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New biologically active epidioxysterols from *Stereum hirsutum*

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Abstract—From the fungus *Stereum hirsutum* have been isolated and identified two new epidioxysterols **1**, **4**, together with two known ones **2** and **3**. Their structures were elucidated on the basis of spectroscopic analysis and chemical reactions. Epidioxysterols **1**–**4** have been shown to possess a significant activity against *Mycobacterium tuberculosis.*

Stereum hirsutum is a basidiomycete involved in esca, one of the most destructive diseases in grapevine[1] and seems to play an important role in the wood deterioration process.[2] From the culture medium of this fungus, different new acetylenic compounds,[3] tricyclic sesquiterpenes,[4] chromene, and aromatic aldehydes derivatives[5] have been isolated and identified.

Recently, we reported the isolation and biological investigation of active principles from *S. hirsutum* on the basis of inhibitory potency on thrombin.[6] A bioassay oriented fractionation of the extract of *S. hirsutum* has led to the isolation of complex mixtures of diacylglycerophospholipids (DAGPs) and diacylglycerols (DGs) responsible for the activity of the extract. Moreover, docking studies on thrombin were performed in order to clarify the binding mode of some isolated compounds. In the course of our investigations on biologically active chemical substances from *S. hirsutum*, preliminary experiments indicated that most of the antimicrobial activity was associated with a fraction containing steroid derivatives; this was examined in detail and four epidioxysterols (**1**–**4**) (Fig. 1) were isolated and identified. Since few epidioxysterols from different sources have been described for antimicrobial activity,[7,8] we studied the antibacterial, antifungal, and antitubercular activity of the complex mixture and of the pure epidioxysterols and detected a significant activity against *Mycobacterium tuberculosis*.

This paper describes the structural elucidation of these compounds and the antitubercular activity against *M. tuberculosis*.

Stereum hirsutum was collected in September 2005 in northeast Slovenia. Within 24 h of collection, it was dried in an air-flow chamber at 30–35 °C and then stored at −20 °C. A mushroom specimen was deposited for evidence. The freshly frozen mushroom (1 kg) was disrupted into small pieces and homogenized with 100 mL of 50% (v/v) methanol. The homogenate was exposed to ultrasound for 10 min, macerated at room temperature for 12 h, and again exposed to ultrasound for 10 min.

Dry extract (10 g) was redissolved in methanol, mixed with 5 g silica gel, and evaporated in vacuo. The dry extract was chromatographed on silica gel using CH_2Cl_2, CH_2Cl_2/MeOH (5:1), and MeOH to give three fractions [F-1: 1.8 g (18%); F-2: 2.05 g (20.5%); F-3: 4.85 g (48.5%)]. The fraction eluted with CH_2Cl_2 was concentrated in vacuo and submitted to column chromatography over silica gel using CH_2Cl_2/*n*-hexane (9:1) and CH_2Cl_2/MeOH (5:0.5) as eluents to afford two fractions

Keywords: *Stereum hirsutum*; Epidioxysterols; *Mycobacterium tuberculosis*; Antitubercular activity.
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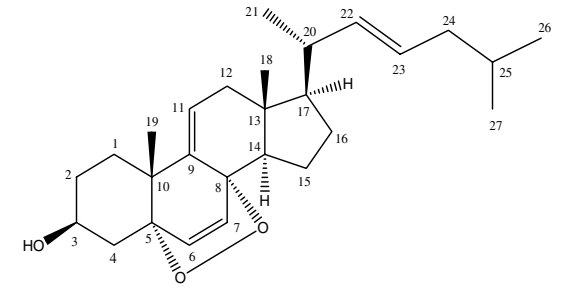

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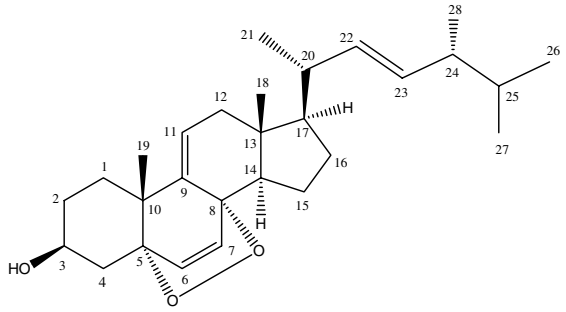

