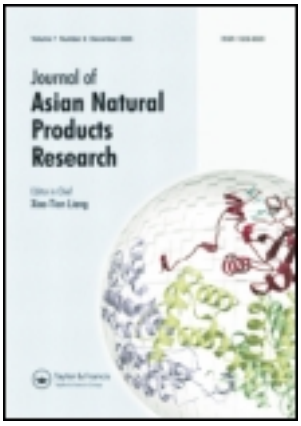

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Depsitinuside: a new depside galactoside from an endophytic fungus isolated from *Viburnum tinus*

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NOTE

Depsitinuside: a new depside galactoside from an endophytic fungus isolated from *Viburnum tinus*

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Chromatographic purification of the extract of an endophytic fungal culture yielded depsitinuside (**1**), a new phenolic ester together with ergosterol (**2**) and (22*E*,24*S*)-24-methyl-5-α-cholesta-7,22-diene-3β,5,6β-triol (**3**). The structure of **1** was elucidated based on 1D, 2D NMR spectroscopy and high-resolution mass spectrometry, whereas the known compounds (**2** and **3**) were identified by ^{1}H NMR, mass spectrometry, and in comparison with the literature values. Compound **1** was evaluated for its enzyme inhibitory potential against acetylcholinesterase, butyrylcholinesterase and lipoxygenase, and was found inactive (10%–40% inhibition at a concentration of 2 mg/ml).

Keywords: *Viburnum tinus*; endophytic fungus; depside; steroids; enzyme inhibition

1. Introduction

Investigation of endophytic fungi is a relatively new and attractive area of natural product chemistry and drug development. Endophytes are wonderful natural machinery to synthesize countless chemicals of diverse structural classes with potential activities [1–4]. Colonization and propagation of endophytes may offer a significant benefit to their host plants by producing a plethora of metabolites that provide protection and survival value to the plants. These compounds could impact the broader ecological community as plant growth regulators, antimicrobials, antivirals, and insecticidals, or even mediate resistance to some types of abiotic stress. Endophytes also could be potential sources of novel natural products with agrochemical, pharmaceutical, and industrial potential [5–8].

In continuation of our research on endophytic fungi to find medicinally important active metabolites, we investigated the culture extract of a nonsporulating endophytic fungus isolated from the leaves of *Viburnum tinus*, and isolated a new phenolic ester depsitinuside (**1**) and two known steroids: ergosterol (**2**) [9] and (22*E*,24*S*)-24-methyl-5-α-cholesta-7,22-diene-3β,5,6β-triol (**3**) [10], which were characterized spectroscopically (Figure 1).

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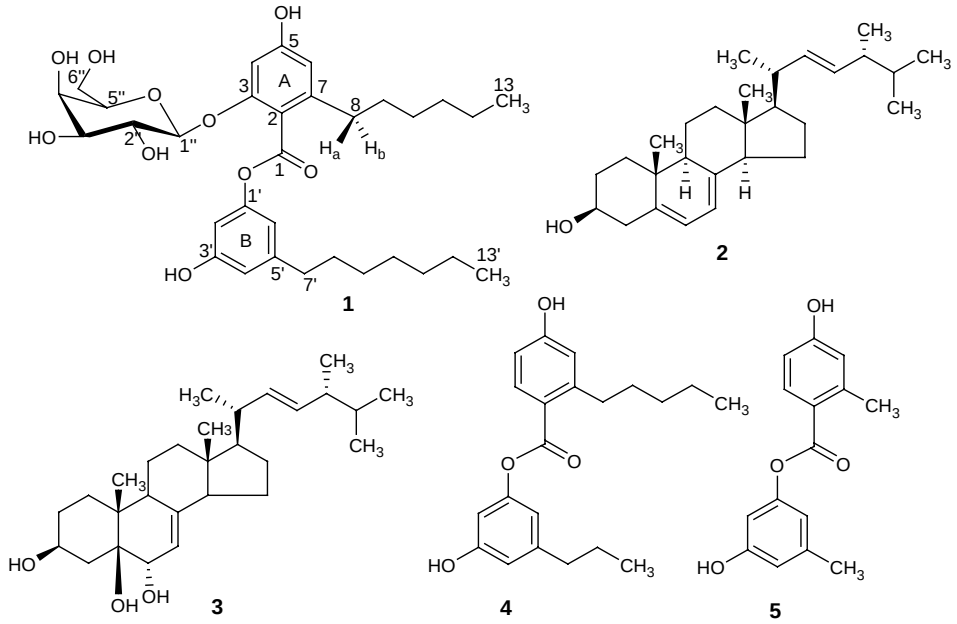

Figure 1. Metabolites (**1**–**3**) isolated from endophytic fungus, strain 8984.

