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SYNTHESIS AND ANTIMICROBIAL ACTIVITY OF GLUCURONOSYL DERIVATIVES OF STEVIOLBIOSIDE FROM *Stevia rebaudiana*

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Glucuronosyl derivatives of the glycoside steviolbioside from Stevia rebaudiana *were synthesized for the first time. Steviolbioside exhibited selective bacteriostatic and bactericidal activity against* S. aureus *ATCC 209p with minimum inhibitory concentration (MIC) of 62.5 μg/mL, which corresponded to the activity of the antibiotic chloramphenicol.*

Keywords: glucuronic acid, glucuronosides, glucuronides, glycosides, steviolbioside, *Stevia rebaudiana*, rebaudiosides, antimicrobial activity.

Xenobiotics, including useful ones (drugs, certain food additives), are known to be removed from the human body via glucuronosylation in the liver by glucuronic acid and elimination with urine as glucuronosides (glucuronides) [1]. For example, calorie-free sugar substitutes based on glucosides (rebaudiosides) from *Stevia rebaudiana* are eliminated from the human body as glucuronosides of their aglycone steviol [2, 3]. In continuation of research on chemical transformations of *S. rebaudiana* glycosides [4–8], we report the synthesis of the first glucuronosides of the *S. rebaudiana* glycoside steviolbioside **2** and their antimicrobial activity.

Steviolbioside **2** was obtained by alkaline hydrolysis of stevioside **1**. Its sophorosyl hydroxyls were protected by acylation with acetic anhydride in Py to form glycoside **3**, which was glucuronosylated using methyl-1-deoxy-1-bromo-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate (**6**) that was obtained in three steps from D-(+)-glucurono-3,6-lactone (**4**). First, lactone **4** was converted by NaOMe [9, 10] to the methyl ester of glucuronic acid and then acylated. The PMR spectrum of the obtained methyl-1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranuronate (**5**) showed an anomeric proton resonating at 5.77 ppm as a single doublet with vicinal SSCC 7.7 Hz. This indicated that lactone **4** opened stereospecifically to give the β-anomer of saccharide **5**, which was converted by the literature method [11] into methyl-1-deoxy-1-bromo-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate (**6**). Hepta-acetylated steviolbioside **3** was glucuronosylated as before [12] using a phase-transfer reaction with the bromide of methyl glucuronate **6** in the presence of tetrabutylammonium bromide (TBAB). Column chromatography produced in 26% yield glucuronoside **7**, the PMR spectrum of which showed resonances for the protons of hepta-acetylated steviolbioside **3** and for the protons of the glucuronate moiety, the anomeric proton of which resonated at 5.89 as a doublet with vicinal SSCC 7.9 Hz. This indicated that a β-D-glycoside bond had formed. Then, the acetyl protection was removed from the glucuronate and sophorosyl moieties using hydrazine monohydrate according to the literature [12].

The PMR spectrum of glucuronoside **8** (61% yield) lacked strong resonances for the acetyls at 1.96–2.06 ppm and the glucuronate methoxycarbonyl moiety at 3.73 ppm that were observed in the spectrum of glucuronoside **7** and exhibited a broad singlet at 9.19 ppm that was characteristic of the hydrazide amide proton. The IR spectrum of hydrazide **8** exhibited bands for stretching vibrations amide I (1654 cm^{-1}) and amide II (1544 cm^{-1}) and the hydrazide NH group (3200–3400 cm^{-1}). This in combination with MALDI mass spectral data indicated that hydrazide **8** had formed.

