86. Regioselective Enzyme-Mediated Glycosylation of Natural Polyhydroxy Compounds

Part 1

Galactosylation of Stevioside and Steviolbioside

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Bovine β-1,4-galactosyltransferase is an efficient catalyst for the regioselective transfer of galactose from UPD-galactose, generated *in situ* with the UDP-glucose/UDP-glucose-4-epimerase system, to the kaurane glycosides stevioside (**1**) and steviolbioside (**2**), affording the corresponding galactosyl derivatives **3** and **4** in high yields. By a combination of 2D NMR techniques (COSY, TOCSY, ROESY, HMQC, and HMBC), the structure of the products is established as 13-[(β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl)oxy]kaur-16-en-19-oic acid β-D-glucopyranosyl ester (**3**) and 13-[(β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl)oxy]kaur-16-en-19-oic acid (**4**).

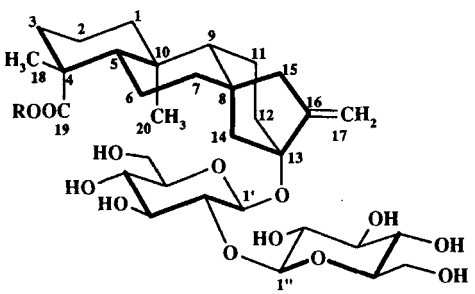
Introduction. – Stevioside (**1**), a diterpenoid glycoside isolated from the South American plant *Stevia rebaudiana* BERTONI (Compositae) is used, either alone or in combination, for sweetening drinks and other foods [1]. Because of a slight bitter residual aftertaste and a low solubility, the modification of the sugar moieties of stevioside is currently under investigation with the purpose of obtaining new derivatives with improved organoleptic properties and increased solubility. To this end, various glycosidases [2] and cyclodextrin-glucosyltransferase [3] have been used to link additional sugar units. These reactions being not selective, a complex mixture of products was usually obtained.

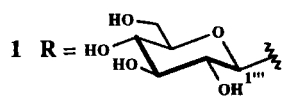
Within the scope of our general interest in applied biocatalysis [4], we recently started a project aimed at exploiting glycosyltransferases for the introduction of a sugar residue into saccharide units. This biocatalytic methodology is highly regio- and stereoselective [5], easy to carry out (activation of the glycosidic bond is performed *in situ* employing an unprotected sugar precursor, thus avoiding the preliminary elaboration of the carbo-

hydrates), and allowed to prepare glycopeptides and specific derivatives of simple carbohydrates [6].

Our attention was drawn to the glycosylation of complex natural glycosides by a recent report by *Kren*, *Augé*, and coworkers [7], in which the alkaloid elymoclavine 2-acetamido-2-deoxy-β-D-glucopyranoside was successfully glycosylated adding a galactose or a glucose unit to its sugar moiety by using the β-1,4-galactosyltransferase from bovine milk (GalT).

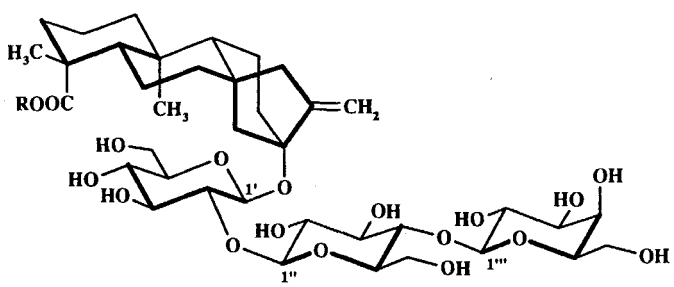
We decided to apply the same methodology to galactosylate stevioside (**1**) and its congener steviolbioside (**2**), and report now the results of our efforts which led to the regioselective synthesis of the galactosyl derivatives **3** and **4** in good yields. By a combination of COSY 45, 2D TOCSY, 2D ROESY, HMQC, and HMBC experiments, we established the structure of these new derivatives and completely assigned their ^{13}C- and ^{1}H-NMR spectra.

