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ANTITUBERCULOSIS ACTIVITY OF GLYCOSIDES FROM *Stevia rebaudiana* AND HYBRID COMPOUNDS OF STEVIOLBIOSIDE AND PYRIDINECARBOXYLIC ACID HYDRAZIDES

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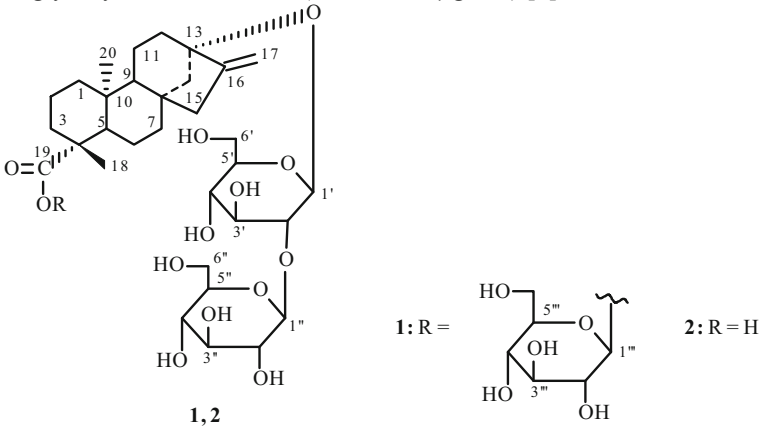
Stevioside and steviolbioside, glycosides from Stevia rebaudiana *Bertoni, in addition to hybrid compounds synthesized from steviolbioside and the antituberculosis drug isoniazid and its isomer nicotinic acid hydrazide exhibited moderate antituberculosis activity against* M. tuberculosis *strain* $H_{37}R_V$ in vitro *(MIC = 7.5, 3.75, 5, and 10 μg/mL, respectively).*

Keywords: glycosides, *Stevia rebaudiana*, stevioside, steviolbioside, isoniazid, hydrazides, hybrid compounds, *M. tuberculosis*, antituberculosis activity.

About 100 metabolites of various structures (lipids, phenols, quinones, peptides, alkaloids, terpenoids, steroids, glycosides) that inhibit the growth of *Mycobacterium tuberculosis* have currently been isolated from natural plant sources [1–4]. Glycosides are the most recent addition to the list of natural tuberculostatics. The literature available to us indicates that they are few in number. They include a saponin isolated from stems of *Colubrina retusa* [minimal inhibiting concentration (MIC) = 10 μg/mL*] [5]; glycosides based on 11-hydroxyhexadecanecarboxylic acid that were isolated from the MeOH extract of *Ipomoea tyrianthina* (MIC = 25 μg/mL) [4]; and glycosides of imberbic acid that were isolated from *Combretum imberbe* (MIC = 100–12.5 μg/mL) [6]. An analysis of the literature reveals an interesting fact: the antituberculosis activity of the glycosides is lower than that of their aglycons. Thus, the MIC = 1.56 μg/mL for the triterpenoid imberbic acid whereas its glycosides of various structure have MIC < 100–12.5 μg/mL [6]. The triterpenoid aegicerin has MIC = 3.1 μg/mL [7] whereas the glycoside that is structurally similar to the aglycon and was isolated from *Scrophularia cryptophila* has much lower activity (MIC > 100 μg/mL) [8]. Likewise, steroids isolated from *Thalia multiflora* are significantly more active (MIC = 1–4 μg/mL) than their glycosylated derivatives (MIC > 100 μg/mL) [9].



1, 2

*All data on antituberculosis activity given herein refer to strain $H_{37}R_V$ *in vitro*.