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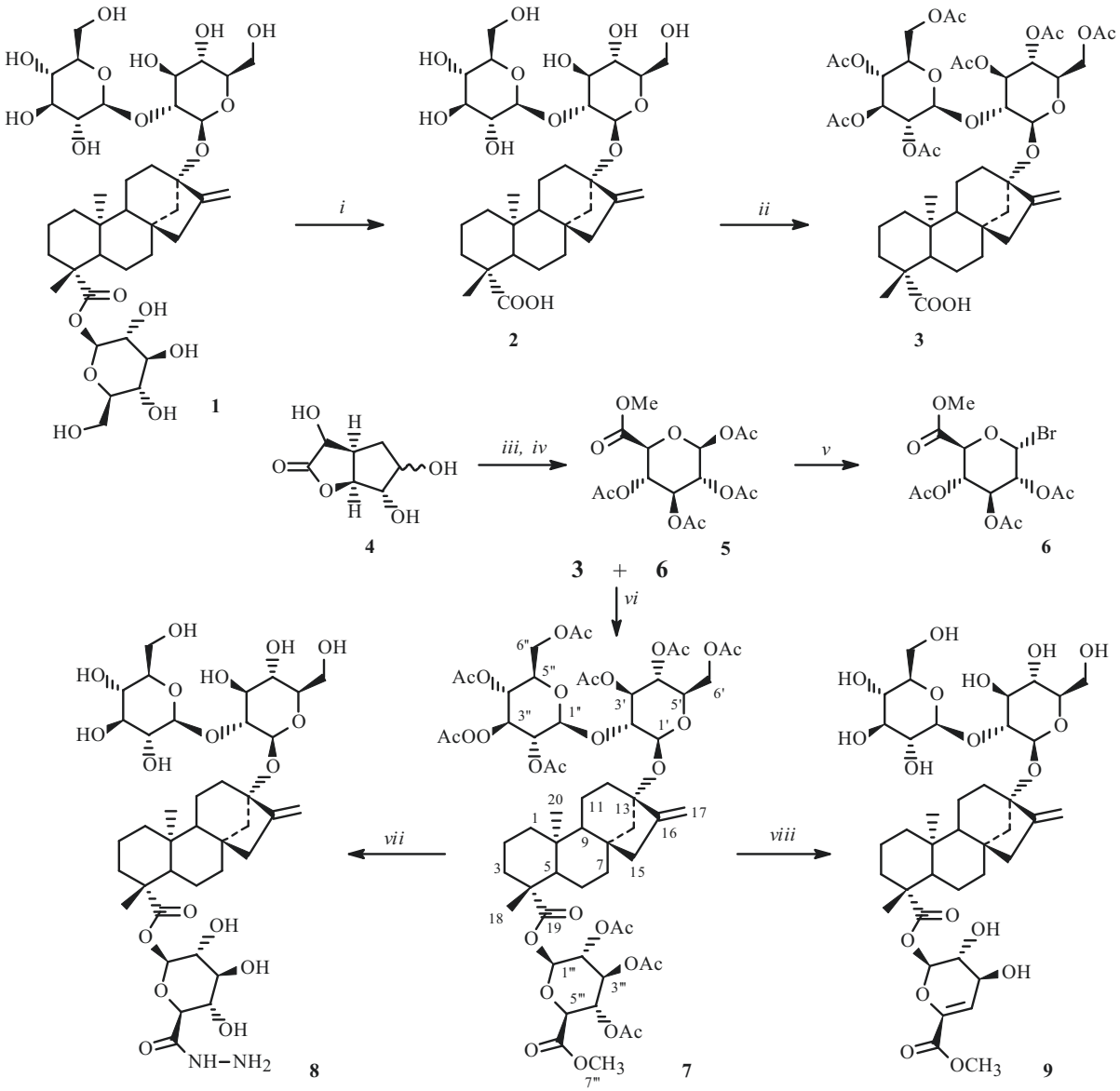

i. 10% KOH; *ii*. Ac_2O–Py; *iii.* MeONa, MeOH, 20°C; *iv*. Ac_2O, $HClO_4$, 0°C; *v*. 33% HBr–AcOH, CH_2Cl_2, 0°C; *vi*. TBAB, K_2CO_3, CH_2Cl_2–H_2O 10 : 1, 40°C; *vii*. $NH_2NH_2 \cdot H_2O$, MeOH, 20°C; *viii*. MeONa, MeOH, 20°C

