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SYNTHESIS AND ANTITUBERCULOSIS ACTIVITY OF DERIVATIVES OF THE DITERPENOID ISOSTEVIOL WITH AZINE, HYDRAZIDE, AND HYDRAZONE MOIETIES

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*Derivatives of the diterpenoid isosteviol (16-oxo-*ent*-beyeran-19-oic acid) with azine, hydrazone, and hydrazide moieties were synthesized. They exhibited high tuberculostatic activity* in vitro *against the strain* Mycobacterium tuberculosis $H_{37}R_V$ *(minimum inhibiting concentration in the range 6.3–1.7 μg/mL).*

Keywords: diterpenoids, *ent*-beyerane, isosteviol, hydrazides, *M. tuberculosis*, antituberculosis activity.

Only streptomycin, capreomycin, and kanamycin of all drugs used today for tuberculosis chemotherapy are isolated from natural sources [1]. All other tuberculosis drugs [1] and agents undergoing preclinical and clinical trials [2, 3] are products from organic syntheses. Nevertheless, over 50 metabolites of various structures that inhibit the growth of *Mycobacterium tuberculosis* in the range of minimum inhibiting concentrations (MIC) 60-3 μg/mL have currently been isolated from natural plant sources [4–17]. Diterpenoids [4, 5, 8–13] and triterpenoids [4, 5, 11, 14–17] are most widely represented among them. The diterpenoid isosteviol (16-oxo-*ent*-beyeran-19-oic acid) (**1**) also exhibits moderate antituberculosis activity (MIC = 50 μg/mL). Our group has investigated for several years chemical modification of **1** and its derivatives in which two isosteviol molecules are linked by a polymethylene spacer with C^{16} atoms [18]. It was found that the length of the spacer affects considerably the activity. Increasing the number of methylenes in the spacer from one to eight decreases MIC from 25 to 12.5 μg/mL [18]. These results were unusual because **1**, its bis-derivatives [18], and the aforementioned di- and triterpenoids of different structures [4, 5, 8–17] are not nitrogenous organic compounds yet they exhibit antituberculosis activity at the level of the N-containing antituberculosis drug pyrazinamide (MIC = 12.5 μg/mL) [19, 20]. This is probably evidence that they have a different mechanism of *M. tuberculosis* growth inhibition. It seemed interesting to determine how the introduction of pharmacophoric N-containing moieties (hydrazide and hydrazone) into **1** and its bis-derivatives [18] affects their antituberculosis activity.

For this we reacted **1** and its acid chloride **4** with hydrazine hydrate and adipic acid hydrazide (Scheme 1) and studied the ability of the resulting products to inhibit the growth of *M. tuberculosis*.

The reaction of **1** and an excess of hydrazine hydrate in MeOH gave not isosteviol hydrazone (**3**) but the azine **2**, which was isolated as the salt (Scheme 1). The reason for this was probably that hydrazone **3** formed and reacted with both starting **1** and that formed via hydrolysis of **3**. Compound **3** was obtained pure via reaction of **1** with a 10-fold excess of anhydrous hydrazine.

The reaction of isosteviol acid chloride **4** with anhydrous hydrazine in CCl_4 produced **5**. Its PMR spectrum showed characteristic resonances for protons of the *ent*-beyerane skeleton in addition to a broad singlet of the hydrazide amine at 3.83 ppm, a broad singlet of the hydrazone amine at 4.75, and a singlet of the hydrazide amide at 6.9. The reaction occurred under biphasic conditions. Compound **5** was found entirely in the organic phase. This isosteviol derivative, like hydrazone **3**, could be synthesized without a solvent in an excess of pure hydrazine. However, it was more convenient to carry out the reaction in the presence of an aprotic solvent that is immiscible with hydrazine. This enabled the excess of hydrazine to be easily separated in a separatory funnel.