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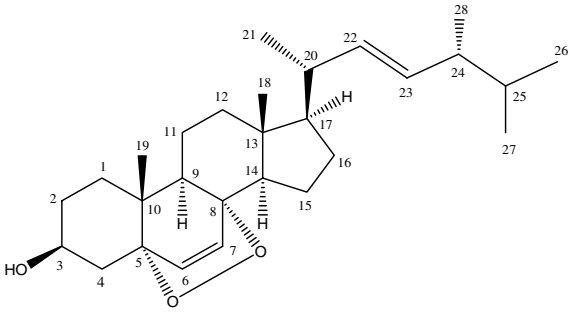

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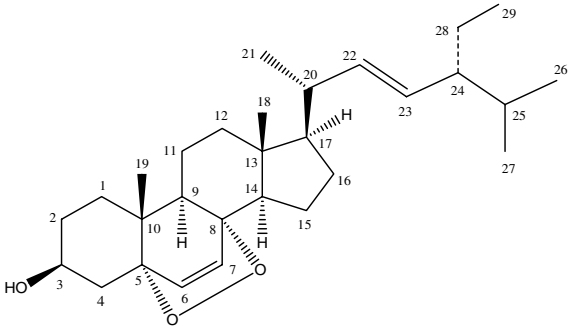

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Figure 1. Chemical structures of epidioxysterols **1**–**4**.

[F-4: 250 mg (13.9%); F-5: 1.38 g (76.7%)]. Successive purification of F-4 by reverse-phase flash chromatography, using MeOH, followed by reverse-phase RP-18 preparative TLC, using CH_3CN/MeOH (7:3) afforded almost pure **1** [40 mg, 16% (purity 94%)]; **2** [60 mg, 24% (purity 94%)]; **3** [36 mg, 14.4% (purity 92%)]; **4** [50 mg, 20% (purity 98%)], respectively.

Compound **1** was obtained as colorless amorphous solid and was shown to have the molecular formula $C_{27}H_{40}O_3$ from the observation of a quasi-molecular ion-peak in high-resolution (HR) FAB-MS. In the EI-MS spectrum of **1** the molecular ion at *m*/*z* 412 was observed, while the fragments at *m*/*z* 394 and 380 were due to the loss of H_2O and O_2 from the molecular ion, respectively. The fragment at *m*/*z* 300 was due to the loss of the side chain C_8H_{15} from the molecular ion. IR absorption bands at 3380, 2962, and 1650 cm^{-1} indicated the presence of hydroxyl group, alkyl moiety, and double bonds, respectively.

Detailed ^1H and ^{13}C NMR data for compounds **1**–**4** are available as a Supplementary Table. ^1H NMR spectrum of **1** showed signals due to five methyl groups: three secondary methyls [δ_H 1.03, 3H, d, J = 6.6 Hz (H_3-21); δ_H 0.85, 3H, d, J = 6.6 Hz (H_3-26); δ_H 0.87, 3H, d, J = 6.6 Hz (H_3-27)] and two tertiary methyls [δ_H 0.82, 3H, s (H_3-18); δ_H 1.11, 3H, s (H_3-19)]. The ^1H NMR aided spectrum, with the ^{13}C NMR spectral data, suggested the presence of three olefins (two disubstituted and one trisubstituted ones). Two-dimensional NMR spectra [^1H–^1H COSY, ^1H-detected heteronuclear multiple quantum coherence (HMQC), and HMBC] indicated that one double bond is located between the C-6 and C-7 positions, one between the C-9 and C-11 position, and the remaining one between the C-22 and C-23 positions. Olefin protons [δ_H 6.60, 1H, d (H-7); δ_H 6.27, 1H, d (H-6)] with *cis*-coupling (J = 8.5 Hz), together with two oxygenated quaternary carbons on C-5 (δ_C 82.2) and C-8 (δ_C 78.3) were suggestive of the presence of a peroxide structure. The HMBC spectrum of **1** afforded long-range ^1H–^{13}C correlations shown in Figure 2.

The relative configuration of **1** was determined based on the following evidence. NOESY analysis focused on the ring junctures of the steroidal skeleton confirmed that **1**

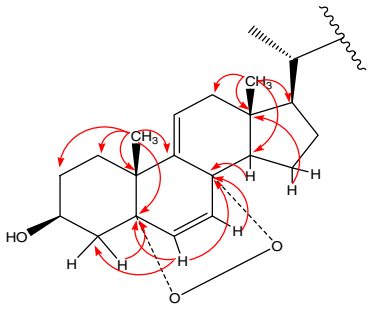


Figure 2. Selected HMBC correlations for **1** and **2**.