The acetyl protection of the sophorosyl and glucuronate moieties in **7** was removed without affecting the glucuronate carboxymethyl by work up with NaOMe in MeOH as before [13]. The PMR spectrum of obtained product **9** (27% yield) lacked a multiplet at 1.94–2.06 ppm that was observed in the spectrum of **7** and corresponded to steviolbioside sophorosyl and glucuronate acetyl resonances. However, the PMR spectrum of **9** contained a singlet at 3.79 ppm for the glucuronate ester protons (this singlet was observed at 3.73 ppm in the spectrum of glucuronoside **7**). These results indicated that the protection was removed from all hydroxyls of **7** without affecting the glucuronate ester. The PMR spectrum of **9** exhibited all characteristic resonances of the *ent*-caurene moiety [4–8], i.e., singlets at 0.95 (H_3-20), 1.19 (H_3-18), 4.85 (H_A-17), and 5.20 ppm (H_B-17). However, "extra" resonances, e.g., a doublet of doublets at 4.61 and a doublet at 6.14 ppm, were observed in the region of the glucuronate proton resonances. Furthermore, the molecular mass of **9** in the MALDI mass spectrum was 18 *m*/*z* units less than expected, indicating that a water molecule was eliminated from the expected glucuronoside **7** when the acetyl protection was removed. This agreed with the literature for β-elimination occurring during alkaline degradation of pectinic polysaccharides (e.g., rhamnogalacturonan) [14] and uronic acids [1, 15] to form the dehydrouronides (dehydroglucuronides) with a C(4)=C(5) double bond. A comparison of the PMR spectra of the product obtained by us and lepidimoic acid, a disaccharide obtained during alkaline degradation of rhamnogalacturonan [14], showed that the doublet of doublets at 4.61 ppm with vicinal SSCC 7.7 and 5.6 Hz corresponded to the C-3 proton resonance; the doublet at 6.14 ppm with SSCC 4.1 Hz, to the glucuronate C-4

proton forming the double bond with C-5. Thus, the product from the reaction of **7** with NaOMe was dehydroglucuronoside **9**, the anomeric protons of which appeared in the PMR spectrum as a doublet at 6.17 ppm with vicinal SSCC 7.7 Hz, which was consistent with the α-L-*threo*-configuration.

The antibacterial and antifungal activities of glycosides **2** and **3**, glucuronosides **7** and **8**, and dehydroglucuronoside **9** were studied *in vitro* against Gram-positive bacterial strains *Staphylococcus aureus* ATCC 209p and *Bacillus cereus* ATCC 803; Gram-negative strains *Escherichia coli* CDCF-50 and *Pseudomonas aeruginosa* ATCC 9027; and fungi *Aspergillus niger* BKMF-1119, *Trichophyton mentagrophytes* var. *gypseum* 1773, and *Candida albicans* 855–653. The reference drugs were the antibiotic chloramphenicol and the antifungal drug ketoconazole. Only steviolbioside **2** of all tested compounds was active. It exhibited selective bacteriostatic and bactericidal activity against *S. aureus* ATCC 209p with a minimum inhibitory concentration (MIC) of 62.5 μg/mL, which corresponded to the activity of chloramphenicol. The other compounds were inactive as antimicrobials.

