were obtained in excellent isolated yields, depending on the reaction conditions.

A series of papers has been published on the regioselective acylation of 2-*O*-glycopyranosylglycerol (i.e. **23**) and on the evaluation of the antitumor promoting activity of these compounds ([23,24], and references therein). Best (and complementary) results were obtained with lipase PS and with Novozym 435. Interestingly, acylation took preferentially place on the glycerol moiety allowing, in the case of **23**, the isolation of compounds **23a** and **23b**, respectively.



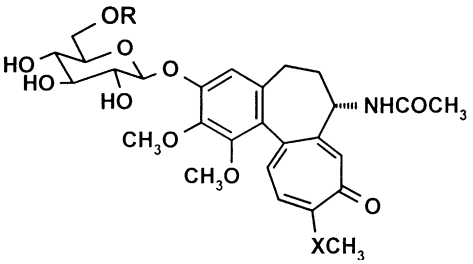
17 , X = H

17a , X = Acyl



18 , X = H

18a , X = Acyl

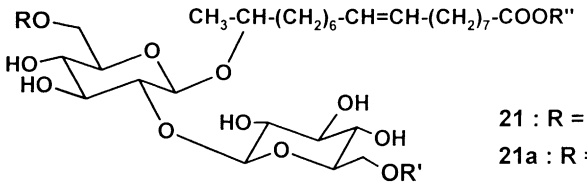


19 , X = O ; R = H

19a , X = O ; R = Acyl

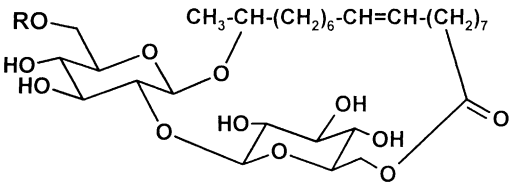
20 , X = S ; R = H

20a , X = O ; R = Acyl



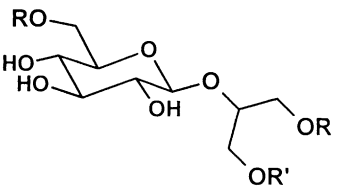
21 : R = R' = H ; R'' = Me

21a : R = R' = Ac ; R'' = Me



22 : R = H

22a : R = Ac



23 : R = R' = H

23a : R = Ac ; R' = H

23b : R = H ; R' = Ac

Regioselective enzymatic esterification of the aglycon moiety of natural glycosides is quite unusual and, to the best of our knowledge, there is just one other example reported, describing the acylation of plant cytokinins [25].

3. Regioselective glycosylation of natural glucosides by action of glycosyltransferases

The β-1,4-galactosyl-transferase (GalT), originally isolated from bovine colostrum and now also available

