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Lanostane-Type Triterpenes and Abietane-Type Diterpene from the Sclerotia of Chaga Medicinal Mushroom, *Inonotus obliquus* (Agaricomycetes), and Their Biological Activities

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ABSTRACT: Three new lanostane-type triterpenes (compounds 1–3), 1 new abietane-type diterpene (compound 4), and 10 known compounds (5–14) were isolated from sclerotia of *Inonotus obliquus*. Their structures were elucidated through a combination of spectrometric techniques, including infrared, 1-dimensional, and 2-dimensional nuclear magnetic resonance and high-resolution electrospray mass spectrometry. In *in vitro* assays, compounds 2 and 4–12 showed hepatoprotective effects against D-galactosamine–induced damage in WB-F344 cells, with inhibitory effects from 35.4% to 83.8%. Compounds 3, 13, and 14 exhibited selective cytotoxicity against Bel-7402, A-549, or KB cell lines. Compounds 13 and 14 showed inhibitory effects against protein tyrosine kinases, with half-maximal inhibitory concentrations of 23.8 and 7.4 μmol/L, respectively.

KEY WORDS: bioactive compounds, *Inonotus obliquus*, medicinal mushrooms, terpenes

ABBREVIATIONS: $[\alpha]_D$, specific optical rotation; **CC**, column chromatography; **ECD**, electronic circular dichroism; **ESIMS**, electrospray ionization mass spectrometry; **EtOAc**, ethyl acetate; **EtOH**, ethanol; **HMBC**, heteronuclear multiple-bond connectivity; **HPLC**, high-performance liquid chromatography; **HRESIMS**, high-resolution electrospray ionization mass spectrometry; **IR**, infrared; **NMR**, nuclear magnetic resonance; **OD**, optical density; **PTK**, protein tyrosine kinase; **ROESY**, rotating frame Overhauser effect spectroscopy; t_R, retention time

I. INTRODUCTION

The Chaga medicinal mushroom, *Inonotus obliquus* (Pers.: Fr.) Pilát (Haemochaetaceae, Agaricomycetes), is a white rot species widely distributed throughout Europe, Asia, and North America. This mushroom is usually found in nature as a sterile conk (sclerotia) on *Betula* species, whereas the fruiting body is rarely found in nature because it grows under the bark of trees and is easily accessed and eaten by insects.[1,2] *I. obliquus* has been used as a folk medicine for cancer treatment in Russia and western Siberia, and has been used to prevent and treat heart, liver, and stomach diseases and tuberculosis. Moreover, it has been traditionally used since the 16th century in Russia, Poland, and many Baltic countries to treat gastrointestinal cancer, cardiovascular disease, and diabetes.[3–6] *I. obliquus* produces a diverse range of metabolites, which are biologically active compounds possessing hypoglycemic,[7] hepatoprotective,[8] antioxidant,[9] anti-inflammatory,[10] antifungal, and antitumor activities.[2,3,11–13] In previous investigations of the chemical constituents of this mushroom, lanostane-type triterpenoids such as inotodiol,[14] trametenolic acid,[15] and inonotsuoxides[16] were reported to have antitumor and antifungal activities. Kukulyanskaya et al.[17] reported that *I. obliquus* produces a diverse range of phenolic constituents, including hispidin analogues and melanins. These compounds are considered to be the active constituents in inhibiting replication of HIV-1 and the influenza viruses H1NI and H3N2.[18] Extracts from sclerotia of *I. obliquus* have been known to have positive effects in controlling cancer, HIV-1,

and stomach ulcers.[19] Antitumor experiments have been conducted with *n*-hexane extracts of *I. obliquus*, and inotodiol has been reported to have a significant anticancer effect *in vitro* on Walker 256 carcinosarcoma cells and human MCF-7 mammary adenocarcinoma cells and *in vivo* against leukemia P388 cells.[12]

In our research for additional bioactive compounds from *I. obliquus*, 3 new lanostane-type triterpenes (compounds 1–3); a new abietane-type diterpene (compound 4); and 10 known compounds—including 4 triterpenes (inotodiol [compound 5], lanosterol [compound 6], 3*β*,22-dihydroxylanosta-8,24-dien-11-one [compound 7], and trametenolic acid [compounds 8]), 3 sterols (ergosta-7,22-dien-3*β*-ol [compound 9], 24*β*-ethylcholest-4-en-3*β*-ol [compound 10], and ergosta-7-en-3*β*-ol [compound 11]), and 3 phenolic compounds (vanillic acid [compound 12], protocatechuic aldehyde [compound 13], and 4-[3,4-dihydroxyphenyl]but-3-en-2-one [compound 14])—were isolated from the ethyl acetate (EtOAc) fraction of an ethanol (EtOH) extract from *I. obliquus* sclerotia through the use of column chromatography (CC) and high-performance liquid chromatography (HPLC). Thin-layer chromatography and HPLC analysis showed that the EtOAc fraction of *I. obliquus* was rich in secondary metabolites, and it exhibited cytotoxicity and inhibitory activity against protein tyrosine kinases (PTKs). We describe herein the isolation and structural elucidation of the new compounds 1–4 and their biological activities (Fig. 1).

