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Effect of organic cosolvents on the stability and activity of the β-1,4-galactosyltransferase from bovine colostrum [1]

Sergio Riva [a,*], Barbara Sennino [a], Francesca Zambianchi [a], Bruno Danieli [b], Luigi Panza [b]

[a] *Istituto di Chimica degli Ormoni, C.N.R., Via Mario Bianco 9, Milano I-20131, Italy*
[b] *Dipartimento di Chimica Organica e Industriale, Centro C.N.R. di Studio sulle Sostanze Organiche Naturali, Universita' degli Studi di Milano, Via Venezian 21, Milano I-20133, Italy*

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Abstract

The influence of various organic cosolvents on the stability and activity of the β-1,4-galactosyltransferase from bovine colostrum (GalT) and of its ancillary enzyme UDP-galactose-4′-epimerase has been investigated using the glucosylated alkaloid colchicoside (**1**) as a model substrate. It has been found that some cosolvents, such as Me_2SO and MeOH, can be used up to 20% v/v without any influence on the performance of these enzymes, while others, such as tetrahydrofuran, rapidly inactivated GalT at concentrations as low as 5% v/v. These results have been exploited for the galactosylation of the poorly water soluble coumarinic glucoside fraxin (**2**). © 1998 Elsevier Science Ltd.

Keywords: β-1,4-Galactosyltransferase; UDP-galactose-4′-epimerase; Organic cosolvents; Colchicoside; Fraxin

1. Introduction

The growing interest in the biological activity of defined oligosaccharides, like the tetrasaccharide Sialyl Lewis x, has stimulated research for new efficient syntheses of these compounds and of their analogues. In addition to chemical approaches, biocatalytic methods and especially enzymes belonging to the transferase class have been investigated. As a consequence, several glycosyltransferases have been isolated, characterized and, in some cases, cloned and overexpressed [1–3].

The β-1,4-galactosyltransferase from bovine colostrum (GalT), which is available from natural source, is by far the most studied enzyme of this class. It has been shown that, besides accepting its natural substrates D-glucose and 2-acetamido-2-deoxy-D-glucose, GalT is quite versatile towards substitutions at the pyranoside moiety of the sugar acceptor, provided that the equatorial C-4 hydroxyl is always present. Various glycosides [4], cyclitols [4,5], *C*-glucopyranosyl analogues [6], and glycopeptides

* Corresponding author. Fax: +39-2-285-000-36; e-mail: rivas@ico.mi.cnr.it.

[1] Regioselective enzyme-mediated glycosylation of natural polyhydroxylated compounds, Part 2. For Part 1, see Ref. [12].

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[7–9] have been galactosylated using this enzyme. Moreover, Augé et al. [10] showed that GalT can use a glucoside of an alkaloid, elymoclavine 17-*O*-(2-acetamido-2-deoxy-β-D-glucopyranoside, as a substrate. This was an interesting observation because natural glycosides often possess pharmacological properties and variations of their sugar composition might offer an easy access to new compounds with increased solubility, bioavailability and biological action.

In the framework of our research on synthetic applications of this enzyme [6,11], the Augé's protocol was applied to other natural glycosides and, up to now, good results have been obtained, both in terms of degree of conversion and selectivity, with the sweetener stevioside and its congener steviolbioside [12], as well as with various saponins extracted from Ginseng roots (unpublished results).

