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Remote control of enzyme selectivity: the case of stevioside and steviolbioside

Giorgio Colombo,[a] Sergio Riva[a,*] and Bruno Danieli[b]

[a]*Istituto di Chimica del Riconoscimento Molecolare, C.N.R., Via Mario Bianco 9, 20131 Milano, Italy*
[b]*Dipartimento di Chimica Organica ed Industriale, Università degli Studi di Milano, Via Venezian 21, 20133 Milano, Italy*

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Abstract—The remote control of lipase PS site- and regioselectivity by substrate modification has been observed in the acetylation of stevioside (**1**) and steviolbioside (**2**): deglucosylation at position C-19 changed the acylation site of the sophorose moiety linked at C-13. In fact, while esterification of **1** gave mainly the corresponding 6″-*O*-acetylated derivative, acylation of **2** gave exclusively the 6′-*O*-monoester. A possible rationale has been suggested, based on the conformational behavior of the substrates in different simulated solvents.

1. Introduction

Selectivity is surely one of the key factors, if not 'the' key factor, of the growing popularity of biocatalysis among organic chemists, as testified by the hundreds of reports describing striking enzymatic performances.[1] Among them, probably the most impressive ones are the few dealing with the ability of enzymes to recognize remotely located stereocenters, as it is presently impossible to match these transformations with classical chemical catalysts. Examples of discrimination of a stereogenic center three or more bonds apart from the reaction center have been reported, describing the use of hydrolases (proteases or lipases)[2] or oxidoreductases.[2a,3] For instance, Hedenstrom and co-workers have recently disclosed the highly enantioselective *Candida rugosa*-catalyzed esterification of a series of methyldecanoic acid with 1-hexadecanol in cyclohexane: using 8-methyldecanoic acid (in which the stereogenic center is located eight bonds apart from the reactive carboxylate) as a substrate, they still measured a significant and unexpectedly high *E* value (25).[4] Remote control of enantioselectivity is the common feature of all these papers, a property that we have also exploited for the kinetic resolution of racemic *N*-Boc-piperidine-2-ethanol, a building block for the stereoselective syntheses of piperidine alkaloids.[5] On the contrary, to our knowledge, there are no reports on the remote control of enzymatic site- and regioselectivity, and this is the topic that we would like to disclose in this paper.

2. Results and discussion

Enzymatic modification of natural glycosides is one of our research interest since the late eighties,[6] as these complex and often labile molecules are ideal targets for mild and selective biotransformations. Specifically, in the past we have reported on the use of hydrolases and glycosyltransferases for the modification of flavonoid, terpene and alkaloid glycosides, and we are presently adding glycosidases to our repertoire.[7] As proposed years ago, the efficient preparation of random libraries of new derivatives (so-called 'combinatorial biocatalysis')[8] is a useful tool to improve the properties of these biologically active compounds.

Stevioside (**1**) is a sweet diterpenoid glycoside isolated from the South American plant *Stevia rebaudiana* Bertoni. The presence of three glucopyranosyl moieties makes this compound an interesting model for enzymatic transformations. Accordingly, few years ago, we submitted **1** as well as its congener steviolbioside (**2**)—easily prepared from **1** by alkaline hydrolysis—to the action of the β-1,4-galactosyltransferase from bovine colostrum (GalT).[9] Despite the presence in **1** of three possible galactosylation sites (HO-4′; HO-4″; HO-4‴), not only was this biotransformation stereo- and regio-selective, but also completely 'site-selective', producing the tetrasaccharide derivative **3** as the only product. Similarly, GalT-catalysis on **2** gave an almost quantitative conversion to the HO-4″-galactosylated compound **4**.

In order to expand the number of stevioside and steviolbioside derivatives in our hands, we investigated the regioselective acylation of these diterpenic glycosides. A

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* Corresponding author. Tel.: +39-02-285-00032; fax: +39-02-2850-0036; e-mail address: sergio.riva@icrm.cnr.it

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preliminary screening indicated that lipase PS was the best catalyst for this biotransformation. Accordingly, stevioside was dissolved in THF containing vinyl acetate, the enzyme was added and the suspension shaken at 45 °C for 3.5 h. An almost 70% conversion to a major product and to a minor less polar by-product was observed and both compounds were isolated by flash chromatography (51 and 4% isolated yields, respectively). At the beginning only the most abundant compound was examined and characterized as 6″-*O*-acetyl stevioside (**6**) by usual spectroscopic techniques.