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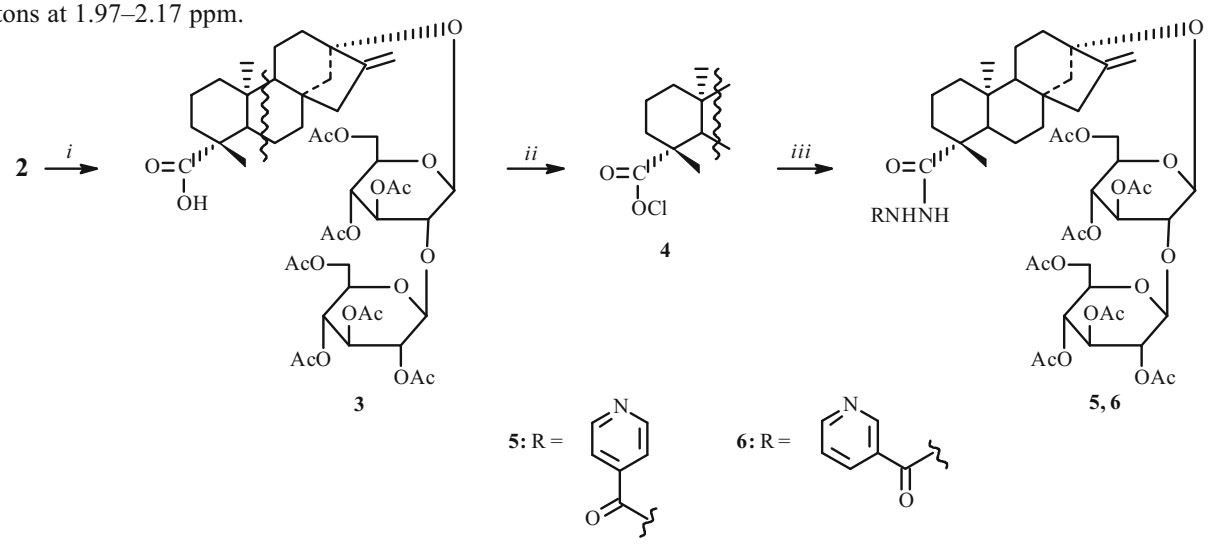
Considering the paucity of publications on the antimycobacterial activity of natural glycosides that do not have *N*-containing groups, we studied the antituberculosis activity of two glycosides from *Stevia rebaudiana*, stevioside (**1**) and steviolbioside (**2**), which are known for their sweetness that is 250 times greater than that of sucrose [10] and for their antihypertensive [11, 12], antihyperglycemic, and insulinotropic [13–15] activity.

We found that glycosides **1** and **2** exhibited moderate tuberculostatic activity against strain $H_{37}R_V$ *in vitro*. The MIC for **1** is 7.5 μg/mL; for **2**, 3.75 μg/mL.

Steviolbioside (**2**) has a reactive carboxylic acid and was modified chemically more than once in order to alter its properties. Thus, a series of 19-*O*-substituted steviolbiosides were synthesized [16, 17]. Among these, 19-*O*-[3,3-*bis*(sodiosulfo)propyl]steviolbioside exhibited better taste quality than **2**. A series of steviolbioside amides were synthesized [18]. Of these, *bis*(steviolbioside)tetramethylene diamide and steviolbioside stearylamide exhibited antitumor activity at the level of doxorubicin; *bis*(steviolbioside)decamethylene diamide and *bis*(steviolbioside)dodecamethylene diamide, antibacterial activity greater than that of penicillin G against *Bacillus subtilis*. Furthermore, steviolbioside esters containing uracil were reported [19].

Keeping in mind the tuberculostatic activity of **2** found by us, it seemed interesting to synthesize its hybrid compounds with the known antituberculosis drug isoniazid (isonicotinic acid hydrazide) and its isomer nicotinic acid hydrazide. Moreover, the synthesis and study of the antituberculosis activity of hybrid compounds based on natural metabolites of antituberculosis activity and synthetic mycostatics was recognized to be promising [2].

We used an approach involving acid chlorides to synthesize steviolbioside hybrids. Hydrazides of isonicotinic and nicotinic acids were reacted with steviolbioside acid chloride (Scheme 1). However, considering that the sugar OH groups would react with thionylchloride, they are protected beforehand by acetyls. Steviolbioside (**2**) was acetylated by acetic anhydride in cold Py as before [20]. The IR spectrum of the resulting product showed an absorption band for OAc stretching vibrations at 1751 cm^{-1}. Its PMR spectrum had characteristic resonances for steviolbioside protons in addition to resonances for acetyl protons at 1.97–2.17 ppm.



i. Ac_2O, Py, 20°C (75%); *ii*. $SOCl_2$, 20°C (95%); *iii*. $RNHNH_2$, Py/C_6H_6, 20°C (**5** – 66%, **6** – 84%)

Scheme 1

The resulting acetylated derivative **3** was reacted with an excess of thionylchloride to produce the corresponding acid chloride **4**, which was immediately reacted with the pyridinecarboxylic acid hydrazides in benzene:pyridine at room temperature. As a result, products **5** and **6** were isolated in yields of 66 and 84%, respectively. The IR spectra of these products contained bands for carboxyl stretching vibrations of **2** at 1690 cm^{-1} and bands in the range 3400–3300 corresponding to stretching vibrations of free NH groups and at 1680–1590 for absorption of amides and aromatic fragments.