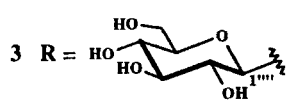




1 R =

2 R = H



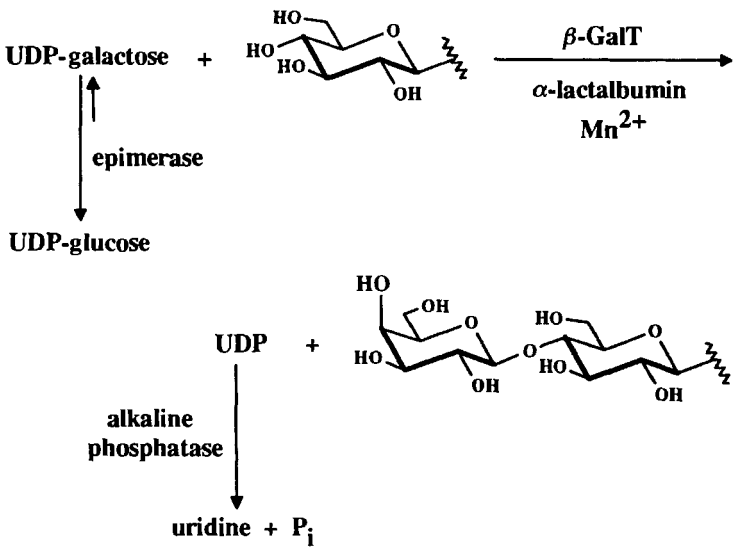


3 R =

4 R = H

Results and Discussion. – 1. *Enzymatic Galactosylation.* The multienzymatic process used for galactosylation is described in the *Scheme* and was performed according to the methodology introduced by *Wong* and *Whitesides* [8] and modified by *Paulson* and coworkers [9]. Being highly expensive, β-1,4-galactosyltransferase (GalT) was partially purified by us from bovine colostrum [6]. UDP-Galactose served as galactosyl donor and was generated *in situ* from the much cheaper UDP-glucose by action of the enzyme UDP-glucose-4-epimerase. Alkaline phosphatase was added to hydrolyze the condensation by-product UDP, which is a strong inhibitor of GalT, to uridine and inorganic phosphate (P_i). Manganese ion and α-lactalbumin were also added, as they are essential cofactors for the catalytic action of GalT. Due to the low solubility of **1** and **2** in the aqueous buffer, the reactions were performed in the presence of 2.5% of DMF.

Scheme



Both stevioside and steviolbioside proved to be substrates for this transferase. The reactions were almost complete in a week and highly regioselective, anal. HPLC showing the formation of a single product in 81 and 95% yield, respectively. Contrary to what was observed by *Kren*, *Augé*, and coworkers [7], no reaction occurred in the absence of UDP-glucose-epimerase, showing that GalT is not able to transfer glucose from UDP-glucose to **1** or **2**.

Usual chromatographic workup allowed the isolation of the pure monogalactosyl derivatives **3** and **4** whose structure determination will be described in the following.

2. *Structure Elucidation.* The MS and NMR spectra of the galactosyl derivatives **3** and **4** were examined in comparison with the ones of the parent compounds stevioside (**1**) and steviolbioside (**2**).

The positive- and negative-mode fast atom bombardment mass spectra (FAB-MS) of all the glycosides were of low intensity and not informative. On the contrary, bombardment of a glycerol solution of the glycosides with Cs ions afforded valuable negative-mode LSI (liquid secondary ionization) MS which contained structurally diagnostic peaks. In the spectrum of **1**, the pseudomolecular-ion peak at *m/z* 803 ($[M - H]^-$) was of low intensity, the dominant ion being at *m/z* 641 ($[M - H - \text{hexose}]^-$), generated by the loss of the hexose residue linked to the carboxy function. The last mentioned ion was the base peak in the spectrum of **2**. It was, therefore, to be expected that, in case the galactosylation of stevioside (**1**) had occurred on the glucose bound to the 4-carboxylic group, an abundant fragment ion at *m/z* 641 resulting from the favored loss of the galactopyranosyl-glucopyranosyl residue could be detected in the mass spectrum of the galactosylated derivative, in addition to a less intense pseudomolecular ion. However, this was not the case: the MS of **3** contained a low-intensity pseudomolecular-ion peak at *m/z* 965 and a very intense peak at *m/z* 803, indicating that one galactose residue had been attached to the disaccharide chain bound at C(13). The same conclusion could be drawn from the spectrum of the galactosylated steviolbioside **4**.