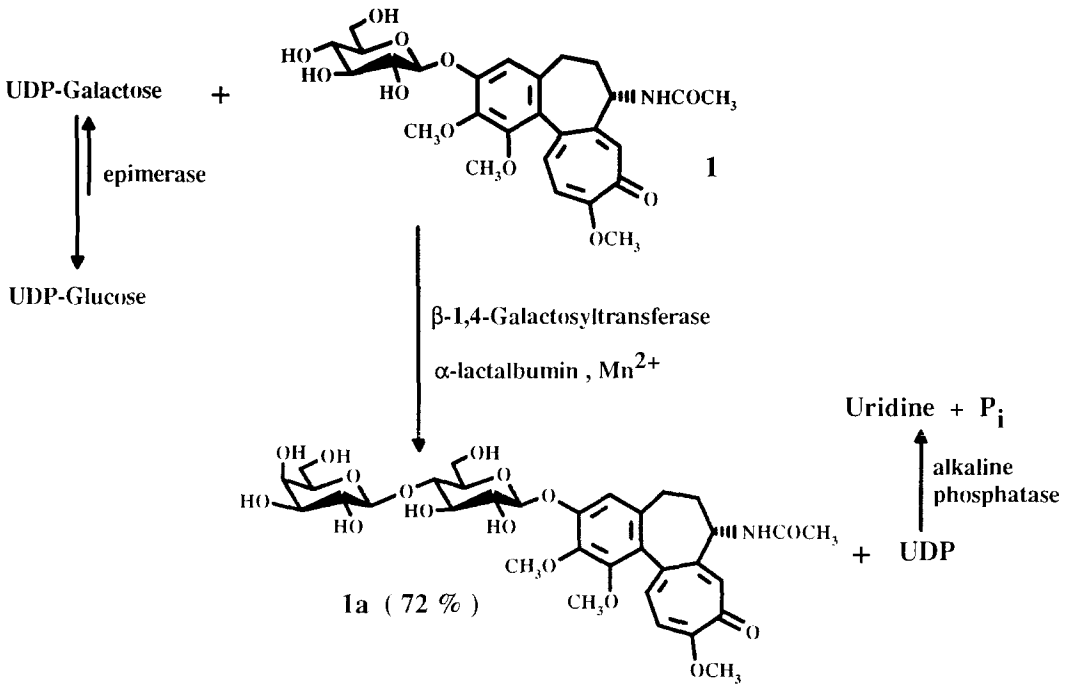
The general exploitation of this enzymatic methodology is hampered, however, by the low solubility of some glycosides, due to the hydrophobic nature of their aglycones. The use of organic cosolvents might overcome this limitation. As only limited data are available on the compatibility of these solvents with this transferase (see, for instance, Ref. [13]), in the following, we report the results of a systematic investigation on the effects of organic cosolvents on the properties of GalT and ancillary enzymes using the alkaloid colchicoside (**1**) as a model compound.

2. Results and discussion

Colchicoside (**1**), a natural alkaloid isolated from the Autumn saffron and other Liliaceae, is the 3-*O*-β-D-glucopyranoside of 3-*O*-demethylcolchicine. It has been reported to be about 100 times less toxic than colchicine, while maintaining a therapeutical action against human gout and other inflammations [14,15]. This compound is soluble in water and in buffered aqueous solutions, therefore, it appeared to be a suitable model for our investigation.

GalT-catalyzed galactosylation of **1** was performed according to a standard protocol (Scheme 1), the progress of the reaction being easily monitored by TLC and reverse phase analytical HPLC. After 5 days, 71% conversion to a single product was observed. This product was isolated by flash chromatography and characterized as the 3-*O*-β-lactosyl derivative of colchicine, **1a**, by FABMS, ^{1}H and ^{13}C NMR spectroscopy.

It has been reported that, in some instances, GalT is also able to use UDP-glucose as a sugar donor [10,16]. However, in our case, no transformation of **1** was observed after 48 h in the absence of the ancillary enzyme UDP-galactose-4′-epimerase (epimerase). Therefore, we increased the ratio between the donor (UDP-glucose, epimerized in situ to the GalT substrate UDP-galactose) and the acceptor **1** (used at a starting concentration of 20 mM) in order to acceler-



Scheme 1. Enzymatic galactosylation of colchicoside (**1**).

ate the reaction. We found that using 20 mM of **1** and 150 mM of UDP-glucose, approximately 70% of the starting colchicoside was galactosylated within the first 24 h. Therefore, this ratio of reagents was used in the subsequent experiments. The degree of conversion observed after 24 h in the presence of 5, 10 and 15% v/v of various water miscible organic cosolvents is shown in Table 1.

From these first data, it was evident that some solvents were fully compatible with our enzymatic system, while others were not. Accordingly, a more detailed investigation was performed in the range of concentrations, different for the various cosolvents, in which the enzymatic activities could be expected to be retained. For instance, the effect of the 'good' cosolvents MeOH and Me_2SO was investigated up to 40% v/v, while the 'poor' cosolvents tetrahydrofuran and *N*,*N*-dimethylformamide were studied in the concentration ranges up to 10–15% v/v in more details. The whole set of data are visualized in Figs. 1–3.