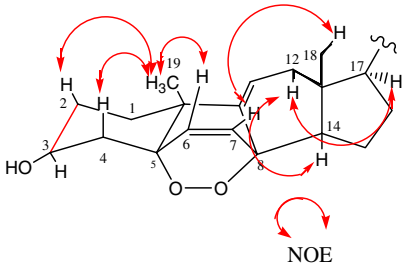
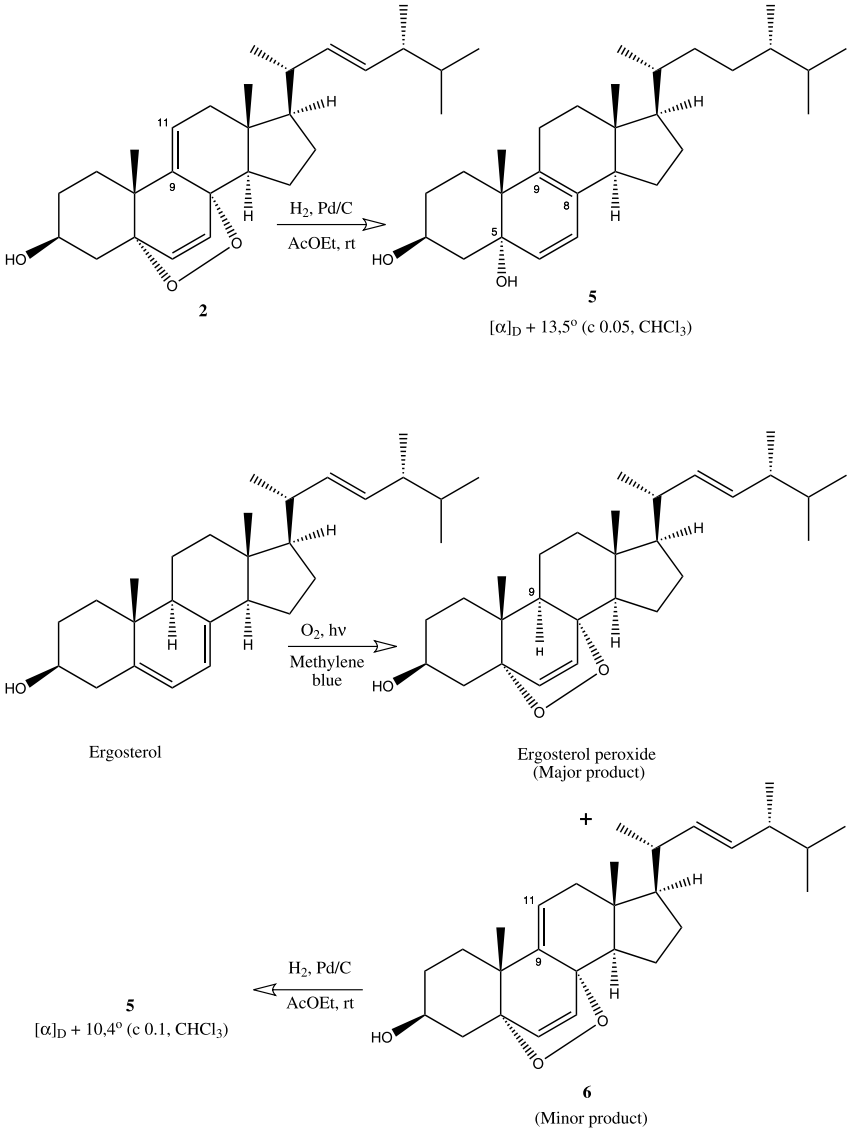


Figure 3. Important NOE correlations and long-range coupling (bold) for **1** and **2**.

has the same relative configuration as that of **2** (Fig. 3). The NOE correlation between H-14 and H-12α, which showed long-range coupling (W-shape) with the angular methyl group (H-18) in the COSY spectrum, as shown in the bold lines in Figure 3, exhibited the *trans* configuration between H-14 and H-18. The relative configuration of H-17 was assigned as an α-orientation due to the NOE correlation between H-12 α and H-17. The α-orientation of the epidioxy group was determined by the NOE correlations between H-18 and H-7 (olefinic proton). The relative configuration of the angular methyl group at C-10 was indicated by the NOE correlation between H-6 (olefinic proton) and H-19, and the stereochemistry at C-3 was deduced from the *J* values of H-3 (*J* = 5.0, 11.2 Hz).