In the absence of an enzymatic method to selectively cleave the β-(2′ → 1″) bond, the position of attack of the newly introduced galactose moiety to the external glucose of the sophorose disaccharide was determined by a comprehensive NMR analysis. The ^{1}H-NMR spectra [10] of **1** and **2**, as well as their ^{13}C-NMR data [11], have been fully analyzed for (D_5)pyridine solutions. However, except for the signals of the anomeric protons, the majority of the sugar-proton resonances of **3** and **4** in this solvent greatly overlapped between 3.4 and 4.4 ppm, thus inhibiting a full assignment of the signals. We found that (D_5)pyridine/CD_3OD 1:1 (*v*/*v*) was a more suitable solvent and allowed to obtain well resolved spectra with a good dispersion of signals. Because of the strong influence of the solvent on the chemical shifts of the sugar protons, the spectra of **1** and **2** had to be reexamined.

Identification of the spin system of the individual monosaccharides and complete assignment of proton resonances were achieved through the TOCSY [12] experiment with long mixing times which ensured the detection of a transfer of magnetization from the anomeric proton to the corresponding CH_2(6). These data were complemented by a COSY-45 experiment which was used to trace each magnetization transfer between vicinal coupled protons. With the proton resonances assigned, the ^{13}C-NMR chemical shifts were readily deduced by a HMQC [13] spectrum.

The sugar sequence and linkage sites were examined by cross-relaxation experiments (ROESY [14]) which revealed intra- and interresidue correlations. Finally, confirmation of the sugar connectivities and assignment for the sugar moieties were obtained by extensive analysis of the HMBC [15] spectra. In a similar way, the aglycon resonances were attributed. The NMR data of **3** are collected in the *Table*, those of the other compounds are reported in the *Exper. Part.*

As an example of structure analysis, the linkage site of the galactosyl moiety in the oligosaccharide chain of **3** consisting of three sugar residues was discovered by first assigning H–C(1′) from the cross-peak to the easily identified C(13) in the HMBC spectrum. Then C(2′) was identified by a combination of COSY and HMBC, and from this it was possible to attribute H–C(1″). The *J*-network of the second glucose residue revealed H–C(4″) at low field attached to a deshielded C(4″), suggesting that glycosylation occurred at this position. In fact, C(4″) showed a cross-peak to H–C(1‴) in the HMBC spectrum, and H–C(4″) and H–C(1‴) were found to be close in space from an interresidue cross-peak in the ROESY spectrum. That the introduced sugar is a β-galactopyranoside was deduced from *J*-coupling information. H–C(1‴) showed a strong coupling to H–C(2″), whereas H–C(4‴) was weakly coupled to the neighboring protons. The resonances of the glucose moiety attached to the C(19) carboxy function were identified starting from the easily assigned H–C(1⁗) at low field.

Therefore, the structures of the products were established as 13-[(β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl)oxy]kaur-16-en-19-oic acid β-D-glucopyranosyl ester (**3**) and 13-[(β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl)oxy]kaur-16-en-19-oic acid (**4**), respectively.

The ROESY data spectra can be used to depict a preferred interresidue conformation of these glycosides in CD_3OD–(D_5)pyridine 1:1. From these informations, the conformation of **1** drawn in the *Figure* can be deduced.

In stevioside (**1**), H–C(1′) has a strong NOE contact to both H–C(12) and H–C(14), H–C(1″) is faced to H–C(2′), and H–C(5″) has a strong interaction to the olefinic proton at δ 5.12. The other olefinic proton at δ 4.82 strongly interacts with both protons at C(15). The anomeric proton of the glucose linked to the carboxy function at C(19) has interactions with H_{ax}–C(6) and CH_3(20). Moreover, the anomeric proton of the galactose moiety is eclipsed to H–C(4″) in the galactosyl derivatives.