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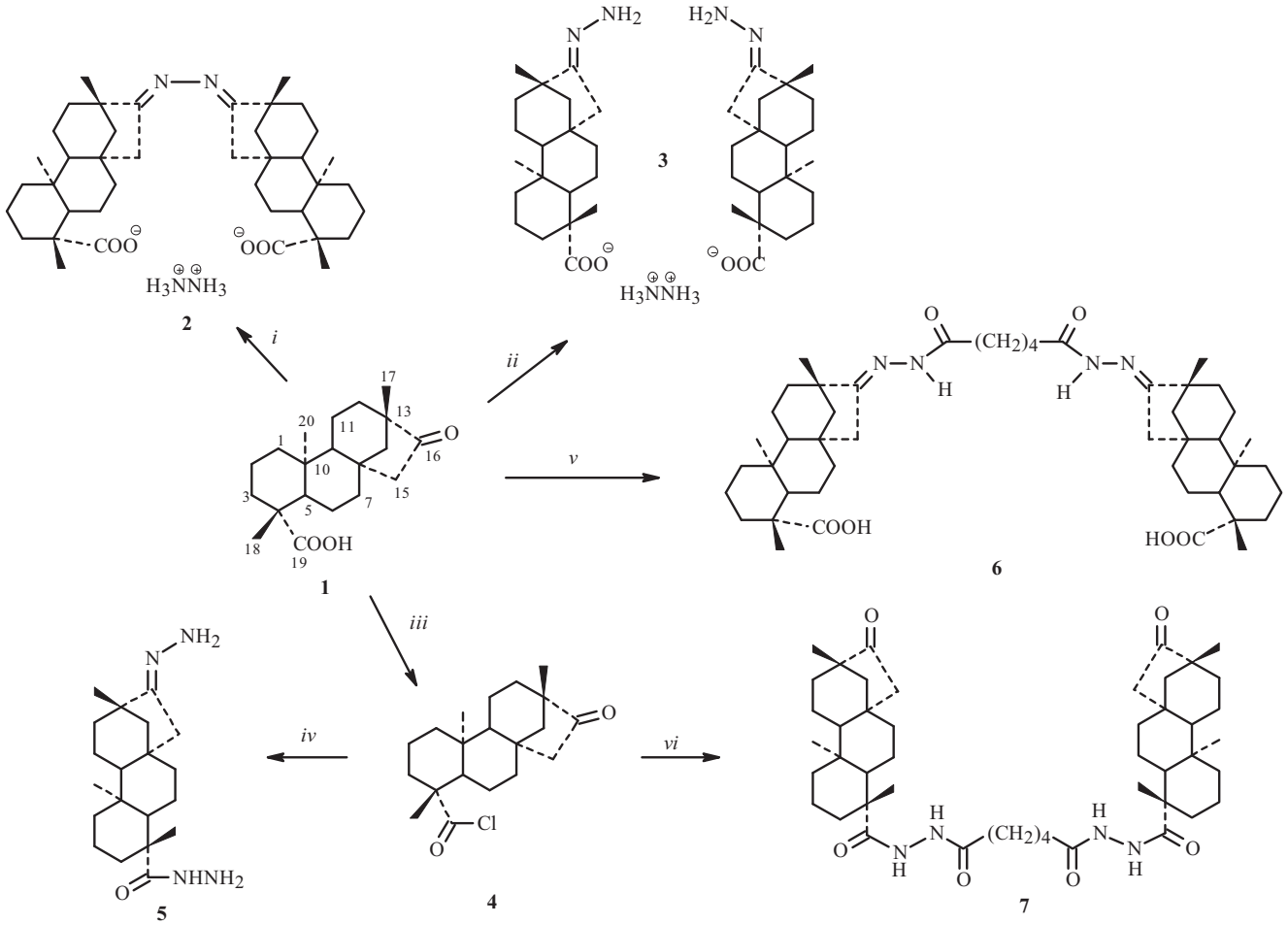

i. $N_2H_4 \cdot H_2O$ (98%), CH_3OH, 65°C, 6 h, 84% yield; *ii*. N_2H_4 (10-fold excess), CH_3OH, 4 h, 68% yield; *iii*. $SOCl_2$ (3-fold excess), 45°C, 1.5 h; *iv*. N_2H_4 (60-fold excess), CCl_4, 60°C, 6 h, 32% yield; *v*. $NH_2NHC(O)(CH_2)_4C(O)NHNH_2$, CH_3OH, 25°C, 39 h, 51% yield; *vi*. $NH_2NHC(O)(CH_2)_4C(O)NHNH_2$, dioxane/pyridine, 50°C, 24 h, 25% yield.

Scheme 1

The reaction of **1** with adipic acid dihydrazide in MeOH produced **6** in which two isosteviol molecules were linked by a dihydrazide spacer through the C^{16}=O groups (Scheme 1). The PMR spectrum of **6** showed characteristic resonances for protons of the *ent*-beyerane skeleton and multiplets at 1.5 and 2 ppm corresponding to methylene protons of adipic acid dihydrazide in addition to a singlet at 10.1 corresponding to the N–H protons. The reaction was carried out at room temperature because heating caused hydrolysis of **6** and further formation of azine **2** (Scheme 1).