The ^{1}H NMR in DMSO-d_6 (^{1}H- and ^{13}C NMR spectra of **1** and **2** were previously examined in pyridine[10] and methanol–pyridine[9]) revealed three anomeric protons at 5.24, 4.42 and 4.37 ppm, respectively. The low field signal was attributed to H-1‴, that is to the anomeric proton of the glucose moiety connected to the carboxyl function. Analysis of the extended spin connections (TOCSY) indicated that the acetate group was not located on this glucose because all proton and carbon resonances were in agreement with those of an unsubstituted saccharide. The anomeric proton at 4.42 ppm was attributed to the H-1′ of the sophorose disaccharide because of its vicinal relationship to H-2′, in turn directly linked to the low field substituted C-2′ at 82.91 ppm, and because of the long range interaction with the quaternary C-13. That the acetate group was located on the external glucose of the sophorose unit was easily deduced by the *J*-network (vicinal, long-range and spatial coupling) connecting the last anomeric proton at 4.37 ppm to the low field C-6″ methylene protons at 4.19 and 3.95 ppm via H-2″ at 3.03, H-3″ at 3.10, H-4″ at 3.19 and H-5″ at 3.30 ppm.

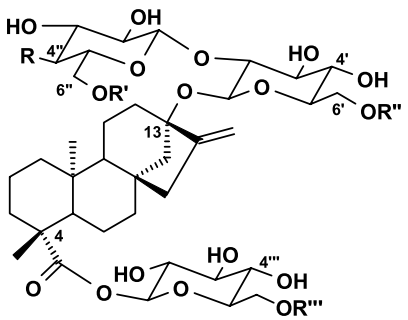
This result was coherent with the data obtained in the GalT-catalyzed reaction, as acylation took place on the external glucose of the sophorose moiety.

Enzymatic acylation of steviolbioside (**2**) was also very efficient: conversion was almost quantitative to a single product that was isolated and characterized. However, to our surprise, lipase PS directed its action to the inner glucose of the sophorose moiety of **2**, producing the monoacetate **7**. A similar result was obtained with the methyl ester **2a**, which was quantitatively converted into the monoacetate **7a**. In this case too the attribution was unequivocally supported by NMR data. The H-1′ at 4.53 ppm was connected to the low field H_a-6′ and H_b-6′ at 4.28 and 3.92 bearing the acetoxy group, whereas H-1″ at 4.36 was connected to an unsusbstitued oxymethylene at 3.59 and 3.48 ppm.

Therefore a significant effect on lipase PS site- and regioselectivity was observed by changing a remote portion of the molecule: deglucosylation at position C-19 changed the acylation site of the sophorose moiety linked at C-13.

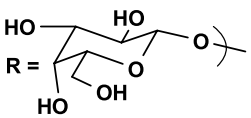
This unexpected result suggested us to examine the structure of the very minor product obtained in the acetylation of stevioside. We found that it contained two acetate groups and that one of these was on the CH_2-6‴ of the glucose attached to the carboxyl function (H-1‴ at 5.32 was connected to CH_2-6‴ at 4.23 and 4.09). In analogy with the previously described monoacetate **6**, we expected the additional acetate to be located on the external glucose of the sophorose moiety. However, in spite of our reluctance, a clear connection of the second low field methylene at 4.27 and 3.99 to H-1′ at 4.47 convinced us that the additional acyl moiety was located on CH_2-6′, and therefore to this diester the structure of 6′,6‴-di-*O*-acetyl stevioside **5** was assigned.

As the surprising structure of this very minor byproduct did not give any clue to explain the puzzling behavior of lipase PS, we decided to start a molecular modeling analysis of the conformation of the two substrates **1** and **2** in different simulation environments.[11] Our goal was to verify whether



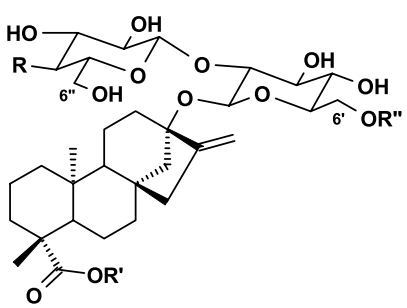
1 : R = OH ; R' = R" = R‴ = H

3 : R' = R" = R‴ = H ; R =



5 : R = OH ; R' = H ; R" = R‴ = Ac

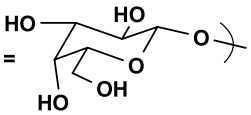
6 : R = OH ; R' = Ac ; R" = R‴ = H



2 : R = OH ; R' = R" = H

2a : R = OH ; R' = Me ; R" = H

4 : R' = R" = H ; R =



7 : R = OH ; R' = H ; R" = Ac

7a : R = OH ; R' = Me ; R" = Ac

the deglucosylation at position C-19 of **1** originated such a significant conformational variation in **2** to justify the different enzymatic selectivity experimentally observed. The solvent in these models is treated as an analytical continuum starting near the van der Waals surface of the solute. The equilibrated continuum solvent is characterized by a dielectric constant typical for the particular solvent, i.e. 78 D for water and 4.8 D for chloroform. The increased accuracy in modeling the solvent effects yields parallel increased accuracy in the conformational searches. Conformational searches on compounds **1** and **2** were performed using the Monte Carlo/Energy Minimization (MC/EM) approach implemented in MacroModel: the dihedrals (one to five at a time) corresponding to simple bonds are changed by random quantities, the new conformations obtained are scored based on the molecular mechanics energy function, and low energy conformations are stored as probable structures for the compound in solution. In this work the SUMM variant of the MC/EM algorithm was used.[11] For each compound in each different solvent, 30,000 steps of the MC/EM procedure were run. All the minima found within 50 kJ/mol of the global minimum were fully reminimized to allow a more accurate determination of the relative energies.