These results show that this multienzymatic system is sensitive to the nature of the organic cosolvent, the 'break point' (defined as the highest concentration at which the enzymatic performance is similar to the blank) being around 20% v/v for Me_2SO and MeOH, 15% v/v for acetone, 10% v/v for EtOH, dioxane and acetonitrile, 5% v/v for *N*,*N*-dimethylformamide and even less for tetrahydrofuran. It is also interesting to note that some cosolvents (Me_2SO, MeOH, and EtOH) seem to have a slight activating effect in concentrations up to 10–15% v/v.

Table 1
Degrees of conversion of colchicoside into its corresponding lactoside in the presence of various amounts of organic cosolvents[a,b]

Cosolvent	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.
[b]Conditions: colchicoside, 20 mM; UDP-glucose, 150 mM; Mn^{2+}, 25 mM; α-lactalbumin, 1 mg/mL; GalT, 1 U/mL; epimerase, 1.8 U/mL; alkaline phosphatase, 5 U/mL, Tris buffer 50 mM, pH = 7.4; 30 °C. Each experiment was repeated at least twice.

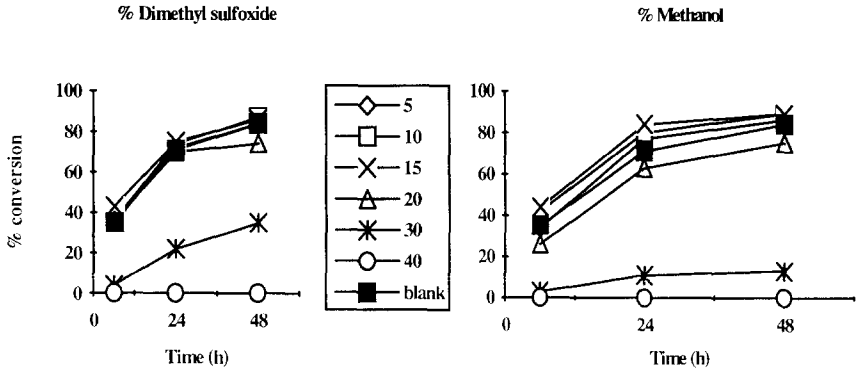


Fig. 1. Influence of various amounts of Me_2SO and MeOH on the GalT-catalyzed galactosylation of colchicoside.