Compound **2** was obtained as a white amorphous solid and was shown to have the molecular formula $C_{28}H_{42}O_3$ from the observation of a quasi-molecular ion-peak in high-resolution (HR) FAB-MS. In the EI-MS spectrum of **2** the molecular ion at *m*/*z* 426 was observed, while the fragments at *m*/*z* 394 and 329 were due to the loss of O_2 and of the side chain C_7H_{13} from the molecular ion. IR absorption bands at 3379, 2960, and 1650 cm^{-1} indicated the presence of hydroxyl group, alkyl moiety, and double bonds, respectively.

^1H NMR spectrum of **2** showed signals due to six methyl groups: four secondary methyls [δ_H 1.03, 3H, d, *J* = 6.6 Hz (H_3-21); δ_H 1.09, 3H, d, *J* = 6.8 Hz (H_3-28); δ_H 0.85, 3H, d, *J* = 6.6 Hz (H_3-26); δ_H 0.87, 3H, d, *J* = 6.6 Hz (H_3-27)] and two tertiary methyls [δ_H 0.82, 3H, s (H_3-18); δ_H 1.11, 3H, s (H_3-19)]. The chemical shift values of carbons in **2** were essentially identical to those of **1**, except for C-24. The side chain was assigned through the ^1H–^1H COSY from H_3-20 to H_3-28. HMBC correlations of the olefinic methine proton H-22 (δ 5.22 ppm) with C-20; C-23 and C-24 and those of H-23 (δ 5.26 ppm) with C-20; C-22; C-24; confirmed these connectivities. The nature of the double bond was assumed to be *trans* by the coupling constant value *J* = 15.2 Hz observed between H-22 and H-23. The stereochemistry of C-20 chiral center in the side chain was assigned by NOESY analysis while the configuration



Scheme 1.

of the methyl group at C-24 was assumed to be R by comparison of the C-26, C-27, and C-28 proton chemical shifts with those observed for the epimer.[9] The chemical conversions from **2** to the known diol **5** were carried out to establish the absolute configuration of **2**, as shown in Scheme 1.[10,11] Catalytic hydrogenation of **2** in the presence of 10% palladium on carbon in ethyl acetate gave **5** {$[\alpha]_D^{25}$ +13.5° (*c* 0.05, $CHCl_3$)}. The authentic diol **5** {$[\alpha]_D^{25}$ +10.4°} was independently prepared from ergosterol in two steps with photosensitized oxidation,[11] in which the desired epidioxysterol **6**, identical to **2**, was obtained as a minor product, followed by catalytic hydrogenation. The spectral data of **5** derived from **2** were identical to those of authentic **5**, including the sign of its optical rotation value. These findings together with the spectral analysis of **6** confirmed the structure of **2**.

Compound **3** was obtained as a white amorphous solid and was shown to have the molecular formula $C_{28}H_{45}O_3$ from the observation of a quasi-molecular ion-peak in high-resolution (HR) FAB-MS. In the EI-MS spectrum of **3** the molecular ion at *m/z* 428 $(M-H)^+$ was observed, while the fragment at *m/z* 410 was due to the loss of H_2O from the molecular ion. IR absorption bands at 3381, 2962, and 1644 cm^{-1} indicated the presence of hydroxyl group, alkyl moiety, and double bonds, respectively.

^1H NMR spectrum of **3** showed signals due to six methyl groups: four secondary methyls [δ_H 1.03, 3H, d, J = 6.6 Hz (H_3-21); δ_H 1.09, 3H, d, J = 6.8 Hz (H_3-28); δ_H 0.85, 3H, d, J = 6.6 Hz (H_3-26); δ_H 0.87, 3H, d, J = 6.6 Hz (H_3-27)] and two tertiary methyls [δ_H 0.82, 3H, s (H_3-18); δ_H 1.11, 3H, s (H_3-19)]. ^1H NMR aided spectrum, with ^{13}C NMR spectral data, suggested the presence of two disubstituted olefins. Olefin protons [δ_H 6.60, 1H, d (H-7); δ_H 6.27, 1H, d (H-6)] with *cis*-coupling (J = 8.5 Hz), together with two oxygenated quaternary carbons on C-5 (δ_C 82.0) and C-8 (δ_C 79.3) were suggestive of the presence of a peroxide structure. The ^1H–^1H COSY spectrum of **3** revealed the following correlations for H-1-H-4, H-6-H-7, H-9-H-12, H-14-H-22, and H-24-H-28. The HMBC spectrum of **3** afforded long-range ^1H–^{13}C correlations shown in Figure 4. Besides, the photosensitized oxidation of ergosterol led to a major product which spectral data were identical to those of **3** (Scheme 1).