EXPERIMENTAL

PMR and ^{13}C NMR spectra were recorded on Avance-400 and Avance-600 spectrometers (Bruker, Germany). MALDI mass spectra were obtained on an UltraFlex III TOF/TOF time-of-flight mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) in linear mode. The Nd:YAG laser had λ = 355 nm. Data were processed using the FlexAnalysis 3.0 program (Bruker Daltonik GmbH, Bremen, Germany). Measurements were made in the range *m*/*z* 200–6000 in positive-ion mode using a metallic target and a matrix of 2,5-dihydroxybenzoic acid and *p*-nitroaniline. The sample was dissolved in CH_2Cl_2–DMSO at a concentration of 10^{-3} mg/mL. The matrix solution in MeCN was prepared at a concentration of 10 mg/mL. Samples were deposited by the dried drop method. Matrix solution was placed onto an Anchor Chip target (Bruker Daltonik GmbH, Bremen, Germany) using a 0.5-μL pipette. After the solvent evaporated, analyte solution (0.5 μL) was placed onto the target. Electrospray ionization (ESI) mass spectra were obtained on an AmazonX mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). Measurements were taken in negative-ion mode in the range *m*/*z* 100–1500 with capillary potential 4500 V and N_2 drying-gas at 200°C and flow rate 8 L/min. Compounds were dissolved in $CHCl_3$ or MeOH at a concentration of 10^{-5} M. Data were processed using the DataAnalysis 4.0 program (Bruker Daltonik GmbH, Bremen, Germany). Optical rotation was measured on a PerkinElmer 341 polarimeter (USA). IR spectra were recorded on a Vector 22 Fourier spectrometer (Bruker) in the range 400–4000 cm^{-1}. Samples were studied as films. The course of reactions and purity of compounds were monitored by TLC on Sorbfil plates (OOO Imid, Krasnodar, Russia). Compounds were detected by treating plates with H_2SO_4 solution (5%) followed by heating to 120°C.

(β-D-Glucopyranosyl)-13-*O*-[β-D-glucopyranosyl-(1″→2α')-β-D-glucopyranosyl]-*ent*-car-16-en-19-oate (1) was prepared from the sweetener Stevioside (OOO Travy Baikala, Irkutsk, Russia) as before [8]. Mp 201–203°C (MeOH) {lit.: mp 196–198°C [16]}, $[\alpha]_D^{22}$ –33.7° (*c* 6.6, H_2O), {$[\alpha]_D^{20}$ –39.3° (*c* 5.7, H_2O) [16]}.

13-*O*-[β-D-Glucopyranosyl-(1″→2α')-β-D-glucopyranosyl]-*ent*-caur-16-en-19-oic acid (2) was prepared from **1** by the literature method [16]. Mp 190°C (MeOH) {lit.: mp 188–192°C [16]}, $[\alpha]_D^{20}$ –32.5° (*c* 0.2, MeOH), {$[\alpha]_D^{20}$ –37.4° (*c* 1.4, dioxane) [16]}.

13-*O*-[(3″,4″,6″-Tri-*O*-acetyl-β-D-glucopyranosyl]-(1″→2α')-(3′,4′,6′-tri-*O*-acetyl-β-D-glucopyranosyl)]-*ent*-caur-16-en-19-oic acid (3) was prepared from **2** by the literature method [5]. Mp 126°C (MeOH) {lit.: mp 125°C (*i*-Pr_2O–pentane) [5]}, $[\alpha]_D^{20}$ –39.5° (*c* 0.2, EtOH), {$[\alpha]_D^{20}$ –35.3° (*c* 1, EtOH) [5]}.

Methyl-1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranuronate (5) was prepared from commercially available D-(+)-glucurono-3,6-lactone **4** according to the literature [9, 10]. Mp 177°C (MeOH) {lit.: mp 176°C [9], 177–178°C [17]}, $[\alpha]_D^{20}$ 8.1° (*c* 1.1, $CHCl_3$), {$[\alpha]_D^{25}$ 7.4° (*c* 2.0, $CHCl_3$) [17]}.

Methyl-1-deoxy-1-bromo-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate (6) was prepared by the literature method [11]. Mp 105–106°C (EtOAc) {lit.: mp 106–107°C (EtOH) [11]}.