An n(OAc) band at 1755 cm^{-1} indicated that acetyls were present. Resonances for aromatic protons at 7.83–8.80 ppm and 7.40–8.75 in PMR spectra of **5** and **6**, respectively, were consistent with the presence of pyridine rings. MALDI-TOF mass spectra of the products showed molecular peaks at 1056 *m/z* $[M]^+$, 1078 $[M + Na]^+$, and 1094 $[M + K]^+$, corresponding to the empirical formula $C_{52}H_{69}N_3O_{20}$. Carrying out the reaction with heating to 60°C caused partial hydrolysis of the acetyls. According to MALDI-TOF mass spectra, a mixture of products was formed and contained the fully acetylated mixed hydrazide of steviolbioside and pyridinecarboxylic acid and the same hydrazide but with partially removed acetyls (usually the two groups on the primary C atoms are lost most readily). The mixture could not be separated by chromatography.

Hybrid compounds **5** and **6** exhibited moderate tuberculostatic activity against strain $H_{37}R_V$ *in vitro*. The MIC for **5** was 5 μg/mL; for **6**, 10 μg/mL. Hence, covalent binding of **2** and the antituberculosis drug isoniazid did not enhance the activity of **2** (MIC = 3.75 μg/mL) or isoniazid (MIC = 0.1 μg/mL) [21].

EXPERIMENTAL

IR spectra were recorded on a UR-20 spectrophotometer (400–3600 cm^{-1}) and Vector 22 Fourier-spectrometer (400–4000 cm^{-1}, Bruker). Crystalline samples were studied as an emulsion in mineral oil. Matrix-assisted laser-desorption/ionization (MALDI) mass spectra were obtained on an Ultraflex III MALDI-TOF instrument (Bruker). The matrix was 2,5-dihydroxybenzoic acid. PMR spectra were recorded on an Avance instrument (Bruker) at operating frequency 600 MHz. Column chromatography was performed over silica gel (Chemapol). Reaction mixtures were analyzed by TLC on Silufol UV-254 plates.

Antituberculosis activity of **1** and **2** against bacteria was studied experimentally *in vitro* using vertical diffusion on thick nutrient medium Novaya. A culture of laboratory strain ($H_{37}R_V$ or *M. avium*) was weighed on a torsion balance. A weighed portion (10 mg) was placed into a ceramic mortar and thoroughly ground. A culture suspension with bacterial standard turbidity 100 million microbes per milliliter (10 units) was prepared. The resulting suspension (0.2 mL) was innoculated into tubes with nutrient medium, into which drug solutions (0.3 mL) that were prepared by serial dilutions were added. The tubes were placed in a vertical position into a thermostat and incubated at 37°C for 10–12 d. The activity of the compounds against the strain was studied in parallel in three tubes at each concentration.

Antituberculosis activity of **5** and **6** was studied on a BACTEC MGIT 960 culture system. Middlebrook 7h9 growth medium with BACTEC MGIT OADC enrichment additive (oleic acid, albumin, dextrose, and catalase) was used. Experiments were performed by serial dilutions against strain $H_{37}R_V$. A culture of the laboratory strain was weighed on a torsion balance. A weighed portion (10 mg) was placed into a ceramic mortar and thoroughly ground. A culture suspension with bacterial standard turbidity 100 million microbes per milliliter (10 units) was prepared. The resulting suspension (0.1 mL) was innoculated into tubes containing growth medium and a solution of the studied compound (5.0 mL) (for each dilution) and incubated in the instrument thermostat at 37°C. The presence or absence of mycobacteria growth was recorded daily for 11 d. The MIC of the synthesized compounds was determined as the lowest concentration at which growth was delayed by a day more than for isoniazid.

Stevioside (**1**) was prepared by the literature method [22]; steviolbioside (**2**), by base hydrolysis as before [23]; nicotinic acid hydrazide, by the literature method [24]. We used commercial isoniazid (Merck).

13-*O*-[β-D-Heptaacetylsophorosyl]-*ent*-cauren-19-oic acid (3) [20]. A cooled (0°C) solution of **2** (0.5 g, 0.7 mmol) in anhydrous Py (5 mL) was treated with acetic anhydride (1.75 mL), left at room temperature for 20 h, heated at 60°C for 4 h, and poured into cooled acetic acid solution (1%, 40 mL). The resulting precipitate was filtered off, washed with acidified water, and dried in air to afford the product (0.55 g, 75%), mp 125°C (MeOH), $[\alpha]_D^{20}$ –39.5° (*c* 0.2, EtOH). IR spectrum (mineral oil, ν, cm^{-1}): 1754 (C=O), 1664 (C=C), 1230 (C–O).