2. Results and discussion

Compound **1** was isolated as a colorless amorphous powder. The HR-FAB-MS (− ve mode) of **1** exhibited the molecular ion peak at *m/z* 589.3009 $[M - H]^-$, corresponding to the molecular formula $C_{32}H_{45}O_{10}$ with 10 double bond equivalences (DBE). The IR spectrum of **1** indicated the presence of carbonyl and hydroxyl groups, besides exhibiting stretching bands for aromatic and aliphatic systems. The ^{1}H and ^{13}C NMR spectral data (see Section 3) were the evidence for two benzene rings in the molecule, compensating the eight DBE; one DBE was attributed to a carbonyl function. The ^{1}H NMR spectrum displayed a methine doublet at δ 4.58 ($J = 7.6$ Hz), which correlated with a carbon in the ^{13}C NMR spectrum at δ 102.8 for an anomeric center of sugar moiety. The presence of the glycon unit accommodated the remaining DBE and led to the idea that the other part of the molecule could be an acyclic aliphatic system.

Five aromatic methines resonated as broad singlets in the ^{1}H NMR spectrum at δ 6.41, 6.38, 6.36, 6.29, and 6.22, whereas the upfield region displayed three signals for two methylene groups at δ 2.57 (1H, dt, $J = 11.5$, 7.5 Hz, H-8$_a$), 2.45 (1H, dt, $J = 11.5$, 7.5 Hz, H-8$_b$), and 2.35 (2H, t, $J = 7.8$ Hz, H-7′). The downfield shift and splitting of two germinal protons H-8$_{a,b}$ were due to the magnetic anisotropic effect of the carbonyl function, helping us to fix CH_2-8 at ring-A (Figure 1), and therefore, CH_2-7′ could be fixed at ring-B. These data indicated that **1** must be a depside-type nucleus and more likely an identical structure to decarboxynorimbricaric acid (**4**), which is actually a 3-hydroxy-5-propylphenyl ester of olivetolic acid [11]. The lengths of the aliphatic chains in **1** were found to be six and seven carbon skeletons. The main difference was the glycone moiety, which was fixed at C-3 on ring-A based on HMBC correlations. The anomeric methine (δ 4.58) showed its HMBC interaction with C-3 (δ 156.6)

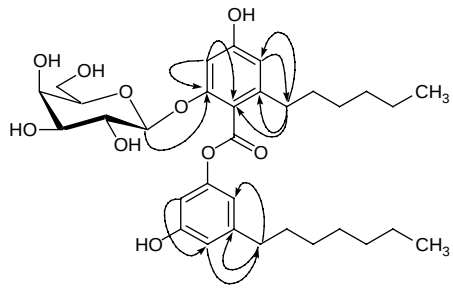

Figure 2. HMBC correlations observed in the spectrum of **1**.

(Figure 2). The HMBC correlations of methylene protons (δ 2.57 and 2.45) with carbon signals at δ 143.7, 110.9, and 114.6 confirmed one aliphatic chain at C-7 of ring-A, whereas the triplet methylene (δ 2.35) showed correlations with carbons at δ 145.3, 113.0, and 112.3 indicating the attachment at C-5′ of ring-B. H-4″ of the sugar unit displayed its position at δ 3.69 as a doublet with a smaller coupling constant ($J = 1.5$ Hz), as an indication of galactose. The idea was further supported by the carbon shifts of the sugar moiety in the ^{13}C NMR spectrum. The acid hydrolysis of **1** provided various products, among which the sugar could be separated and identified as β-D-galactose through its optical rotation sign and comparison of the retention time of its trimethylsilyl (TMS) ether with that of the standard in gas chromatography (GC). The structure of **1** was finally confirmed by the combination of COSY, HMQC, and HMBC data and was named as depsitinuside.

Depsides with a phenyl benzoate nucleus form the largest group of lichen metabolites with minor structural variations [12–15]. Of this class of molecules, lecanorin (**5**) has the lowest number of carbons and is known as a phytotoxic agent [14], whereas other depsides are also reported to possess antiproliferative and cytotoxic activities [15].

Compound **1** was evaluated for its enzyme inhibitory properties against acetylcholinesterase, butyrylcholinesterase and lipoxygenase, and was found to be inactive (10%–40% inhibition) at a concentration of 2 mg/ml.

3. Experimental

3.1 General experimental procedures

IR spectra were recorded as KBr pellets on a JASCO 320-A infrared spectrophotometer. Mass spectra were recorded on Finnigan (Varian MAT, Waldbronn, Germany) 112 and Finnigan (Varian MAT) 312 and 112S double-focusing mass spectrometers connected to MASPEC data system-1 on PC base or on a Jeol JMX-HX 110 mass spectrometer. Linked scan and peak matching experiments were also carried out on the same instruments. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer; chemical shifts δ are in parts per million, while coupling constants J are in hertz. The GC was carried out on a Shimadzu GC (GC-9A) (3% OV-1 silanized chromosorb W, column temperature 180°C, injection port and detector temperature 275–300°C, flow rate 35 ml/min, flame ionization detector). Optical rotations were measured on a JASCO DIP-360 polarimeter. Column chromatography was carried out using silica gel (GF_{254} type 60 of 70–230 mesh E. Merck, Darmstadt, Germany) as a stationary phase packed in glass columns with organic solvents as the mobile phase. Thin layer chromatography (TLC) was done on pre-coated aluminum TLC plates (GF_{254}, 0.25 mm E. Merck). Ceric sulfate solution was prepared in 65% sulfuric acid and used as the spraying agent. The TLC plates were visualized by spraying this reagent with subsequent heating under UV light at 254 and 366 nm.