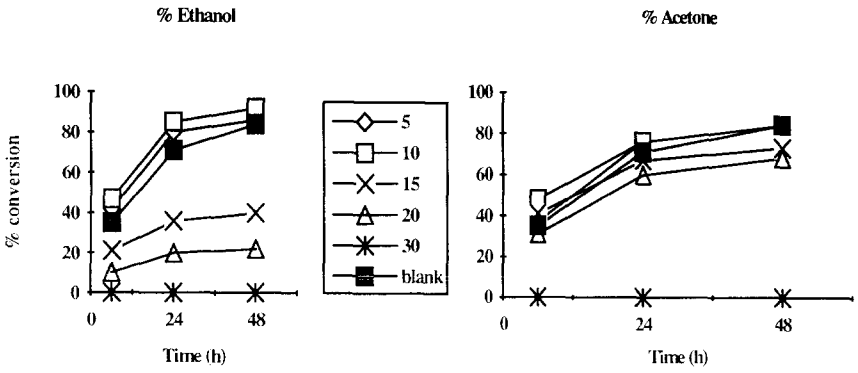
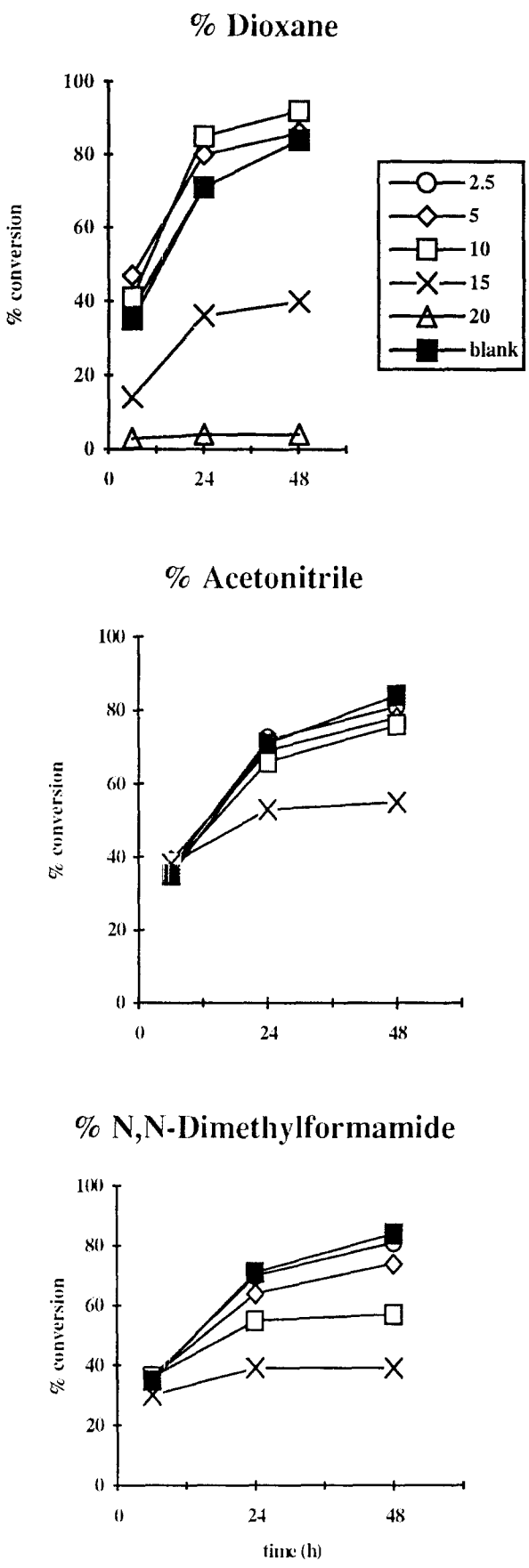


Fig. 2. Influence of various amounts of EtOH and acetone on the GalT-catalyzed galactosylation of colchicoside.



It was important to ascertain which enzyme was affected by the organic cosolvent. To this end, GalT was initially incubated in the reaction buffer containing 25 mM of manganese(II) chloride and 1 mg/mL of α-lactalbumin, together with 10% of the organic cosolvents but in the absence of the substrates. The residual activity was measured spectrophotometrically using a standard enzymatic coupled assay [17]. Under these non-operative conditions, GalT was quite rapidly inactivated even in the absence of cosolvents, its activity being reduced to 36% and 13% after 24 and 48 h of incubation, respectively. We found that the addition of some cosolvents (Me_2SO, acetone, dioxane, and EtOH) increased the stability of this enzyme, while other cosolvents (*N*,*N*-dimethylformamide, acetonitrile and tetrahydrofuran) enhanced the inactivation process. Therefore, we decided to compare the stability of GalT under reaction conditions, that is in the presence of UDP-glucose and of the substrate **1**. Since it was not possible to use the spectrophotometric assay, the reaction solutions (in which all the components but the epimerase were present) containing 10% v/v of the different cosolvents were incubated for 24 h. The epimerase was then added and the degree of conversions were monitored after 6 and 24 h. GalT confirmed to be unstable in tetrahydrofuran with less than 4% conversion after 24 h, while in the presence of (*N*,*N*-dimethylformamide and acetonitrile the conversions were lower than in the blank. All the other cosolvents gave results that were comparable to or slightly better than the blank. When the epimerase was treated in the same way (incubation under reaction conditions for 24 h, followed by the addition of GalT), the conversions obtained in the presence of all the cosolvents were similar, or slightly better, to the values previously obtained (see Table 1 and Figs. 1–3). These data indicate that GalT, being quite unstable under operative conditions, is the enzyme of the multienzymatic system of Scheme 1 that has to be added portionwise to force the reaction to complete conversion.