(Methyl-2‴,3‴,4‴-tri-*O*-acetyl-β-D-glucopyranosyluronate)-13-*O*-[(3″,4″,6″-tri-*O*-acetyl-β-D-glucopyranosyl)-(1‴→2α')-(3′,4′,6′-tri-*O*-acetyl-β-D-glucopyranosyl)]-*ent*-caur-16-en-19-oate (7). Bromide **6** (0.8 g, 2 mmol) and hepta-acetylated steviolbioside **3** (1.44 g, 1.5 mmol) were dissolved in CH_2Cl_2–H_2O (10:1, 22 mL); treated with K_2CO_3 (0.51 g, 3.75 mmol) and TBAB (0.19, g, 2 mmol); refluxed under Ar for 34 h; diluted with $CHCl_3$ (50 mL); and washed successively with H_2O (3 × 70) and NaCl solution (3 × 70). The organic layer was separated, dried over $MgSO_4$, and evaporated at reduced pressure. The residue was chromatographed over silica gel (CH_2Cl_2–MeOH, 100:1) to isolate **7** as a white powder

(0.5 g, 26%), mp 136–138°C, $[\alpha]_D^{20}$ –37° (*c* 1, MeOH). ^{1}H NMR spectrum (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.74–2.25 (20H, m, *ent*-caurane framework), 0.81 (3H, s, H_3-20), 1.17 (3H, s, H_3-18), 1.94–2.06 (30H, m, 10 AcO), 3.61–5.33 (19H, m, sophorosyl and glucuronate residues), 3.73 (3H, s, H_3-7‴), 4.78 (1H, s, H_A-17), 5.05 (1H, s, H_B-17), 5.88 (1H, d, J = 7.9, H-1‴). ^{13}C NMR spectrum (100 MHz, $CDCl_3$, δ, ppm): 16.34 (CH_3, C-20), 19.21 (CH_2, C-2), 20.57 (CH_2, C-11), 20.66–20.95 [CH_3, 10 $\underline{C}H_3C(O)$], 21.87 (CH_2, C-6), 28.83 (CH_3, C-18), 37.47 (CH_2, C-3), 38.05 (CH_2, C-12), 39.56 (C, C-10), 40.76 (CH_2, C-1), 41.46 (CH_2, C-7), 42.76 (C, C-8), 43.09 (C, C-4), 44.47 (CH_2, C-14), 47.47 (CH_2, C-15), 52.99 (CH_3, C-7‴), 53.64 (CH, C-9), 56.99 (CH, C-5), 62.14 (CH_2, C-6′), 62.56 (CH_2, C-6″), 68.46–77.56 (CH, C-2′, 3′, 4′, 5′, 2″, 3″, 4″, 5″, 2‴, 3‴, 4‴, 5‴), 86.45 (C, C-13), 90.98 (CH, C-1‴), 96.52 (CH, C-1″), 100.71 (CH, C-1′), 104.73 (CH_2, C-17), 152.88 (C, C-16), 166.89 (C, C-6‴), 169.45–170.77 (C, $CH_3\underline{C}(O)$), 175.47 (C, C-19). Mass spectrum (ESI), *m/z* 1275.6 $[M + Na]^+$ (calcd for $C_{59}H_{80}NaO_{29}$, 1275.5).