The reaction of isosteviol acid chloride **4** with adipic acid dihydrazide in the presence of pyridine produced **7** in which two isosteviol molecules were linked by a dihydrazide spacer through the C^{19}OOH carboxyls (Scheme 1). The PMR spectrum of **7** contained characteristic resonances for protons of the *ent*-beyerane skeleton and singlets at 8.91 and 9.46 ppm corresponding to N–H resonances of the hydrazine spacer.

It was found that **2**, **3**, and **5**–**7** exhibited high tuberculostatic activity *in vitro* against strain $H_{37}R_V$. The MIC values were 3.1, 6.3, 6.3, 3.1, and 1.7 μg/mL, respectively.

EXPERIMENTAL

IR spectra were recorded in KBr pellets on a Bruker Vector 22 Fourier spectrometer in the range 400–4,000 cm^{-1}. Mass spectra were obtained in an MX-1310 instrument at ionizing potential 60 eV and electron-collector current 30 μA using direct sample introduction into the ion source at 120°C. The ampul-vaporizer was heated in the range 120–250°C. Matrix-

activated laser desorption/ionization (MALDI) mass spectra were obtained in a Bruker Ultraflex III MALDI TOF time-of-flight mass spectrometer. PMR spectra were taken on an Avance-600 spectrometer. The completeness of reactions and purity of products were monitored by TLC on Silufol UV-254 plates. Isosteviol (**1**) and its acid chloride (**4**) were prepared by the literature methods [21, 22].

Hydrazinium 16,16′-Azino-di-*ent*-beyeran-19-oate (2). A solution of **1** (0.4 g, 1.2 mmol) in MeOH (10 mL) was treated with hydrazine hydrate (0.63 mL, 12 mmol) and refluxed for 6 h. The MeOH and excess of hydrazine hydrate were removed. The solid was dried in vacuo over P_2O_5. Yield 0.32 g (84%), mp 129–131°C. IR spectrum (ν, cm^{-1}): 1182, 1264, 1661 (C=N).

PMR spectrum (600 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.76–1.90 (18H, m, isosteviol skeleton), 0.84 (3H, s, H-20), 1.06 (3H, s, H-17), 1.23 (3H, s, H-18), 2.16 (1H, d, J = 13.7, H-3), 2.65 (1H, dd, J = 18.6, 3.7, H-15α), 5.72 (4H, s, NH_2). $C_{40}H_{64}N_4O_4$. Mass spectrum (*m*/*z*): 632.5 $[M]^+$. $C_{40}H_{60}N_2O_4$.

Hydrazinium 16-Hydrazono-*ent*-beyeran-19-oate (3). A solution of **1** (0.3 g, 1 mmol) in anhydrous MeOH (20 mL) was treated with anhydrous hydrazine (0.3 mL, 10 mmol) and stirred until complete conversion of **1** (5 h). The MeOH and unreacted hydrazine were removed. The resulting solid was dried in vacuo over P_2O_5 for 1 d. Yield 0.30 g (91%), mp 189–191°C, $C_{40}H_{64}N_4O_4$. IR spectrum (ν, cm^{-1}): 1167, 1453, 1662 (C=N), 3366 (NH_2).

PMR spectrum (600 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.68–1.91 (18H, m, isosteviol skeleton), 0.85 (3H, s, H-20), 1.06 (3H, s, H-17), 1.23 (3H, s, H-18), 2.17 (1H, d, J = 13.7, H-3), 2.66 (1H, dd, J = 18.6, 3.7, H-15α), 3.91 (8H, s, NH_2). $C_{40}H_{68}N_6O_4$. Mass spectrum (*m*/*z*): 332.3 $[M]^+$. $C_{20}H_{32}N_2O_2$.

19-Nor-4α-hydrazinocarbonyl-16-hydrazono-*ent*-beyerane (5). Isosteviol acid chloride (**4**) was dissolved in CCl_4 (20 mL), stirred, treated with anhydrous hydrazine (2 mL), and refluxed for 4.5 h. The layer of unreacted hydrazine was separated in a separatory funnel. The organic phase was evaporated to dryness. The dry solid was dried for 1 d in vacuo over P_2O_5. Yield 0.30 g (79%), mp 234–236°C. IR spectrum (ν, cm^{-1}): 754, 982, 1451, 1629 (C=N), 3354 (NH_2).