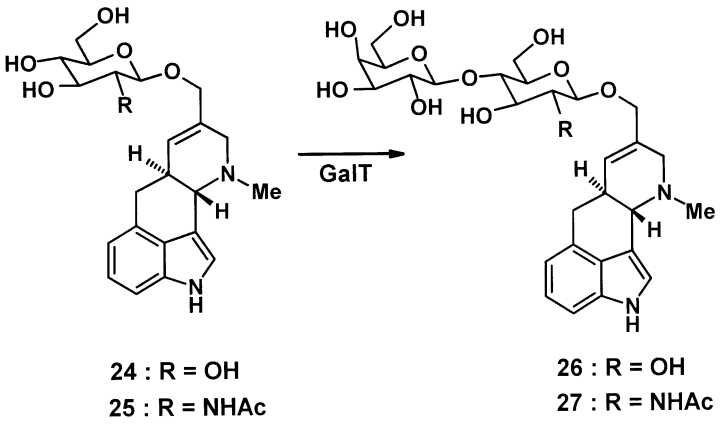
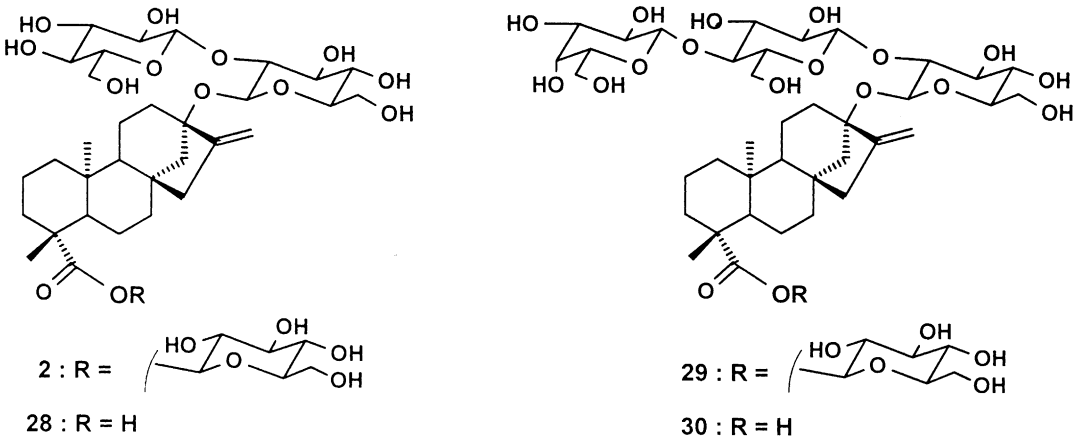


Fig. 2. First GalT-catalyzed galactosylation of natural glycosides [26].

from recombinant sources, is by far the most studied enzyme belonging to the glycosyltransferases group. Besides accepting its natural substrates D-glucose and 2-acetamido-2-deoxy-D-glucose, GalT was proved to be quite versatile towards modification of the sugar acceptor, provided that a free equatorial C-4 hydroxyl is present.

As far as the topic of this review concern, it has to be acknowledged the pioneering work of Kren et al. [26]. They have shown that GalT can use glycopyranosyl derivatives of an alkaloid, elymoclavine 17-*O*-β-D-glucopyranoside (**24**) and elymoclavine 17-*O*-(2-acetamido-2-deoxy-β-D-glucopyranoside) (**25**), as a substrate to give the corresponding lactose, and lactosamine derivative **26** and **27** (Fig. 2). This was an interesting observation because natural glycosides often possess pharmacological activity and variations of their sugar composition might offer an easy access to new compounds with increased solubility, bio-availability and biological action.

Danieli et al. have applied the Kren and Augé's protocol to other natural glycosides. In a first communication [27], good results were obtained, both in terms of degree of conversion and selectivity, with the sweetener stevioside (**2**) and its congener steviolbioside (**28**). Despite the fact that stevioside and steviolbioside possess three and two glucopyranosides in their molecules (and therefore three or two possible galactosylation sites), GalT showed an absolute regioselectivity and site-selectivity, and only their monogalactosyl derivatives **29** and **30** were formed and isolated.