Since a large excess of UDP-glucose (7.5 eq) was used, we compared the performance of our multien-

Fig. 3. Influence of various amounts of dioxane, acetonitrile and *N*,*N*-dimethylformamide on the GalT-catalyzed galactosylation of colchicoside.

Table 2
Degrees of conversion of colchicoside (**1**) into its lactoside (**1a**) with or without 15% v/v Me_2SO[a,b]

% v/v of Me_2SO ([**1**] = 20 mM)	[UDP-glucose]mM	% Conversion		
		6 h	24 h	48 h
0	150	49	89	95
15	150	49	91	95
0	100	50	86	91
15	100	49	85	91
0	50	45	70	74
15	50	45	70	73
0	25	39	50	50
15	25	38	47	48

[a]Determined by HPLC.
[b]Conditions: see Table 1.

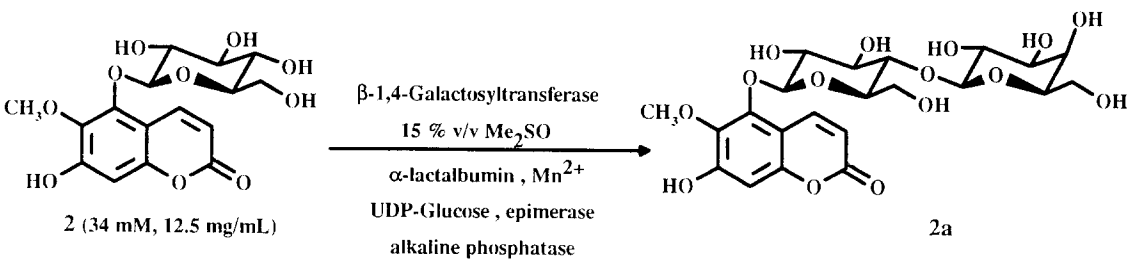
zymatic system in 15% Me_2SO at various ratios of donor and **1**. The results, reported in Table 2, show that the degrees of conversion observed with or without the cosolvent were comparable. Therefore, in cases where the direct transfer of glucose from UDP-glucose to a specific substrate is not negligible as it has been reported in Ref. [10], it is possible to lower the donor:acceptor ratio to eliminate this side reaction, having the system working even in the presence of significant amount of cosolvent.

As an application of our findings, we chose the coumarinic glucoside fraxin (**2**), as a model substrate, a compound that is almost insoluble in water (< 0.5 mg/mL). Fraxin was solubilized in the reaction buffer containing 15% v/v Me_2SO and a 98% conversion to the corresponding β-lactoside **2a** was obtained in 48 h (Scheme 2). The general applicability of the results described in this paper with other natural glucosides that are poorly soluble in buffered solutions is currently under investigation and the results will be reported in due course.

3. Experimental

General methods.—Colchicoside (**1**) was a generous gift from Indena, Milano (Italy). Fraxin (**2**) was purchased from Aldrich. UDP-glucose, α-lactalbumin from bovine milk, UDP-galactose-4′-epimerase (EC 5.1.3.2, from galactose-adapted yeast), and alkaline phosphatase (EC 3.1.3.1, from bovine intestinal mucose, type VII S) were from Sigma. β-1,4-Galactosyltransferase (EC 2.4.1.22, from bovine colostrum) was purified as described elsewhere [11], and its activity was checked by a spectrophotometric assay [17]. HPLC used a Jasco 880/PU pump connected to a Jasco 870/UV/VS detector and a Licrospher 100 RP-18 (5 μm, E. Merck, analytical); UV/VIS Spectrophotometer: Jasco V-530; NMR: Bruker 300 AC; FABMS: VG 7070 EQ-HF spectrometer equipped with its own source, operating at 8 keV with xenon gas and in diethanolamine as matrix. Melting points were determined using a Kofler apparatus and are uncorrected. Optical rotations were measured using a Perkin-Elmer 141 polarimeter. TLC used precoated Silica Gel $60F_{254}$ plates (E. Merck); Flash chromatography: Silica Gel 60 (70–230 mesh, E. Merck).