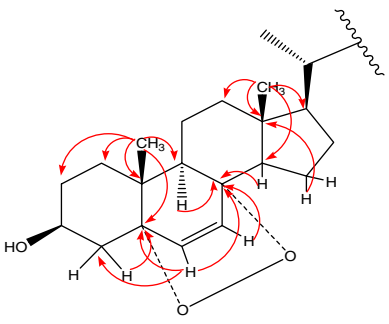


Figure 4. Selected HMBC correlations for **3** and **4**.

Compound **4** was obtained as a white amorphous solid and was shown to have the molecular formula $C_{29}H_{46}O_3$ from the observation of a quasi-molecular ion-peak in high-resolution (HR) FAB-MS. In the EI-MS spectrum of **4** the molecular ion at *m/z* 443 was observed, while the fragments at *m/z* 411 and 410 were due to the loss of O_2 and H_2O from the molecular ion, respectively. The fragment at *m/z* 303 was due to the loss of the side chain $C_{10}H_{19}$ from the molecular ion. IR absorption bands at 3380, 2962, and 1645 cm^{-1} indicated the presence of hydroxyl group, alkyl moiety, and double bonds, respectively.

^1H and ^{13}C NMR spectra of **4** were quite similar to those of **3**, suggesting that differences between **3** and **4** were due to the presence of an ethyl group in **4** instead of a methyl group at the C-24 position. The relative configuration of **3** and **4** was determined on the basis of NOESY analysis as reported above for compound **1**.

All the isolated compounds **1**–**4** were evaluated for antibacterial activity against a set of Gram-positive and Gram-negative reference strains, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27753; for antifungal activity against 11 *Candida* spp. clinical isolates; for antitubercular activity against the reference strain *M. tuberculosis* H37Rv. Antimicrobial activity was always evaluated by reference methods.[12–14] Ciprofloxacin was chosen as a standard in both antibacterial and antitubercular activity measurements, as it is an antibiotic employed in the treatment of a wide range of infections and as it is a drug known to have an excellent activity against most Gram-negative and Gram-positive bacteria. Miconazole was chosen as a standard in antifungal activity measurements. Antitubercular activity was evaluated by MRA, a recently developed, one-week duration, micro-dilution Resazurin assay.[12] The minimum inhibitory concentration, MIC, was defined as the lowest drug concentration that prevented Resazurin color change from blue to pink and was determined by visual inspection twice in duplicate experiments; viable counting from control wells and from test wells performed into agar plates confirmed bactericidal and bacteristatic activity of the compounds. Isoniazid and Rifampicin were always included as a standard in antitubercular activity measurements, having a MIC of 0.05 μg/mL and of 0.1 μg/mL, respectively. The antimicrobial activity of F-4 and of the pure compounds **1**–**4** is reported in Table 1.

No antibacterial nor antifungal activity was detected. Epidioxysterols **1** and **2** exhibited a killing activity with MIC of 16 μg/mL against *M. tuberculosis* H37Rv reference strain, while compounds **3** and **4** exhibited a MIC of 64 μg/mL.

From the obtained data, it is possible to deduce that killing activity of the new epidioxysterols is specific for *M. tuberculosis* and that the MIC of F-4, containing the complex mixture of epidioxysterols **1**–**4**, is not due to a synergic effect of the isolated compounds **1**–**4**. 5α, 8α-Epidioxysterols were previously isolated from an edible mushroom *Lentinula edodes*[15] and from sea hare

Table 1. Antimicrobial activity (MIC, μg/mL) of fraction 4 and of purified epidioxysterols **1**–**4**; reference drugs are also reported

	Mycobacterium tuberculosis H37Rv	*Staphylococcus aureus* ATCC 25923	*Escherichia coli* ATCC 25922	*Pseudomonas aeruginosa* ATCC 27753	*Candida* spp. (11 strains)
F-4	16	>512	>512	>512	>512
1	16	>512	>512	>512	>512
2	16	>512	>512	>512	>512
3	64	>512	>512	>512	>512
4	64	>512	>512	>512	>512
Ciprofloxacin	0.5	0.5	0.25	1	—
Miconazole	—	—	—	—	0.25–32

Aplysia juliana.[16] To our knowledge, compounds **2** and **3** have been isolated and identified from *Pellia epiphylla* (L.) Corda, a common thalloid European liverwort, while compounds **1** and **4** are new.[17] Nevertheless, this is the first example of the isolation and structure elucidation of epidioxysterols from *S. hirsutum*.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.08.072.

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