PMR spectrum (400 MHz, C_5D_5N, δ, ppm): 1.19 (3H, s, H_3-20), 1.34 (3H, s, H_3-18), 1.98–2.17 (21H, m, 7 $OC(O)CH_3$], 3.97–5.76 (12H, m, sophorosyl H), 5.05 (1H, s, H-17), 5.66 (1H, s, H-17). $C_{46}H_{64}O_{20}$.

13-*O*-[β-D-Heptaacetylsophorosyl]-*ent*-cauren-19-oylchloride (4). Heptaacetylated steviolbioside (**3**, 0.3 g) was treated dropwise with freshly distilled thionylchloride (1 mL) and left at room temperature for 24 h. The excess of thionylchloride was distilled off. The solid was ground with petroleum ether and dried in air to afford a yellow powder (0.29 g, 95%). IR spectrum (mineral oil, ν, cm^{-1}): 1814 (ClC=O), 1755 [$OC(O)CH_3$], 1230 (C–O).

General Method for Preparing Hydrazides 5 and 6. Freshly prepared acid chloride (**4**, 0.3 g, 0.3 mmol) in anhydrous benzene (3 mL) was treated dropwise with a solution of nicotinic or isonicotinic hydrazide (0.3 mmol) in anhydrous Py (3 mL) and stirred at room temperature for 48 h. Solvent was distilled at reduced pressure and temperature less than 50°C. The solid was washed several times with water, dried, and recrystallized from MeOH.

19-Nor-4α-(carbazoylcarboxo-4-pyridinyl)-13-*O*-(β-D-heptaacetylsophorosyl)-*ent*-caurene (5). Yield 0.22 g (66%), mp 140°C (MeOH), $[\alpha]_D^{20}$ –36.5° (*c* 0.5, MeOH). IR spectrum (mineral oil, ν, cm^{-1}): 3392, 3504 (NH), 1755 (C=O), 1645 [C=C + C(O)NH], 1592 (Ar), 1502 (Ar), 1232 (C–O).

PMR spectrum (400 MHz, DMSO-d_6, δ, ppm): 0.96 (3H, s, H_3-20), 1.17 (3H, s, H_3-18), 1.55–2.08 [21H, m, 7 $OC(O)CH_3$], 3.94–5.24 (12H, m, sophorosyl + 2H, C-1′ anomer. H, C-1″ anomer. H + 2H, H_2-17), 7.83 (2H, s, Ar-H-3‴,5‴) , 8.80 (2H, br.s, Ar-H-2‴,6‴), 9.32 (1H, s H–N^2), 10.48 (1H, s, H–N^1).

Mass spectrum (MALDI-TOF, m/z, I_{rel}, %): 1056 (100) $[M]^+$, 1078 (51) $[M + Na]^+$, 1094 (85) $[M + K]^+$. $C_{52}H_{69}N_3O_{20}$.

19-Nor-4α-(carbazoylcarboxo-3-pyridinyl)-13-*O*-(β-D-heptaacetylsophorosyl)-*ent*-caurene (6). Yield 0.28 g (84%), mp 135°C (MeOH), $[\alpha]_D^{20}$ –33° (*c* 0.5, MeOH). IR spectrum (mineral oil, ν, cm^{-1}): 3469, 3371 (NH), 1754 [$OC(O)CH_3$], 1652 [C=C + C(O)NH], 1592 (Ar), 1231 (C–O).

PMR spectrum (400 MHz, $CDCl_3$, δ, ppm): 1.00 (3H, s, H_3-20), 1.31 (3H, s, H_3-18), 1.66–2.06 [21H, m, 7 $OC(O)CH_3$], 3.67–5.16 (12H, m, sophorosyl + 2H, C-1′ anomer. H, C-1″ anomer. H + 2H, H_2-17), 7.39–7.41 (1H, m, Ar-H-5‴), 8.18 (1H, s, Ar-H-4‴), 8.21 (1H, d, J = 1.58, Ar-H-6‴), 8.75 (1H, br.s, Ar-H-2‴), 9.08 (1H, s, H–N^2), 9.42 (1H, s, H–N^1).

Mass spectrum (MALDI-TOF, m/z, I_{rel}, %): 1056 (20) $[M]^+$, 1078 (100) $[M + Na]^+$, 1094 (85) $[M + K]^+$. $C_{52}H_{69}N_3O_{20}$.

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