(S)-N-{3-[4-O-(β-D-galactopyranosyl)-β-D-glucopyranosyloxy]-5,6,7,9-tetrahydro-1,2,10-trimethoxy-9-oxobenzo[a]heptalen-7-yl}-acetamide (3-demethylcolchicine-3-O-lactoside, **1a**).—A soln of 50 mM Tris buffer pH 7.4 containing colchicoside (**1**, 44 mg, 0.08 mmol, 40 mM), UDP-glucose (57 mg, 0.1 mmol, 50 mM), manganese(II) chloride (2 mM), 0.35 U/mL GalT, 2 U/mL epimerase, 7 U/mL alkaline phosphatase, 1 mg/mL α-lactalbumin was incubated at 30 °C adjusting the pH with 0.25 M NaOH daily and following the reaction by TLC and HPLC (92:8 acetonitrile–water containing 0.01% CF_3COOH), flow rate 1 mL/min, λ = 254 nm). After 5 days, 71% conversion to a single product was observed. Water was evaporated and the crude residue was purified by flash chromatography to give 31 mg (0.044 mmol, 55% isolated yield) of **1a**; mp 220 °C; $[\alpha]_D$ −104.6 (*c* 0.5, water); R_f 0.24 (8:4:1 AcOEt–MeOH–water); ^1H NMR (Me_2SO-d_6 80 °C): δ 8.35 (d, 1 H, $J_{NH,7}$ 7.5 Hz, –NHCO), 7.18 (s, 1 H,



Scheme 2. Enzymatic galactosylation of fraxin (**2**).

H-8), 7.12 (d, 1 H, $J_{11,12}$ 9.5 Hz, H-12), 7.01 (d, 1 H, H-11), 6.88 (s, 1 H, H-4), 5.01 (d, 1 H, $J_{1',2'}$ 7.5 Hz, H-1'), 4.39 (dt, 1 H, $J_{6ax,7}$ 7, $J_{6eq,7} = J_{NH,7}$ 7.5, H-7), 4.33 (d, 1 H, $J_{1,2}$, H-1''), 3.90, 3.88 and 3.60 (s, 3 H each, CH_3O), 1.88 (s, 3 H, CH_3CO). ^{13}C NMR (Me_2SO-d_6): δ 178.4 (C-9), 169.2 (NHCO), 163.9 (C-10), 151.2, 151.0 and 150.7 (C-7a, C-3 and C-1), 141.5 (C-2), 135.5 (C-12a), 135.0 (C-12), 134.3 (C-4a), 130.5 (C-8), 127.0 (C-1a), 112.6 (C-11), 111.3 (C-4), 104.0 (C-1''), 100.3 (C-1'), 80.3 (C-4'), 75.7 (C-5''), 75.4 and 75.2 (C-5' and C-3'), 73.3 (C-2'), 73.2 (C-3''), 70.8 (C-2''), 68.3 (C-4''), 61.3 (CH_3O), 61.2 (CH_3O), 60.6 (C-6''), 60.3 (C-6'), 56.4 (CH_3O), 51.6 (C-7), 35.8 (C-5), 29.4 (C-6), 22.6 (CH_3). FAB-MS: 709 ([M–H]$^-$, 8), 677 ([M–MeOH–H]$^-$, 10), 547 ([M–Gal–H]$^-$, 60), 531 (23), 515 ([M–Gal–MeOH–H]$^-$, 22), 385 ([M–Gal–Glc–H]$^-$, 100), 371 (52).

Galactosylation of colchicoside (**1**) *in the presence of various amounts of organic cosolvents.*—In a total volume of 450 μL, various amounts (v/v) of organic cosolvents were added to a 50 mM Tris buffer pH 7.4 containing 20 mM **1**, 150 mM UDP-glucose, 25 mM manganese(II) chloride, 1 mg/mL α-lactalbumin, 1 U/mL GalT, 1.8 U/mL epimerase, 5 U/mL alkaline phosphatase, 0.01% NaN_3, and 1 mM dithiothreitol. The reactions were incubated at 30 °C and samples were removed after 6, 24 and 48 h, diluted 20-fold and 1 μL was injected for HPLC analysis.