II. MATERIALS AND METHODS

A. General Procedures

Melting points were determined with a Yanagimoto micromelting point apparatus and are uncorrected. Optical rotations were measured with a JASCO P-2000 polarimeter. Infrared (IR) spectra were recorded with a Nicolet 5700 spectrometer through the use of Fourier transform IR transmission electron microscopy. UV spectra were collected on a JASCO V-650 spectrophotometer. Electronic circular dichroism (ECD) spectra were recorded on a JASCO J-815 spectrometer. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with VNS-600, Bruker AV 500-III, MP-400, and Mercury-300 spectrometers. High-resolution electrospray ionization mass spectrometry (HRESIMS) was recorded with an Agilent 1100 LC/

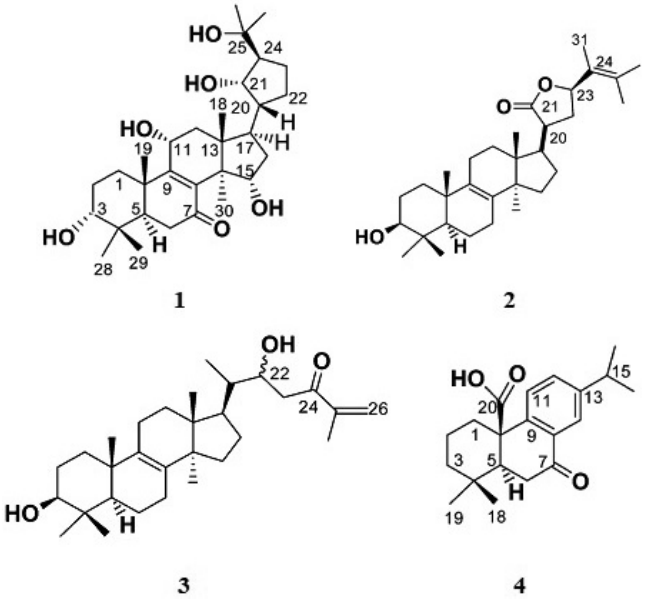

FIG. 1: Structures of compounds 1–4 isolated from the sclerotia of *Inonotus obliquus*

MSD ion trap mass spectrometer. Preparative HPLC was performed on a Lumtech instrument equipped with an Alltech 500 Evaporative Light Scattering Detector and a YMC-Pack ODS-A column (250 × 20 mm, 5 μm; YMC, Kyoto, Japan). Purification was performed using silica gel (160–200 mesh; Qingdao Marine Chemical Factory, Qingdao, China), Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and a YMC-Pack ODS column (50 μm). Fractions obtained from CC were monitored with thin-layer chromatography (silica gel GF254; Qingdao Marine Chemical Factory).

B. Mushroom Material

I. obliquus sclerotia were collected from native forests around Sulaymaniyah, Iraq, in November 2016 and were identified by the Department of Biology at the University of Sulaimani.

C. Extraction and Isolation

I. obliquus sclerotia (20.0 kg) were powdered and extracted 4 times with 95% EtOH under reflux for 1 hour. The solvent was evaporated under reduced pressure to provide an EtOH extract (680.3 g), which was successively partitioned with $CHCl_3$ and H_2O. The aqueous phase was further extracted with EtOAc and 1-butanol to produce an EtOAc fraction (44.0 g), a 1-butanol fraction (149.1 g), and an aqueous fraction (66.2 g). The EtOAc fraction (44.0 g) was subjected to silica gel CC and eluted with a petroleum ether–acetone gradient system (50:1 to 1:1), providing 9 fractions. Each fraction was subjected to silica gel CC and eluted with petroleum ether–acetone or $CHCl_3$-acetone, or a $CHCl_3$-MeOH gradient solvent system (100:0 to 0:100). Fraction 1 (6.1 g) yielded compounds 6 (471 mg), 9 (41 mg), 10 (17 mg), and 11 (18 mg). Fraction 2 (16.8 g) yielded compounds 5 (2.2 g), 6 (94 mg), and 8 (290 mg). Fraction 3 (1.6 g) yielded compounds 7 (17 mg) and 13 (780 mg). Fraction 4 (1.7 g) was purified by reverse-phase HPLC (MeOH-H_2O-HOAc [60:40:1, v/v/v]) to yield compound 1 (41 mg; retention time [t_R] = 25 min). Fraction 5 (3.1 g) was purified by reverse-phase HPLC (MeOH-H_2O-HOAc [80:20:1, v/v/v]) to yield compounds 2 (21 mg; t_R = 38 min) and 3 (16 mg; t_R = 22 min). Fraction 6 (2.3 g) yielded compounds 12 (23 mg) and 14 (71 mg). Fraction 7 (0.9 g) was purified by reverse-phase HPLC (MeOH-H_2O-HOAc [70:30:1, v/v/v]) to yield compound 4 (28 mg; t_R = 33 min).