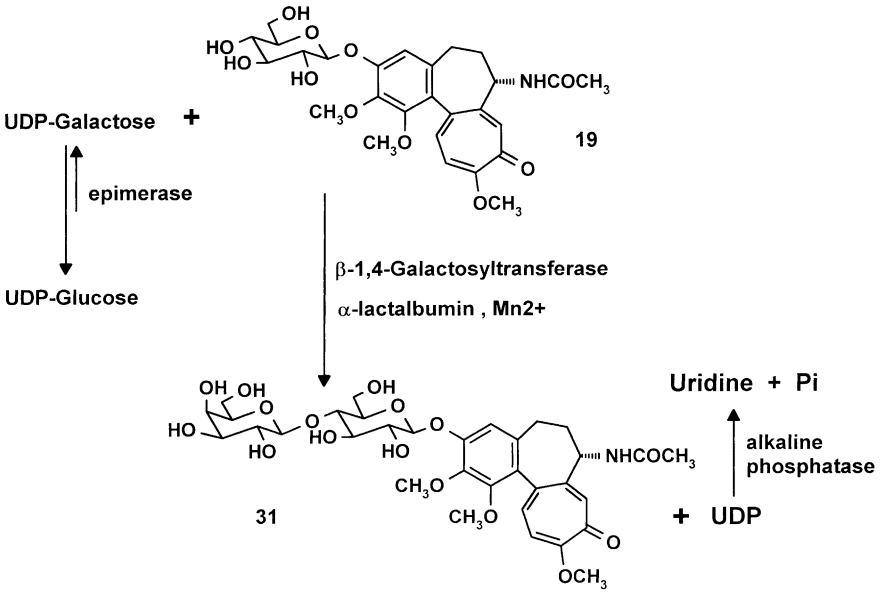
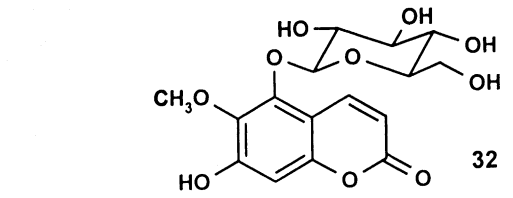
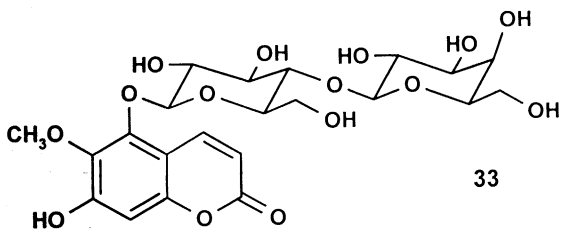


Fig. 3. Multi-enzymatic galactosylation of colchicoside [28].

Due to the hydrophobic nature of most aglycon moieties, a general exploitation of this enzymatic methodology might be hampered by the low solubility of some glycosides. The use of organic co-solvents might overcome this limitation. As only limited data were available on the compatibility of organic solvents with this glycosyltransferase, a systematic investigation on the effects of organic co-solvents on the properties of GalT and of its ancillary enzymes was performed using the alkaloid colchicoside (**19**) as a model compound [28]. GalT-catalyzed galactosylation of **19** was performed according to a standard protocol (Fig. 3) and a 71% conversion to a single product was observed. This compound was isolated by flash chromatography and characterized as the 3-*O*-β-lactosyl derivative of colchicine, **31**. The influence of various organic co-solvents on the stability and activity of GalT and of its ancillary enzyme UDP-galactose-4′-epimerase were then determined. As an example, Table 1 shows the degrees of conversion observed after 24 h in the presence of 5, 10, and 15% (v/v) of various water miscible organic co-solvents. More detailed experiments with various amounts of different co-solvents showed that some of them, like dimethyl sulfoxide and methanol, can be used up to 20% (v/v) without any influence on the performance of these enzymes, while others, such as tetrahydrofuran, rapidly inactivate GalT at concentrations as low as 5% (v/v).

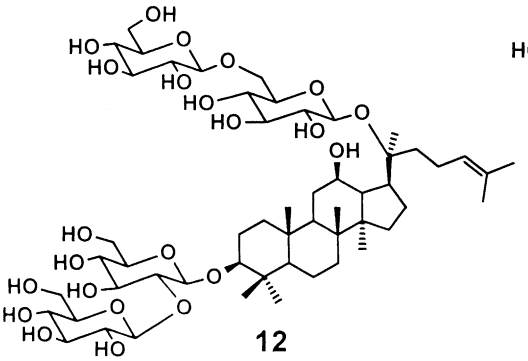
These results were initially exploited for the galactosylation of the coumarinic glucoside fraxin (**32**), a compound that is almost insoluble in water (less than 0.5 mg/ml). Fraxin was solubilized up to 12.5 mg/ml (34 mM) in the reaction buffer containing 15% (v/v) dimethyl sulfoxide, and a 98% conversion to the corresponding β-lactoside **33** was obtained in 48 h [28].