PMR spectrum (600 MHz, $CDCl_3$, δ, ppm, J/Hz): 0.73–1.96 (18H, m, isosteviol skeleton), 0.78 (3H, s, H-20), 1.05 (3H, s, H-17), 1.18 (3H, s, H-18), 2.02 (1H, d, J = 13.7, H-3), 2.63 (1H, dd, J = 18.6, 3.7, H-15α), 3.83 (2H, s, NH_2), 4.75 (2H, s, NH_2), 6.90 (1H, s, NH). Mass spectrum (*m*/*z*): 346.3 $[M]^+$. $C_{20}H_{34}N_4O$.

Adipic Acid *N*,*N*′-*bis*(19-Nor-4α-carboxy-*ent*-beyeran-16-ylidene)dihydrazide (6). Adipic acid dihydrazide (0.1 g, 0.6 mmol) was dissolved with heating in MeOH (50 mL). The solution was cooled, stirred, treated with **1** (0.38 g, 1.2 mmol), and stirred for 39 h. The solid unreacted adipic acid dihydrazide was filtered off. The filtrate was evaporated. The solid was stirred with $CHCl_3$ (20 mL). The undissolved part was filtered off. The filtrate was evaporated to dryness and stored in vacuo over P_2O_5 for 1 d. Yield 0.24 g (51%), mp >300°C. IR spectrum (ν, cm^{-1}): 753, 1125, 1453, 1635 (C=N), 1687 (COOH).

PMR spectrum (600 MHz, C_5D_5N, δ, ppm, J/Hz): 0.75–1.86 (36H, m, isosteviol skeleton), 0.83 (6H, s, H-20), 1.14 (6H, s, H-17), 1.25 (6H, s, H-18), 1.53 (4H, m, C$\underline{H}_2$), 2.01 (4H, m, C$\underline{H}_2$), 2.33 (2H, d, J = 13.7, H-3), 2.91 (2H, dd, J = 18.6, 3.7, H-15α), 10.05 (1H, s, NH), 10.31 (1H, s, NH). $C_{46}H_{70}N_4O_6$. Mass spectrum (*m*/*z*, MALDI): 775.7 $[M + H]^+$.

Adipic Acid *N*,*N*′-*bis*[(19-Nor-16-oxo-*ent*-beyeran-4α-yl)carbonyl]dihydrazide (7). A suspension of adipic acid dihydrazide (0.11 g, 0.6 mmol) in pyridine (10 mL) was heated to 50°C under Ar, stirred, treated dropwise with a solution of **4** (0.2 g, 0.6 mmol) in dioxane (3 mL), and stirred at 50°C under Ar for 24 h. Unreacted adipic acid dihydrazide was filtered off. Pyridine was evaporated from the filtrate in vacuo. The resulting product was recrystallized from *i*-PrOH. Yield 0.06 g (25%), mp 251–253°C. IR spectrum (ν, cm^{-1}): 1449, 1550 (amide II), 1659, 1737 (COOH), 3244.

PMR spectrum (600 MHz, DMSO-d_6, δ, ppm, J/Hz): 0.69–1.95 (18H, m, isosteviol skeleton), 0.72 (3H, s, H-20), 0.87 (3H, s, H-17), 1.12 (3H, s, H-18), 2.13 (1H, d, J = 13.7, H-3), 2.86 (1H, dd, J = 18.6, 3.7, H-15α), 8.91 (1H, s, NH), 9.46 (1H, s, NH). $C_{46}H_{70}N_4O_6$.

The antituberculosis activity of **2**, **3**, and **5**–**7** was studied *in vitro* using bacteriological vertical diffusion on Novaya dense nutrient medium. A culture of laboratory strain $H_{37}R_V$ was weighed on a torsion balance. A weighed portion (10 mg) was placed into a porcelain mortar and thoroughly ground. A suspension of the culture was prepared using bacterial standard turbidity 100 million microbes per millimeter (10 units). The resulting suspension (0.2 mL) was inoculated into tubes with growth medium. The drugs prepared by serial dilutions were added dropwise (0.3 mL) to the tubes, which were placed vertically in a thermostat and incubated at 37°C for 10–12 d. The activity of the compounds against *M. tuberculosis* was studied in parallel in three tubes at each concentration.

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