The global minimum structures of both **1** and **2** in chloroform (a model for a generic hydrophobic organic solvent) and in vacuo were substantially indistinguishable. Steviolbioside was found to adopt an extended conformation in water (Fig. 1, top) and a folded one in the organic solvent (Fig. 1, bottom). In water, the extended conformation allows the molecule to expose the hydroxyl groups of the sugar moieties and the COOH group to the bulk solvent, giving rise to favorable hydrogen bond type solvating interactions. On the other hand, in the organic solvent chloroform, the molecule tends to adopt a folded conformation, due to the H-bond formation between the carboxylate group at C-19 and the OH moieties on C-6″ and C-4″. This conformation causes the hydroxyl moiety on C-6′ to point out towards the solvent, and the lower steric hindrance of this group might be the reason why it suffers selective acylation when steviolbioside enters into the active site of lipase PS.

On the other hand stevioside showed a similar extended conformation in water (Fig. 2, top) and in chloroform (Fig. 2, bottom). In water the extended conformation is favored because of the high number of hydrogen bonding interactions with the bulk solvent. In chloroform, the presence of the glucopyranosyl ester moiety at C-19 prevents the formation of the stabilizing hydrogen bonds and forces the molecule to adopt again a completely extended conformation in order to minimize unfavorable steric clashes. Therefore the hydroxyl group on C-6″ is forced to point outside the molecule, thus minimizing the steric hindrance at this center and directing the acylation mainly on this group.

In conclusion, these calculations correlate well with the experimental results and suggest that the substrate conformation might be the main factor influencing and determining the recognition by lipase PS: the less sterically hindered hydroxyl group enters the active site of the catalyst more favorably, thus directing the site- and regioselective acetylation in organic solvent alternatively to the 6′-OH in **2** and to 6″-OH in **1**. Obviously, contrary to what has been proposed in the very few literature examples on simulated sugars docking inside a protease active site,[12] we were focusing on the substrate that has to be acylated by the

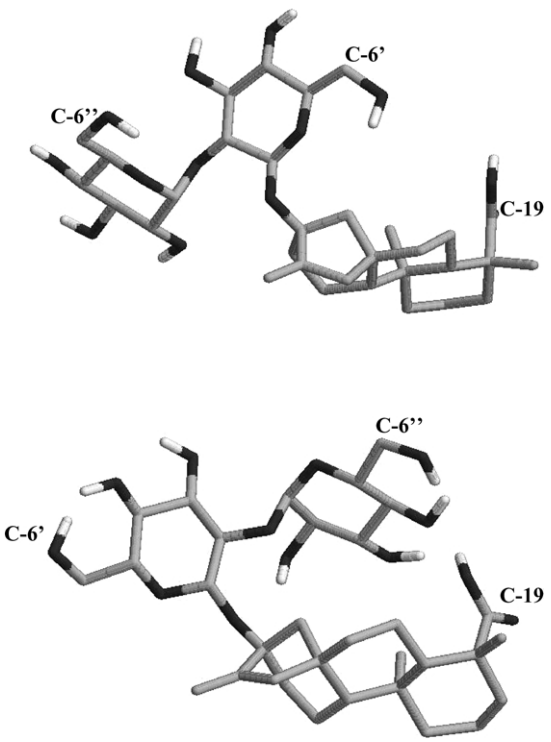


Figure 1. Conformational energy minima of steviolbioside (**2**) in water (top) and $CHCl_3$ (bottom).