Table. *^{1}H- and ^{1}C-NMR Data of* **3** *in CD_3OD/(D_5)Pyridine 1:1*

	$\delta(^{13}C)$ [ppm]	$\delta(^1H)$[a]) [ppm] (J(H, H)[b]) [Hz]		$\delta(^{13}C)$ [ppm]	$\delta(^1H)$[a]) [ppm] (J(H, H)[b]) [Hz])
Aglycone:					
CH_2(1)	41.3	0.64, 1.62	CH_2(11)	21.2	1.42–1.57
CH_2(2)	19.8	1.95, 1.28	CH_2(12)	37.2	1.95, 1.59
CH_3(3)	38.7	0.88, 2.10	C(13)	87.1	
C(4)	44.7		CH_2(14)	44.9	2.16, 1.50
H–C(5)	58.1	0.92	CH_2(15)	48.3	1.84–1.97
CH_2(6)	22.6	2.15, 1.70	C(16)	154.7	
CH_2(7)	42.2	1.15–1.26	CH_2(17)	105.2	5.29, 4.82
C(8)	43.2		Me(18)	28.7	1.08
H–C(9)	54.7	0.78	C(19)	177.9	
C(10)	40.5		Me(20)	16.1	1.00
β-D-Glucopyranose:				β-D-Glucopyranose:	
H–C(1′)	97.9	4.73 (^3J(1′,2′) = 7.8)	H–C(1″)	106.6	4.77 (^3J(1″,2″) = 7.8)
H–C(2′)	85.0	3.64 (^3J(2′,3′) = 9.6)	H–C(2″)	76.3	3.70 (^3J(2″,3″) = 9.3)
H–C(3′)	78.4	3.83 (^3J(3′,4′) = 8.3)	H–C(3″)	76.5	3.78 (^3J(3″,4″) = 8.1)
H–C(4′)	72.1	3.56 (^3J(4′,5′) = 9.8)	H–C(4″)	81.9[c])	3.85 (^3J(4″,5″) = 9.6)
H–C(5′)	78.0	3.46 (^3J(5′,6′a) = 2.5), ^3J(5′,6′b) = 4.1)	H–C(5″)	77.0	3.53 (^3J(5″,6″a) = 2.2), ^3J(5″,6″b) = 3.8)
CH_2(6′)	63.0	4.10, 3.81 (^2J(6′a,6′b) = 11.2)	CH_2(6″)	62.5	4.13, 4.05 (^2J(6″a,6″b) = 11.7)
β-D-Galactopyranose:				β-D-Glucopyranose:	
H–C(1‴)	105.8	4.67 (^3J(1‴,2‴) = 7.8)	H–C(1⁗)	96.0	5.64 (^3J(1⁗,2⁗) = 8.2)
H–C(2‴)	72.7	3.97 (^3J(2‴,3‴) = 9.6)	H–C(2⁗)	74.1	3.67 (^3J(2⁗,3⁗) = 9.6)
H–C(3‴)	75.1	3.75 (^3J(3‴,4‴) = 3.9)	H–C(3⁗)	79.0	3.73 (^3J(3⁗,4⁗) = 8.3)
H–C(4‴)	70.3	4.03 (^3J(4‴,5‴) = 3.7)	H–C(4⁗)	71.0	3.73 (^3J(4⁗,5⁗) = 9.4)
H–C(5‴)	77.4	3.82 (^3J(5‴,6‴a) = 2.4), ^3J(5‴,6‴b) = 3.6)	H–C(5⁗)	79.2	3.58 (^3J(5⁗,6⁗a) = 2.3, ^3J(5⁗,6⁗b) = 3.9)
CH_2(6‴)	62.3	4.04, 3.92 (^2J(6‴a,6‴b) = 10.5)	CH_2(6⁗)	62.2	4.01, 3.70 (^2J(6⁗a,6⁗b) = 11.3)

[a]) The first mentioned δ refers to the axial, the second to the equatorial H-atom (aglycone).
[b]) ΔJ(H, H) = 0.1 Hz.
[c]) Stevioside (**1**): δ 71.4.

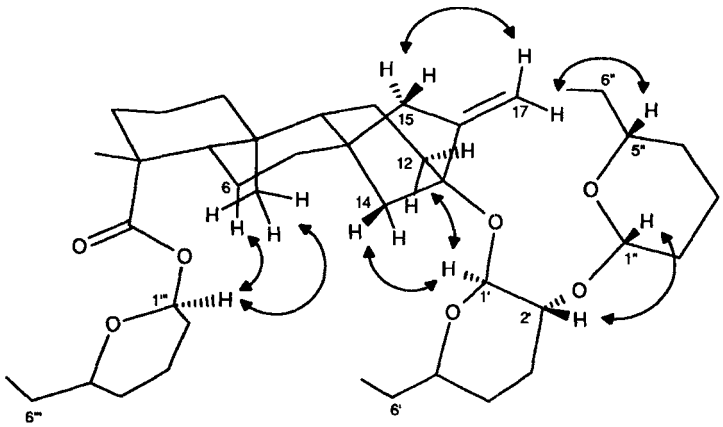


Figure. *Interresidue conformation of stevioside* (**1**) *as suggested by ROE interactions* (arrows)