(β-D-Glucopyranosyluronhydrazide)-13-*O*-[(3″,4″,6″-tri-*O*-acetyl-β-D-glucopyranosyl)-(1″→2α′)-(3′,4′,6′-tri-*O*-acetyl-β-D-glucopyranosyl)]-*ent*-caur-16-en-19-oate (8). A solution of **7** (0.1 g, 0.079 mmol) in MeOH (1 mL) was treated dropwise with hydrazine monohydrate (0.076 mL, 1.572 mmol) and stirred for 12 h at 20°C. The resulting precipitate of hydrazine acetate was filtered off. The solvent was distilled at reduced pressure to afford **8** (0.04 g, 61% yield) as a yellow oil, $[\alpha]_D^{20}$ –30° (*c* 0.1, MeOH). IR spectrum (film, ν, cm^{-1}): 3200–3400 (NH), 1726 [C(O)OC], 1654 (C=C and amide I), 1544 (and amide II). ^{1}H NMR spectrum (600 MHz, CD_3OD, δ, ppm, J/Hz): 0.76–2.19 (20H, m, *ent*-caurane framework), 0.96 (3H, s, H_3-20), 1.19 (3H, s, H_3-18), 3.10–5.08 (23H, m, sophorosyl and glucuronate residues, H_A-17, H_B-17, NH_2), 5.41 (1H, d, J = 7.5, H-1‴), 9.19 (1H, br.s, NH). ^{13}C NMR spectrum (100 MHz, CD_3OD, δ, ppm): 13.65 (CH_3, C-20), 16.18 (CH_2, C-2), 19.07 (CH_2, C-11), 23.13 (CH_2, C-6), 28.71 (CH_3, C-18), 38.97 (CH_2, C-3), 39.25 (CH_2, C-12), 40.65 (CH_2, C-10), 41.80 (CH_2, C-1), 43.51 (CH_2, C-7), 44.28 (C, C-8), 44.85 (C, C-4), 45.02 (CH_2, C-14), 47.71 (CH_2, C-15), 56.59 (CH, C-9), 58.44 (CH, C-5), 62.69–62.90 (CH_2, C-6′, C-6″), 71.45–78.36 (CH, C-2′, 3′, 4′, 5′, 2″, 3″, 4″, 5″, 2‴, 3‴, 4‴, 5‴), 82.42 (C, C-13), 87.48 (CH, C-1‴), 95.45 (CH, C-1″), 97.14 (CH, C-1′), 105.17 (CH_2, C-17), 169.75 (C, C-16), 172.15 [C, C-6‴], 178.21 (C, C-19). Mass spectrum (MALDI), *m/z* 857.6 $[M + Na]^+$ (calcd for $C_{38}H_{60}N_2NaO_{18}$, 857.4).

(Methyl-4‴-deoxy-α-L-*threo*-hex-4-enepyranosyluronate)-13-*O*-[(3″,4″,6″-tri-*O*-acetyl-β-D-glucopyranosyl)-(1″→2α′)-(3′,4′,6′-tri-*O*-acetyl-β-D-gluycopyranosyl)]-*ent*-caur-16-en-19-oate (9). A solution of **7** (0.05 g, 0.04 mmol) in anhydrous MeOH (10 mL) was treated dropwise with freshly prepared NaOMe (0.02 g, 0.4 mmol), stirred for 3 h at 20°C, and neutralized with HCl solution (0.1%, 3 mL). The resulting precipitate was filtered off. The solvent was evaporated at reduced pressure. The residue was chromatographed over silica gel (CH_2Cl_2–MeOH, 7:1) to produce **9** (0.01 g, 27% yield) as a yellow oil, $[\alpha]_D^{20}$ –35° (*c* 0.3, MeOH). ^{1}H NMR spectrum (600 MHz, CD_3OD, δ, ppm, J/Hz): 0.80–2.30 (20H, m, *ent*-caurane framework), 0.95 (3H, s, H_3-20), 1.19 (3H, s, H_3-18), 3.14–3.89 (12H, m, sophorosyl residue), 3.79 (3H, s, H_3-7‴), 4.21 (1H, t, J = 4.3, H-2‴), 4.61 (1H, br.dd, J = 7.7, 5.6, H-3‴), 4.85 (1H, s, H_A-17), 5.20 (1H, s, H_B-17), 6.14 (1H, d, J = 4.1, H-4‴), 6.17 (1H, d, J = 7.7, H-1‴). Mass spectrum (ESI), *m/z*: 837.5 $[M + Na]^+$, 853.5 $[M + K]^+$ (calcd for $[M + Na]^+$ 837.4, $[M + K]^+$ 853.3). $C_{39}H_{58}O_{18}$.

The antibacterial and antifungal activities were studied using serial dilutions in liquid growth media and published methods [18, 19].

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