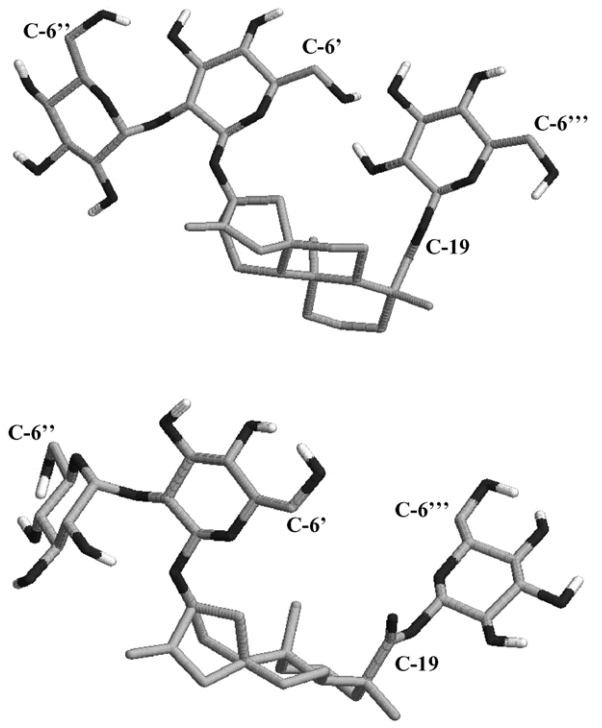


Figure 2. Conformational energy minima of stevioside (**1**) in water (top) and $CHCl_3$ (bottom).

biocatalyst and therefore the enzyme environment has not been taken into consideration. However, in the absence of a high-level ab initio calculation of the whole reaction pathway describing all the intermediates at an electronic level, useful and sound rationales to specific experimental outcomes can be more easily extrapolated using classical mechanics-based approaches.

Needless to say, the observed remote control of lipase PS selectivity by substrate modification makes stevioside and steviolbioside a peculiar case among the reported examples of biocatalyzed-site- and regioselective elaboration of natural glycosides.

3. Experimental

3.1. Materials and methods

Stevioside (**1**) and steviolbioside (**2**) were a gift from Indena, Milano, Italy. Lipase PS was adorbed on celite following a standard protocol.[13] Enzymatic transesterifications were followed by TLC on precoated silica gel 60 F_{254} plates (Merck); compounds were detected with the Komarowsky reagent.

^{1}H and ^{13}C NMR spectra at 300 MHz and 75.2 MHz were recorder in DMSO-d_6+D_2O at 80 °C. NMR spectra at 600 MHz (^{1}H homonuclear DQF-COSY, TOCSY, HMQC, HMBC, ^{1}H–^{13}C COSY and 2D-NOESY) and at 150 MHz (^{13}C) were run in DMSO-d_6 at 35 °C using standard pulse programs. FABMS spectra were recorded on a VG 70-70 EQ-HF instrument in the negative mode using Xe as a gas and 3-nitrobenzylalcohol as the matrix.

3.1.1. Stevioside (1, 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-19-oic acid, β-D-glucopyranosyl ester]. Amorphous solid, mp 235–238 °C; $[\alpha]_D$=−37.5 (*c*=0.2, MeOH). R_f 0.25 (eluent AcOEt–MeOH–H_2O 70:30:5). ^{1}H NMR (300 MHz): 5.09 and 4.77 (2H, m, CH_2-17); 1.19 (3H, s, CH_3-18); 0.92 (3H, s, CH_3-20); 4.53 (1H, d, $J_{1',2'}$=7.7 Hz, H-1′); 3.30 (1H, t, $J_{2',3'}$=7.7 Hz, H-2′); 4.47 (1H, d, $J_{1',2'}$=7.7 Hz, H-1″); 3.09 (1H, t, $J_{2'',3''}$=7.7 Hz, H-2″); 5.37 (1H, d, $J_{1''',2'''}$=7.7 Hz, H-1‴); 3.21 (1H, m, H-2‴); 3.70–3.60 (3H, m, H_a-6′, H_a-6″, H_a-6‴); 3.55–3.45 (3H, m, H_b-6′, H_b-6″, H_b-6‴). ^{13}C NMR (75.2 MHz): 39.95 (C-1); 18.75 (C-2); 37.51 (C-3); 43.34 (C-4); 56.60 (C-5); 21.20 (C-6); 41.10 (C-7); 42.03 (C-8); 53.30 (C-9); 39.13 (C-10); 20.02 (C-11); 35.79 (C-12); 84.80 (C-13); 43.59 (C-14); 47.05 (C-15); 153.51 (C-16); 103.96 (C-17); 28.60 (C-18); 175.62 (C-19); 15.23 (C-20); 96.40 (C-1′); 82.58 (C-2′); 76.40[a] (C-3′); 70.47[b] (C-4′); 76.23[a] (C-5′); 61.20[c] (C-6′); 104.53 (C-1″); 75.24 (C-2″); 76.23[a] (C-3″); 69.99[b] (C-4″); 76.92[a] (C-5″); 61.00[c] (C-6″); 94.20 (C-1‴); 72.66 (C-2‴); 77.65[a] (C-3‴); 69.80[b] (C-4‴); 77.01[a] (C-5‴); 60.75[c] (C-6‴). ([a,b,c] values with the same superscript might be exchanged). FABMS: 803 (7%, $[M-H]^-$), 641 (100%,), 479 (18%,), 317 (13%,).