Later on the same methodology has been applied for the galactosylation of ginsenosides. As an example, ginsenoside Rg_1 (**1**) was submitted to the action of GalT in the presence of UDP-glucose and of the ancillary enzyme UDP-galactose-4′-epimerase [29]. The enzyme showed the well-known specificity for the formation of a β-linkage with the C-4 OH of the glucose acceptor, but it was not able to discriminate between the two glucose moieties of **1**, giving a mixture of mono- and digalactosylated derivatives. Other natural Rg_1-analogues such as ginsenosides F_1, Rh_1, Re, as well as the synthetic derivative ginsenoside 6′-*O*-acetyl-Rg_1 have been also galactosylated, giving the corresponding monolactosyl derivatives. GalT was also able to accept UDP-glucose as an activated sugar donor, giving rise to the corresponding cellobiosyl derivatives of Rg_1.

Enzymatic galactosylation was also performed on ginsenosides which are much less soluble in water (i.e. ginsenoside Rb_1 **12**). Exploiting the previously described results [28], these reactions were run in the presence of 20% (v/v) of DMSO, giving a complex HPLC chromatogram. Five mono- and digalactosylated derivatives of **12** have been isolated by preparative HPLC and characterized, compound **34** being the most abundant of them [15].



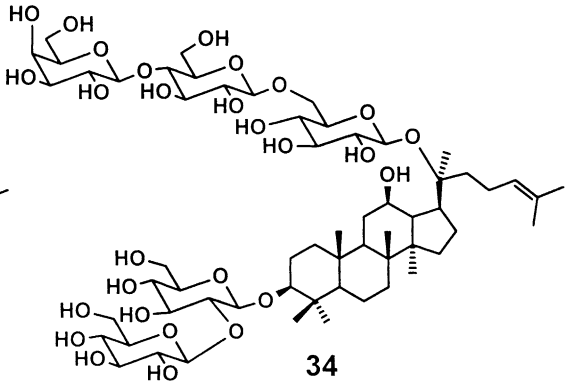


Table 1
Conversion (%) of colchicoside into its corresponding lactoside in the presence of various amounts of organic co-solvents[a]

Co-solvent	5% (v/v)	10% (v/v)	15% (v/v)
Blank	71	71	71
Dimethylsulfoxide	72	74	75
Methanol	77	80	84
Ethanol	80	85	36
Acetone	76	76	67
Dioxane	72	67	25
Acetonitrile	69	66	55
N,*N*-Dimethylformamide	64	55	39
Tetrahydrofuran	55	1	0

[a] Determined by HPLC after 24 h [28].

4. Closing remarks

It has been shown that enzymes from different classes (hydrolases and transferases) can be exploited for the regioselective modification of complex natural glycosides. Both these enzymatic methodologies seem to be highly valuable for the preparation of new derivatives to be investigated for their biological activity.

More information is available on the acylation catalyzed by lipases and/or proteases, while only scant data have been reported on the use of glycosyltransferases (additionally, they are presently limited to a single enzyme). With both classes of enzymes, no sound docking simulations with molecular modeling have been reported so far, despite the fact that the structures of all the biocatalysts described in this review have been determined by X-ray crystallography.

As far as the use of hydrolases concern, it is now well assessed that lipases and proteases are usually not affected by the nature of the aglycon moiety. Therefore, acylation sites can be predicted from the results obtained with similar glycosides: for instance esterification of α-L-rhamnopyranosyl units catalyzed by Novozym 435 will take place at the C-4 OH, acylation of a β-D-glucopyranosyl derivative by action of subtilisin will occur at the primary C-6 OH and then at the

C-3 OH, and so on (for systematic investigations on the regioselectivity of specific hydrolases see, for instance [9] and [30]. On the other hand, no predictions can be presently made with confidence on the site of glycosylation of poly-glucosylated substrates.

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