3. *Conclusion.* We have shown that the β-1,4-galactosyltransferase (GalT) from bovine colostrum is able to galactosylate in good yield the diterpene glucosides stevioside (**1**) and steviolbioside (**2**) at OH–C(4) of the external glucose of the disaccharide moiety attached at C(13) of the aglycone skeleton. No other isomers could be detected, thus confirming the high regioselectivity of this enzymatic system. At variance to previous observations with another substrate [7], GalT is not able to transfer glucose to **1** and **2**.

The solubility in H_2O of the galactosyl derivative **3** was increased to 1.4 g/l from 1.25 g/l for stevioside. The relative sweetness, evaluated by a professional human sensory panel [16], was nearly × 200 with respect to sucrose (stevioside is × 160). The quality of taste was also significantly improved, with a decrease of aftertaste and increase of deliciousness. These results are in agreement with the accepted structure-sweetness relationships which indicate that transglycosidation at the glucosyloxy residue at C(13) results in better intensity and quality of sweetness.

Experimental Part

General. Stevioside (**1**) and steviolbioside (**2**) were a generous gift from *Indena*, Milano. UDP-Glucose, lactalbumin from bovine milk, UDP-glucose-4-epimerase (EC 5.1.3.2, from galactose-adapted yeast), 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 milk) was purified as described elsewhere [6a], and activity checked according to a spectrophotometric assay [17]. HPLC: *Jasco 880/PU* pump, *Jasco 870/UV/VS* detector; *Lichrospher 100 RP-18* (5 μm, *Merck*; anal.) and *Partisil 10 ODS-3* (*Whatman*, prep). NMR Spectra: *Bruker-Advance-500* spectrometer, at 500 MHz for ^1H and 100.3 MHz for ^{13}C; 5-mm reverse probe head (temp. 303 K, ^{13}C 90° pulse width = 9.4–10.5 μs, ^1H 90° pulse width = 9.1–9.6 μs); samples in $CD_3OD/(D_5)$pyridine 1:1, c = 20–40 mg ml^{-1}; chemical shifts δ in ppm rel. to CH_3OH as internal standard (^1H: δ 3.3 ppm; ^{13}C: δ 49.0 ppm). Further pulses: WALTZ ^1H-decoupling pulse: 110 ms; GARP ^{13}C-decoupling pulse: 65 ms; MLEV-17 pulse: 225 ms. COSY: 45° mixing pulse. TOCSY: phase sensitive using TPPI, mixing time 80–110 ms (100 MLEV-17 cylces plus two trim pulses of 2.5 ms each). ROESY: phase-sensitive using TPPI, spinlock cw pulse (250 ms). HMQC: phase-sensitive using TPPI, BIRD sequence, GARP-decoupled. HMBC: phase-sensitive using TPPI, delay to achieve long-range couplings 71 ms (J(C,H) = 7 Hz). LSI-MS: *MAT 8500* (*Finnigan*), matrix glycerol, 4.5 kV Cs beam, negative-ion mode.

13-[(2-O-β-D-Glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-19-oic Acid β-D-Glucopyranosyl Ester (**1**). ^1H-NMR: 0.64(H_{ax}–C(1)); 1.62(H_{eq}–C(1)); 1.95(H_{ax}–C(2)); 1.29(H_{eq}–C(2)); 0.89(H_{ax}–C(3)); 2.10(H_{eq}–C(3));