3.1.2. Enzymatic acetylation of stevioside (1). Stevioside (**1**) (100 mg) was dissolved 4.75 ml of anhydrous THF, vinyl acetate (250 μl) and lipase PS on celite (200 mg) were added and the suspension was shaken at 45 °C. After 3,5 h an almost 70% conversion was observed to a main product accompanied by traces of another less polar product. The suspension was filtered, the solvent evaporated and the crude residue purified by flash chromatography (eluent AcOEt–MeOH–H_2O, from 90:15:3 to 80:15:5) to give 4 mg of 6′,6‴-di-*O*-acetyl stevioside (**5**) and 51 mg of 6″-*O*-acetyl stevioside (**6**).

Compound 6′,6‴-di-*O*-acetyl stevioside (**5**). Vitreous solid, R_f 0.35 (eluent AcOEt–MeOH–H_2O 70:30:5). ^{1}H NMR (600 MHz): 5.01 and 4.73 (2H, m, CH_2-17); 1.07 (3H, s, CH_3-18); 1.00 (3H, s, CH_3-20); 4.47 (1H, d, $J_{1',2'}$=8.0 Hz, H-1′); 3.26 (1H, t, $J_{2',3'}$=8.0 Hz, H-2′); 3.38 (1H, t, $J_{3',4'}$=8.0 Hz, H-3′); 3.07 (1H, t, $J_{4',5'}$=8.0 Hz, H-4′); 3.30 (1H, ddd, $J_{5',6'a}$=2.1 Hz, $J_{5',6'b}$=6.5 Hz, H-5′); 4.27 (1H, dd, ^2J=11.9 Hz, H-6′a); 3.99 (1H, dd, H-6′b); 4.36 (1H, d, $J_{1',2'}$=7.9 Hz, H-1″); 3.02 (1H, t, $J_{2'',3''}$=7.9 Hz, H-2″); 3.15–3.20 (2H, m, H-3″ and H-4″); 3.05 (1H, m, H-5″); 3.57 (1H, dd, ^2J=12.0 Hz, $J_{5'',6''a}$=2.0 Hz, H-6″a); 3.49 (1H, dd, $J_{5'',6''b}$=6.5 Hz, H-6″b); 5.32 (1H, d, $J_{1''',2'''}$=8.1 Hz, H-1‴); 3.14–3.18 (H-2‴ and H-4‴); 3.28 (1H, dd, $J_{3''',4'''}$=8.1 Hz, H-3‴); 3.46 (1H, dd, $J_{4''',5'''}$=8.2 Hz, $J_{5''',6'''a}$=2.0 Hz; $J_{5''',6'''b}$=7.0 Hz, H-5‴); 4.23 (1H, dd, ^2J=12.1 Hz, H-6‴a); 4.09 (1H, dd, H-6‴b). ^{13}C NMR (150 MHz): 153.50 (C-16); 104.00 (C-17); 28.07 (C-18); 176.18 (C-19); 15.20 (C-20); 96.1 (C-1′); 82.2 (C-2′); 76.13[a] (C-3′); 70.31[b] (C-4′); 73.4 (C-5′); 63.8 (C-6′); 104.6 (C-1″); 75.3 (C-2″); 76.33[a] (C-3″); 70.40[b] (C-4″); 76.71[a] (C-5″); 63.8 (C-6″); 94.30 (C-1‴); 72.70 (C-2‴); 77.5[a] (C-3‴); 69.80 (C-4″); 74.4 (C-5‴); 63.3 (C-6‴). ([a,b] values with the same superscript might be exchanged). FABMS: 887 (4%, $[M-H]^-$), 725 (100%), 683 (28%,), 521 (14%), 479 (8%), 317 (23%).