Reaction were repeated at least twice and the mean values obtained were the following, reported as: organic cosolvents, % v/v, conversion at 6 h, conversion at 24 h, conversion at 48 h. Blank, 0, 35, 71, 84. Me_2SO, 5, 35, 72, 84; Me_2SO, 10, 36, 74, 87; Me_2SO, 15, 43, 75, 86; Me_2SO, 20, 36, 70, 74; Me_2SO, 30, 4, 22, 35; Me_2SO, 40, 0, 0, 0. MeOH, 5, 34, 77, 86; MeOH, 10, 42, 80, 89; MeOH, 15, 44, 84, 89; MeOH, 20, 26, 63, 75; MeOH, 30, 3, 11, 13; MeOH, 40, 0, 0, 0. EtOH, 5, 42, 80, 86; EtOH, 10, 47, 85, 92; EtOH, 15, 21, 36, 40; EtOH, 20, 10, 20, 22; EtOH, 30, 0, 0, 0. Acetone, 5, 37, 76, 84; acetone, 10, 48, 76, 84; acetone, 15, 41, 67, 73; acetone, 20, 31, 60, 68; acetone, 30, 0, 0, 0. Dioxane, 2.5, 38, 71, 84; dioxane, 5, 47, 80, 86; dioxane, 10, 41, 85, 92; dioxane, 15, 14, 36, 40; dioxane, 20, 3, 4, 4. Acetonitrile, 2.5, 37, 72, 81; acetonitrile, 5, 39, 69, 78; acetonitrile, 10, 37, 66, 76; acetonitrile, 15, 38, 53, 55. *N,N*-Dimethylformamide (DMF), 2.5, 36, 70, 81; DMF, 5, 36, 64, 74; DMF, 10, 36, 55, 57; DMF, 15, 30, 39, 39.

8-[4-O-(β-D-galactopyranosyl)-β-D-glucopyranosyloxy]-7-hydroxy-6-methoxy-2H-1-benzopyran-2-one (fraxetin-8-O-lactoside, **2a**).—In a total volume of 4 mL, 50 mg (0.135 mmol, 34 mM) of **2** dissolved in 600 μL of Me_2SO were added to a 50 mM Tris buffer solution, pH 7.4, containing 360 mg of UDP-glucose (final concentration 158 mM), 25 mM manganese(II) chloride, 1 mg/mL α-lactalbumin, 0.5 U/mL GalT, 2 U/mL epimerase, 3.5 U/mL alkaline phosphatase, 0.01% NaN_3, 1 mM dithiothreitol. The reaction was incubated at 30 °C for 48 h. After this time, a 98% conversion to a single product was monitored by HPLC (92:8 acetonitrile–water (containing 0.01% CF_3COOH), flow rate 1 mL/min, λ = 254 nm). The solvent was evaporated and the residue was purified by flash chromatography (10:3:0.8 AcOEt, MeOH, water) to give 61 mg (0,115 mmol, 85% isolated yield, of **2a**. mp 218 °C, $[\alpha]_D$ 52.4 ($c \neq 0.5$, Me_2SO); R_f 0.19; ^1H NMR (Me_2SO-d_6): δ 7.90 (d, 1 H, $J_{3,4}$ 10.5 Hz, H-4), 7.08 (s, 1 H, H-5), 6.27 (d, 1 H, H-3), 5.00 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1'), 4.27 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1''), 3.83 (s, 3 H, CH_3O). ^{13}C NMR (Me_2SO-d_6): δ 160.9 (C-2), 145.9, 144.0 and 142.9 (C-6, C-8 and C-8a), 145.4 (C-4), 131.7 (C-7), 112.6 (C-3), 110.7 (C-4a), 105.5 (C-5), 104.0 (C-1' and C-1''), 80.3 (C-4'), 75.9 (C-5''), 75.5 (C-5'), 74.9 (C-3'), 73.8 (C-2'), 73.4 (C-3'), 70.9 (C-2''), 68.5 (C-4''), 60.8 and 60.5 (C-6' and C-6'), 56.5 (CH_3O). FAB-MS: *m/z* 531 ([M–H]$^-$, 20) 369 ([M–Gal–H]$^-$, 55), 207 ([M–Gal–Glc]$^-$, 100).

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