0.92 (H_{ax}–C(5)); 1.70 (H_{eq}–C(6)); 2.16 (H_{ax}–C(6)); 1.14–1.26 (2H–C(7)); 0.76 (H_{ax}–C(9)); 1.40–1.49 (2H–C(11)); 1.97 (H_{ax}–C(12)); 1.61 (H_{eq}–C(12)); 2.38 (H_{ax}–C(14)); 1.51 (H_{eq}–C(14)); 1.85–1.96 (2H–C(15)); 5.35 (H–C(17)); 4.84 (H′–C(17)); 1.07 (Me(18)); 1.00 (Me(20)); 4.76 (*d*, *J*(1′,2′) = 7.8, H–C(1)); 3.72 (*dd*, *J*(2′,3′) = 9.3, H–C(2′)); 3.84 (*dd*, *J*(3′,4′) = 8.3, H–C(3′)); 3.55 (*dd*, *J*(4′,5′) = 10.2, H–C(4′)); 3.48 (*m*, *J*(5′,6′a) = 2.4, *J*(5′6′b) = 4.8, H–C(5′)); 4.13 (*dd*, ^2J = 11.9, H_a–C(6′)); 3.83 (*dd*, H_b–C(6′)); 4.86 (*d*, *J*(1″,2″) = 7.7, H–C(1″)); 3.68 (*dd*, *J*(2″,3″) = 8.9, H–C(2″)); 3.75 (*dd*, *J*(3″,4″) = 8.1, H–C(3″)); 3.81 (*dd*, *J*(4″,5″) = 9.5, H–C(4″)); 3.52 (*m*, *J*(5″,6″a) = 2.7, *J*(5″,6″b) = 4.9, H–C(5″)); 4.08 (*dd*, ^2J = 11.8, H_a–C(6″)); 3.97 (*dd*, H_b–C(6″)); 5.65 (*d*, *J*(1‴,2‴) = 8.2, H–C(1‴)); 3.68 (*dd*, *J*(2‴,3‴) = 9.6, H–C(2‴)); 3.76 (*dd*, *J*(3‴,4‴) = 8.1, H–C(3‴)); 3.74 (*dd*, *J*(4‴,5‴) = 9.8, H–C(4‴)); 3.56 (*m*, *J*(5‴,6‴a) = 2.3, *J*(5‴,6‴b) = 7.3, H–C(5‴)); 4.02 (*dd*, ^2J = 12.1, H_a–C(6‴)); 3.91 (*dd*, H_b–C(6‴)). ^{13}C-NMR: 41.5 (C(1)); 19.9 (C(2)); 38.9 (C(3)); 44.6 (C(4)); 58.1 (C(5)); 22.8 (C(6)); 42.3 (C(7)); 43.2 (C(8)); 54.6 (C(9)); 40.4 (C(10)); 21.2 (C(11)); 37.4 (C(12)); 87.0 (C(13)); 45.1 (C(14)); 48.3 (C(15)); 154.7 (C(16)); 105.2 (C(17)); 28.7 (C(18)); 177.8 (C(19)); 16.1 (C(20)); 98.1 (C(1′)); 84.0 (C(2′)); 78.4 (C(3′)); 72.2 (C(4′)); 78.0 (C(5′)); 63.0 (C(6′)); 106.4 (C(1″)); 76.9 (C(2″)); 79.1 (C(3″)); 71.4 (C(4″)); 78.7 (C(5″)); 62.8 (C(6″)); 96.0 (C(1‴)); 74.2 (C(2‴)); 78.1 (C(3‴)); 71.1 (C(4‴)); 79.3 (C(5‴)); 62.3 (C(6‴)): LSI-MS: 803 (7, [*M* – H]$^-$), 641 (100, [*M* – H – hexose]$^-$), 479 (18, [*M* – H – hexose – hexose]$^-$), 317 (12, [aglycone – H]$^-$).