Compound 6″-*O*-acetyl stevioside (**6**). Amorphous solid, mp 228–231 °C; $[\alpha]_D$=−33.5 (*c*=0.1, MeOH); R_f 0.29 (eluent AcOEt–MeOH–H_2O 70:30:5). ^{1}H NMR (600 MHz): 0.74 (H_{ax}-1); 1.78 (H_{eq}-1); 1.37 (H_{ax}-2); 1.78 (H_{eq}-2); 0.97 (H_{ax}-3); 2.03 (H_{eq}-3); 1.02 (H_{ax}-5); 1.70 (H_{eq}-6); 1.83 (H_{ax}-6); 1.34–1.43 (CH_2-7); 0.92 (H_{ax}-9); 1.46–1.65 (CH_2-11); 1.40 (H_{ax}-12); 1.82 (H_{eq}-12); 2.12 and 1.37 (CH_2-14); 2.05–1.95 (CH_2-15); 4.68 and 4.98 (CH_2-17); 1.11 (CH_3-18); 0.84 (CH_3-20); 4.42 (1H, d, $J_{1',2'}$=8.0 Hz, H-1′); 3.19 (1H, dd, $J_{2',3'}$=8.2 Hz, H-2′); 3.07 (1H, dd, $J_{3',4'}$=8.3 Hz, H-3′); 3.36–3.38 (H-4′ and H-5′); 3.41 (1H, dd, ^2J=11.9 Hz; $J_{5',6'a}$=6 Hz, H-6′a); 3.66 (1H, dd, $J_{5',6'b}$=1.5 Hz, H-6′b); 4.37 (1H, d, $J_{1'',2''}$=8.0 Hz, H-1″); 3.03 (1H bdd, $J_{2'',3''}$=8.1 Hz, H-2″); 3.10 (1H, dd, $J_{3''4''}$=8.2 Hz, H-3″); 3.19 (1H, dd, $J_{4'',5''}$=8.2 Hz, H-4″); 3.30 (m, $J_{5'',6''a}$=1.5 Hz, $J_{5'',6''b}$=6.0 Hz, H-5″); 4.19 (1H, dd, ^2J=11.5 Hz, H-6″a); 3.95 (1H, dd, H-6″b); 5.24 (1H, d, $J_{1''',2'''}$=8.0 Hz, H-1‴); 3.12 (1H, dd, $J_{2''',3'''}$=8.1 Hz, H-2‴); 3.24 (1H, dd, $J_{3''',4'''}$=8.2 Hz, H-3‴); 3.14 (1H, dd, $J_{4''',5'''}$=8.2 Hz, H-4‴); 3.18 (1H, ddd, $J_{5''',6'''a}$=1.5 Hz; $J_{5''',6'''b}$=6.0 Hz, H-5‴); 3.61 (1H, dd, ^2J=12.0 Hz, H-6‴a); 3.45 (1H, dd, H-6‴b). ^{13}C NMR (150 MHz): 40.05 (C-1); 18.85 (C-2); 37.30 (C-3); 43.49 (C-4); 56.13 (C-5); 21.15 (C-6); 40.91 (C-7); 42.08 (C-8); 53.15 (C-9); 39.65 (C-10); 19.95 (C-11); 35.85 (C-12); 84.78 (C-13); 43.50 (C-14); 47.08 (C-15); 153.60 (C-16); 103.85 (C-17); 28.02 (C-18); 175.58 (C-19); 15.10 (C-20); 96.31 (C-1′); 82.91 (C-2′); 76.60[a] (C-3′); 70.51[b] (C-4′); 76.70[a] (C-5′); 61.10[c] (C-6′); 104.61 (C-1″); 75.25 (C-2″); 76.10[a] (C-3″); 69.95[b] (C-4″); 74.18 (C-5″); 63.85 (C-6″);

94.10 (C-1‴); 72.98 (C-2‴); 77.48[a] (C-3‴); 69.80[b] (C-4″); 77.15[a] (C-5‴); 60.75[c] (C-6‴); 20.55 (OCO*C*H$_3$); 169.95 (O*C*OCH$_3$) ([a,b,c]values with the same superscript might be exchanged). FABMS: 845 (8%, [M−H]$^-$), 683 (100%,), 641 (5%), 479 (18%), 317 (21%).

3.1.3. Steviolbioside (2, 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-19-oic acid). Amorphous solid, mp 193–198 °C; $[\alpha]_D$=−32.5 (*c*=0.2, MeOH); R_f 0.22 (eluent AcOEt–MeOH–H$_2$O 80:15:5). ^{1}H NMR (300 MHz): 5.13 and 4.78 (2H, m, CH$_2$-17); 1.15 (3H, s, CH$_3$-18); 0.92 (3H, s, CH$_3$-20); 4.54 (1H, d, $J_{1',2'}$=8.0 Hz, H-1′); 4.47 (1H, d, $J_{1'',2''}$=8.0 Hz, H-1″). ^{13}C NMR (75.2 MHz): 39.95 (C-1); 18.91 (C-2); 37.70 (C-3); 42.89 (C-4); 56.16 (C-5); 21.68 (C-6); 41.04 (C-7); 41.85 (C-8); 53.42 (C-9); 39.13 (C-10); 19.95 (C-11); 36.36 (C-12); 85.08 (C-13); 43.75 (C-14); 47.28 (C-15); 153.11 (C-16); 104.23 (C-17); 28.69 (C-18); 178.47 (C-19); 15.31 (C-20); 96.23 (C-1′); 82.58 (C-2′); 76.42[a] (C-3′); 70.26[b] (C-4′); 76.42[a] (C-5′); 61.18[c] (C-6′); 104.48 (C-1″); 75.25 (C-2″); 76.42[a] (C-3″); 70.17[b] (C-4″); 76.87[a] (C-5″); 61.18[c] (C-6″). ([a,b,c]values with the same superscript might be exchanged). FABMS: 641 (8%, [M−H]$^-$), 479 (100%), 317 (28%).