3.2 Isolation of fungi and culture

Following surface sterilization, the endophytic fungus, internal strain No. 8984, was isolated from the leaves of *V. tinus* [16]. It was cultivated for 28 days at 20°C on biomalt agar medium [17].

3.3 Isolation and purification

Twelve liters of the culture medium (fungus and agar medium) were extracted with ethyl acetate to afford a crude extract (2 g) after the removal of the solvent under reduced pressure. The combined concentrated extract (2 g) was column-chromatographed on silica gel by eluting with a gradient of *n*-hexane/ethyl acetate (100:0–0:100) to obtain seven fractions (A–G). Fraction F (35.0 mg) on further repeated silica gel column chromatography eluted with an isocratic mobile phase system of 30% ethyl acetate in *n*-hexane yielded **1** (22.0 mg). Fraction C (45.0 mg) on further repeated silica gel column chromatography with 8% ethyl acetate in *n*-hexane yielded **2** (11 mg), whereas **3** (2.5 mg) was purified from fraction D (24.0 mg) by silica gel column chromatography eluting with 15% ethyl acetate in *n*-hexane.

3.3.1 Depsitinuside (*1*)

Colorless amorphous powder (22.0 mg); IR (KBr) ν_{max}: 3475 (OH), 3120 and 2920 (C—H), 1720 (C═O) and 1610, 1560 (aromatic ring); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.41 (1H, br s, H-4), 6.38 (1H, br s, H-6′), 6.36 (1H, br s, H-2′), 6.29 (1H, br s, H-4′), 6.22 (1H, br s, H-6), 4.58 (1H, d, $J = 7.6$ Hz, H-1″), 3.69 (1H, d, $J = 1.5$ Hz, H-4″), 3.66 (1H, dd, $J = 10.5$, 3.5 Hz, H_b-6″), 3.56 (1H, t, $J = 7.6$ Hz, H-2″), 3.54 (1H, dd, $J = 10.5$, 4.8 Hz, H_a-6″), 3.42 (1H, dd, $J = 7.6$, 1.5, H-3″), 3.36 (1H, m, H-5″), 2.57 (1H, dt, $J = 11.5$, 7.5 Hz, H-8_a), 2.45 (1H, dt, $J = 11.5$, 7.5 Hz, H-8_b), 2.35 (2H, t, $J = 7.8$ Hz, H-7′), 1.50–1.41 (4H, m, H-9 and H-8′), 1.32–1.10 (14H, br s, H-10-12 and H-9′-12′), 0.67 (6H, t, $J = 7.0$ Hz, H-13 and H-13′); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 167.4 (C-1), 114.6 (C-2), 156.6 (C-3), 101.3 (C-4), 159.6 (C-5), 110.9 (C-6), 143.7 (C-7), 33.7 (C-8), 31.5 (C-9), 28.9 (C-10), 31.1 (C-11), 22.3 (C-12), 13.5 (C-13), 157.4 (C-1′), 106.0 (C-2′), 151.2 (C-3′), 112.3 (C-4′), 145.3 (C-5′), 113.0 (C-6′), 35.5 (C-7′), 31.5 (C-8′), 29.2 (C-9′), 28.8 (C-10′), 31.1 (C-11′), 22.3 (C-12′), 13.5 (C-13′), 102.8 (C-1″), 70.9 (C-2″), 75.5 (C-3″), 68.6 (C-4″), 73.0 (C-5″), and 61.3 (C-6″); HR-FAB-MS (−ve mode): *m/z* 589.3009 $[M - H]^-$ (calcd for $C_{32}H_{45}O_{10}$, 589.3012).

3.3.2 Acid hydrolysis of *1*

A solution of **1** (4 mg) in MeOH (5 ml) containing 1 N HCl (4 ml) was refluxed for 4 h, concentrated under reduced pressure, and diluted with H_2O (8 ml). It was extracted with EtOAc and the residue recovered from the organic phase was found to be a mixture of products. The aqueous phase was concentrated and D-galactose was identified by the sign of its optical rotation ($[\alpha]_D^{20}$ +79.5). It was also confirmed based on the retention time of its TMS ether (α-anomer 3.8 min, β-anomer 5.2 min) with that of standard [18] in GC.

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