*13-[(2-*O*-β-*D*-Glucopyranosyl-β-*D*-glucopyranosyl)oxy]kaur-16-en-19-oic Acid* (**2**). ^{1}H-NMR: 0.66 (H_{ax}–C(1)); 1.65 (H_{eq}–C(1)); 1.92 (H_{ax}–C(2)); 1.31 (H_{eq}–C(2)); 0.89 (H_{ax}–C(3)); 2.16 (H_{eq}–C(3)); 0.89 (H–C(5)); 1.88 (H_{ax}–C(6)); 1.72 (H_{eq}–C(6)); 1.16–1.27 (2H–C(7)); 0.79 (H–C(9)); 1.49 (H_{ax}–C(11)); 1.58 (H_{eq}–C(11)); 1.93 (H_{ax}–C(12)); 1.62 (H_{eq}–C(12)); 2.28 (H_{ax}–C(14)); 1.50 (H_{eq}–C(14)); 1.89–1.98 (2H–C(15)); 4.87 (H–C(17)); 5.38 (H′–C(17)); 1.11 (Me(18)); 0.95 (Me(20)); 4.79 (*d*, *J*(1′,2′) = 7.7, H–C(1′)); 3.75 (*dd*, *J*(2′,3′) = 8.8, H–C(2′)); 3.87 (*dd*, *J*(3′4′) = 8.3, H–C(3′)); 3.70 (*dd*, *J*(4′,5′) = 10.2, H–C(4′)); 3.39 (*m*, *J*(5′,6′a) = 2.5, *J*(5′,6′b) = 4.4, H–C(5′)); 3.98 (*dd*, ^2J = 11.9, H_a–C(6′)); 3.85 (*dd*, H_b–C(6′)); 4.88 (*d*, *J*(1″,2″) = 7.8, H–C(1″)); 3.62 (*dd*, *J*(2″,3″) = 9.0, H–C(2″)); 3.74 (*dd*, *J*(3″,4″) = 8.2, H–C(3″)); 3.70 (*dd*, *J*(4″,5″) = 9.6, H–C(4″)); 3.52 (*m*, *J*(5″,6″a) = 2.7, *J*(5″,6″b) = 5.3, H–C(5″)); 4.06 (*dd*, ^2J = 11.8, H_b–C(6″)); 3.94 (*dd*, H_a–C(6″)). ^{13}C-NMR: 41.6 (C(1)); 20.2 (C(2)); 39.1 (C(3)); 44.4 (C(4)); 57.7 (C(5)); 23.1 (C(6)); 42.3 (C(7)); 43.1 (C(8)); 54.8 (C(9)); 40.4 (C(10)); 21.2 (C(11)); 38.0 (C(12)); 87.2 (C(13)); 45.3 (C(14)); 48.5 (C(15)); 154.4 (C(16)); 105.5 (C(17)); 29.6 (C(18)); 180.9 (C(19)); 16.4 (C(20)); 97.9 (C(1′)); 83.7 (C(2′)); 78.3 (C(3′)); 71.6 (C(4′)); 78.0 (C(5′)); 62.7 (C(6′)); 106.2 (C(1″)); 76.9 (C(2″)); 78.0 (C(3″)); 71.9 (C(4″)); 78.7 (C(5″)); 63.0 (C(6″)). LSI-MS: 641 (100, [*M* – H]$^-$), 479 (15, [*M* – H – hexose]$^-$), 317 (10, [aglycone – H]$^-$).

General Procedure for the Enzymatic Galactosylation of Stevioside (**1**) *and Steviolbioside* (**2**). The glucoside (0.05 mmol) dissolved in 50 µl of DMF and UDP-glucose (56 mg, 0.1 mmol) were added to 2 ml of 50 mM *Tris* buffer pH 7.4, containing $MnCl_2$ (2 mM), *threo*-1,4-dimercaptobutane-2,3-diol (DTT; 1 mM) and NaN_3 (0.01%). Lactalbumin (1 mg), β-1,4-galactosyltransferase (1 U), UDP-glucose-4-epimerase (4 U), and alkaline phosphatase (6 µl, 15 U) were added, and the soln. was left at 37° for a week, adjusting the pH daily.

13-[(β-D-Galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl)oxy]kaur-16-en-19-oic Acid β-D-Glucopyranosyl Ester (**3**). The reaction was monitored by reversed-phase HPLC (eluent H_2O/MeCN 75:25, flow rate 1 ml/min, λ 200 nm). The peak corresponding to the only product (81%; t_R 14 min) was isolated by prep. reversed-phase HPLC to give 33 mg (69% isolated yield) of **3**. ^{1}H- and ^{13}C-NMR: *Table*. LSI-MS: 965 (6, [*M* – H]$^-$), 803 (100, [*M* – H – hexose]$^-$), 641 (35, [*M* – H – hexose – hexose]$^-$), 479 (20, [*M* – hexose – hexose – hexose]$^-$), 317 (10, [aglycone – H]$^-$).