3.1.4. Enzymatic acetylation of steviolbioside. Steviolbioside (100 mg) was dissolved in 4.75 ml of anhydrous THF, vinyl acetate (250 μl) and lipase PS on celite (250 mg) were added and the suspension was shaken at 45 °C. After 14 h an almost 90% conversion to a product was observed. After filtration and solvent evaporation the new compound was isolated by flash chromatography (eluent AcOEt–MeOH–H$_2$O, 80:15:5) to afford 75 mg of 6′-*O*-acetyl steviolbioside (**7**).

Compound 6′-*O*-acetyl steviolbioside (**7**). Amorphous solid, mp 180–183 °C; $[\alpha]_D$=−29.5 (*c*=0.1, MeOH); R_f 0.37 (eluent AcOEt–MeOH–H$_2$O 80:15:5). ^{1}H NMR (600 MHz): 0.78 (H$_{ax}$-1); 1.79 (H$_{eq}$-1); 1.37 (H$_{ax}$-2); 1.75–1.80 (H$_{eq}$-2); 0.92 (H$_{ax}$–C3); 2.02 (H$_{eq}$-3); 1.00 (H$_{ax}$-5); 1.70–1.75 (CH$_2$-6); 1.39–1.49 (CH$_2$-7); 0.92 (H$_{ax}$-9); 1.49–1.70 (CH$_2$-11); 1.48 (H$_{ax}$-12); 1.88 (H$_{eq}$-12); 1.32 (H$_{ax}$-14); 2.06 (H$_{eq}$-14); 1.98–2.04 (CH$_2$-15); 4.74 and 5.03 (CH$_2$-17); 1.10 (CH$_3$-18); 0.88 (CH$_3$-20); 4.53 (1H, d, $J_{1',2'}$=8.1 Hz, H-1′); 3.24 (1H, t, $J_{2',3'}$=8.1 Hz, H-2′); 3.41 (1H, t, $J_{3',4'}$=8.1 Hz, H-3′); 3.06 (H-4′); 3.34 (H-5′); 4.28 (1H, dd, ^2J=11.9 Hz, $J_{5',6'a}$=1.5 Hz, H-6′a); 3.92 (1H, dd, $J_{5',6'b}$=8.2 Hz, H-6′b); 4.36 (1H, d, $J_{1'',2''}$=8.1 Hz, H-1″); 2.99 (1H, t, $J_{2'',3''}$=8.1 Hz, H-2″); 3.15 (1H, t, $J_{3'',4''}$=8.1 Hz, H-2″); 3.12 (1H, t, $J_{4'',5''}$=8.1 Hz, H-4″); 3.06 (1H, m, H-5″); 3.59 (1H, dd, ^2J=11.5 Hz, $J_{5'',6''a}$=2.0 Hz, H-6″a); 3.48 (1H, dd, $J_{5'',6''b}$=4.0 Hz, H-6″b); 2.01 (C*H*$_3$COO). ^{13}C NMR (150 MHz): 40.27 (C-1); 18.91 (C-2); 37.50 (C-3); 43.03 (C-4); 55.89 (C-5); 21.59 (C-6); 40.87 (C-7); 41.53 (C-8); 53.03 (C-9); 39.70 (C-10); 19.92 (C-11); 36.49 (C-12); 85.98 (C-13); 43.37 (C-14); 46.81 (C-15); 153.02 (C-16); 104.12 (C-17); 28.69 (C-18); 178.46 (C-19); 15.40 (C-20); 96.04 (C-1′); 82.54 (C-2′); 76.10[a] (C-3′); 70.45[b] (C-4′); 73.44[a] (C-5′); 63.89[c] (C-6′); 104.55 (C-1″); 75.27 (C-2″); 76.22[a] (C-3″); 70.20[b] (C-4″); 76.91[a] (C-5″); 61.14[c] (C-6″) ([a,b,c]values with the same superscript might be exchanged). FABMS: 683 (7%, [M−H]$^-$), 641 (5%), 479 (100%), 317 (14%).