13-[(β-D-Galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranosyl)oxy]kaur-16-en-19-oic Acid (**4**). The reaction was monitored by reversed-phase HPLC (H_2O/MeCN 70:30, flow rate 1 ml/min, λ 200 nm). The peak corresponding to the only product (95%; t_R 7 min) was isolated by flash chromatography (AcOEt/MeOH/H_2O 8:3:1): 25 mg (65% isolated yield) of **4**. ^{1}H-NMR: 1.20 (H_{ax}–C(1)); 2.08 (H_{eq}–C(1)); 4.08 (H_{ax}–C(2)); 4.08 (H_{eq}–C(2)); 3.97 (H_{ax}–C(3)); 3.97 (H_{eq}–C(3)); 1.59 (H–C(5)); 1.68 (H_{ax}–C(6)); 1.33 (H_{eq}–C(6)); 1.52 (H_{ax}–C(7)); 1.32 (H_{eq}–C(7)); 1.59 (H–C(9)); 2.02 (H_{ax}–C(11)); 1.92 (H_{eq}–C(11)); 5.28 (H_{ax}–C(12)); 5.28 (H_{eq}–C(12)); 1.72 (H_{ax}–C(14)); 1.18 (H_{eq}–C(14)); 1.72 (H–C(15)); 1.18 (H′–C(15)); 2.00 (H–C(17)); 1.70 (H′–C(17)); 2.82 (Me(18)); 1.68 (Me(20)); 4.70 (*d*, *J*(1′,2′) = 7.7, H–C(1′)); 3.59 (*dd*, *J*(2′,3′) = 9.2, H–C(2′)); 3.73 (*dd*, *J*(3′,4′) = 8.3, H–C(3′)); 3.54 (*dd*, *J*(4′,5′) = 10.1, H–C(4′)); 3.31 (*m*, *J*(5′,6′a) = 2.5, *J*(5′,6′b) = 4.8, H–C(5′)); 3.90 (*dd*, ^2J = 12.0, H_a–C(6′)); 3.77 (*dd*, H_b–C(6′)); 4.74 (*d*, *J*(1″,2″) = 7.8, H–C(1″)); 3.50 (*dd*, *J*(2″,3″ = 9.1, H–C(2″)); 3.68 (*dd*, *J*(3″,4″) = 8.1, H–C(3″)); 3.68 (*dd*, *J*(4″,5″) = 9.8, H–C(4″)); 3.48 (*m*, *J*(5″,6″a) = 2.8, *J* = (5″,6″b) = 4.3, H–C(5″)); 4.02 (*dd*, ^2J = 11.9, H_a–C(6″)); 3.97 (*dd*, H_b–C(6″)); 4.50 (*d*, *J*(1‴,2‴) = 7.8, H–C(1‴)); 3.81 (*dd*, *J*(2‴,3‴) = 9.2, H–C(2‴)); 3.63 (*dd*, *J*(3‴,4‴) = 3.4, H–C(3‴)); 3.93 (*dd*, *J*(4‴,5‴) = 3.2, H–C(4‴)); 3.77 (*m*, *J*(5″,6″a) = 2.5, *J*(5″,6″b) = 4.1, H–C(5‴)); 3.95 (*dd*, ^2J = 10.5, H_a–C(6‴)). ^{13}C-NMR: 41.6 (C(1)); 21.2 (C(2)); 39.1 (C(3)); 44.5 (C(4));

57.9(C(5)); 23.1(C(6)); 43.2(C(7)); 43.2(C(8)); 55.0(C(9)); 40.5(C(10)); 21.2(C(11)); 38.1(C(12)); 87.4(C(13)); 45.4(C(14)); 48.5(C(15)); 154.3(C(16)); 105.4(C(17)); 29.6(C(18)); 181.3(C(19)); 16.5(C(20)); 97.8(C(1')); 83.7(C(2')); 78.2(C(3')); 71.5(C(4')); 77.8(C(5')); 62.7(C(6')); 105.8(C(1'')); 76.4(C(2'')); 76.2(C(3'')); 81.7(C(4'')); 77.0(C(5'')); 62.4(C(6'')); 105.7(C(1''')); 72.5(C(2''')); 75.0(C(3''')); 70.3(C(4''')); 77.2(C(5''')); 62.5(C(6''')). LSI-MS: 803 (100, $[M - H]^-$), 641 (33, $[M - H -$ hexose$]^-$), 479 (18, $[M - H -$ hexose $-$hexose$]^-$), 317 (15, [aglycone $- H]^-$).

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