3.1.5. Steviolbioside methylester (2a, 13-[(2-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]kaur-16-en-19-oic acid methyl ester). Prepared by methylation of steviolbioside with trimethysilyl- diazomethane in MeOH/THF 1:1. Amorphous solid, mp 199–202 °C; $[\alpha]_D$=−30.5 (*c*=0.15, MeOH); R_f 0.32 (eluent AcOEt–MeOH–H$_2$O 80:15:5). ^{1}H NMR (300 MHz) 5.15 and 4.78 (2H, m, CH$_2$-17); 1.12 (3H, s, CH$_3$-18); 0.78 (3H, s, CH$_3$-20). ^{13}C NMR (75.2 MHz): 39.93 (C-1); 18.51 (C-2); 37.30 (C-3); 42.95 (C-4); 55.74 (C-5); 21.42 (C-6); 40.13 (C-7); 41.27 (C-8); 52.92 (C-9); 39.93 (C-10); 19.67 (C-11); 36.34 (C-12); 85.05 (C-13); 43.57 (C-14); 46.83 (C-15); 152.47 (C-16); 104.06 (C-17); 27.96 (C-18); 176.96 (C-19); 14.78 (C-20); 95.78 (C-1′); 82.05 (C-2′); 76.30[a] (C-3′); 69.98 (C-4′); 74.96[a] (C-5′); 60.79 (C-6′); 104.06 (C-1″); 75.92[a] (C-2″); 76.30[a] (C-3″); 69.98 (C-4″); 76.65[a] (C-5″); 60.79 (C-6″); 50.91 (COO*Me*) ([a] values with the same superscript might be exchanged). FABMS: 655 (13%, [M−H]$^-$), 493 (100%), 331 (23%).

3.1.6. Enzymatic acetylation of steviolbioside methyl ester (7). Steviolbioside methyl ester (**7**) (50 mg) was dissolved in 2.5 ml of anhydrous THF, vinyl acetate (80 μl) and lipase PS on celite (300 mg) were added and the suspension was shaken at 45 °C. After 11 h an almost complete conversion was observed to a single product, which, after filtration and solvent evaporation, was isolated by flash chromatography (eluent AcOEt–MeOH–H$_2$O, 80:15:5) to afford 38 mg of 6′-*O*-acetyl steviolbioside methylester (**7a**).

Compound 6′-*O*-acetyl steviolbioside methylester (**7a**). Amorphous solid, mp 185–190 °C; $[\alpha]_D$=−28.0 (*c*=0.1, MeOH); R_f 0.46 (eluent AcOEt–MeOH–H$_2$O 80:15:5). ^{1}H NMR (300 MHz): 5.05 and 4.72 (CH$_2$-17); 1.10 (CH$_3$-18); 0.88 (CH$_3$-20); 4.53 (1H, d, $J_{1',2'}$=8.0 Hz, H-1′); 3.24 (1H, dd, $J_{2',3'}$=8.1 Hz, H-2′); 3.47 (1H, dd, $J_{3',4'}$=8.2 Hz, H-3′); 3.15–3.20 (H-4′); 3.37 (1H, ddd, $J_{4',5'}$=8.2 Hz, $J_{5',6'a}$= 1.5 Hz, $J_{5',6'b}$=8.0 Hz, H-5′); 4.33 (1H, dd, ^2J=11.2 Hz, H-6′a); 4.03 (1H, dd, H-6′b); 4.48 (1H, d, $J_{1'',2''}$=8.0 Hz, H-1″); 3.00 (1H, dd, $J_{2'',3''}$=8.1 Hz, H-2″); 3.26 (1H, dd, $J_{3'',4''}$=8.2 Hz, H-3″); 3.15–3.20 (H-4″); 3.06 (1H, ddd, $J_{4'',5''}$=8.0 Hz, $J_{5'',6''a}$=4.0 Hz, $J_{5'',6''b}$=1.5 Hz, H-5″); 3.45 (1H, dd, ^2J=11.5 Hz, H-6″a); 3.68 (1H, dd, H-6″b); 2.01 (3H, s, C*H*$_3$COO); 3.62 (3H, s, COO C*H*$_3$)). ^{13}C NMR (75.2 MHz): 39.73 (C-1); 18.27 (C-2); 37.13 (C-3); 42.90 (C-4); 55.77 (C-5); 21.04 (C-6); 40.53 (C-7); 41.20 (C-8); 52.93 (C-9); 39.73 (C-10); 19.34 (C-11); 36.03 (C-12); 84.84 (C-13); 43.20 (C-14); 46.78 (C-15); 152.26 (C-16); 103.52 (C-17); 27.72 (C-18); 176.34 (C-19); 14.60 (C-20); 95.60 (C-1′); 82.65 (C-2′); 75.94[a] (C-3′); 70.07[b] (C-4′); 73.07 (C-5′); 63.36 (C-6′); 103.68 (C-1″); 74.61 (C-2″); 75.94[a] (C-3″); 70.29[b] (C-4″); 76.24[a] (C-5″); 61.12 (C-6″); 50.32 (COO*Me*); 19.93 (OCO*C*H$_3$); 169.49 (O*C*OCH$_3$) ([a,b]values with the same superscript might be exchanged). FABMS: 697 (7%, [M−H]$^-$), 655 (5%), 535 (100%), 493 (18%), 331 (13%).

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