Compound 1 was a white amorphous solid. The following data were obtained: specific optical rotation ($[\alpha]^{25}_D$), −114.4 (concentration, 0.1; MeOH); IR (KBr) ν_{max} = 754, 997, 1031, 1056, 1172, 1270, 1378, 1455, 1571, 1644, 1714, 2877, 2967, and 3386 cm^{-1}; UV (MeOH) λ_{max}, 258 nm (log ε, 4.02); ECD (MeOH), 335 ($\Delta\varepsilon$ + 4.65) and 254 ($\Delta\varepsilon$ − 12.87); positive-ion electrospray ionization mass spectrometry (ESIMS) *m/z*, 505 $[M + H]^+$, 527 $[M + Na]^+$, and 543 $[M + K]^+$; positive-ion HRESIMS *m/z*, 527.3350 $[M + Na]^+$ (calculated for $C_{30}H_{48}O_6Na$, 527.3343). See Table 1 for ^1H NMR (pyridine-d_5, 500 MHz) and ^{13}C NMR (pyridine-d_5, 125 MHz) data.

Compound 2 was also a white amorphous solid. The following data were obtained: $[\alpha]^{25}_D$, +102.1 (concentration, 0.1; $CHCl_3$); IR ν_{max}, 762, 1039, 1129, 1371, 1454, 1601, 1652, 1701, 2836, 2876, 2952, 3235, and 3482 cm^{-1}; UV (MeOH) λ_{max}, 227 nm (log ε, 4.06); positive-ion ESIMS *m/z*, 469 $[M + H]^+$, 491 $[M + Na]^+$ and 507 $[M + K]^+$; positive-ion HRESIMS *m/z*, 491.3507 $[M + Na]^+$ (calculated for $C_{31}H_{48}O_3Na$, 491.3496). See Table 1 for ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data.

Compound 3 was a white amorphous solid as well. The following data were obtained: $[\alpha]^{25}_D$, +76.2 (concentration, 0.1; $CHCl_3$); IR ν_{max}, 836, 1031, 1099, 1372, 1411, 1451, 1578, 1624, 1665, 1708, 2874, 2932, and 3409 cm^{-1}; UV (MeOH) λ_{max}, 210 nm (log ε, 3.77); positive-ion ESIMS *m/z*, 457 $[M + H]^+$; positive-ion HRESIMS *m/z*, 457.3662 $[M + H]^+$ (calculated for $C_{30}H_{49}O_3$, 457.3676). See Table 2 for ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data.

TABLE 1: Nuclear Magnetic Resonance Spectroscopy Data for Compounds 1 and 2

Position	Compound 1		Compound 2	
	δ_C, Type	δ_H (*J* in Hz)	δ_C, Type	δ_H (*J* in Hz)
1	35.1, CH_2	2.57, dt (5.5, 12.5), 2.12[a]	35.6, CH_2	1.73, m, 1.23[a]
2	28.9, CH_2	1.97 (2H)	27.9, CH_2	1.66, m, 1.58, m
3	77.7, CH	3.47, m	79.0, CH	3.23, dd (4.5, 11.5)
4	40.0, C		38.9, C	
5	50.9, CH	2.01, d (3.5)	50.4, CH	1.05, dd (2.0,13.0)
6	37.9, CH_2	2.74 (2H)	18.2, CH_2	1.69,[a] 1.51, m
7	204.7, C		27.1, CH_2	1.77, m, 1.41, m
8	141.6, C		134.8, C	
9	167.7, C		134.0, C	
10	41.8, C		37.1, C	
11	64.9, CH	4.82, dd (4.5, 9.5)	21.0, CH_2	2.03 (2H)[a]
12	44.4, CH_2	3.27, dd (9.5, 13.5), 2.48, dd (4.5, 13.5)	26.5, CH_2	2.03 (2H)[a]
13	49.9, C		45.0, C	
14	52.3, C		49.5, C	
15	74.0, CH	4.64, dd (6.0, 9.0)	30.9, CH_2[a]	1.69,[a] 1.23[a]
16	36.6, CH_2	2.21, m, 2.12[a]	30.9, CH_2[a]	1.69,[a] 1.23[a]
17	49.3, CH	2.36, dd (9.0, 18.0)	46.5, CH	1.50, m
18	19.0, CH_3	1.07, s	15.6, CH_3	0.73, s
19	20.2, CH_3	1.25, s	19.1, CH_3	0.98[a]
20	49.2, CH	2.08, m	39.4, CH	2.03[a]
21	79.5, CH	4.09, t (8.0)	167.1, C	
22	24.9, CH_2	1.73, m, 1.48, dt (3.5, 6.0)	29.8, CH_2	2.45, t (15.0), 1.90, d (3.5)
23	28.7, CH_2	1.85, m, 1.42, m	78.6, CH	4.39, dt (3.5, 15.0)
24	58.8, CH	2.23, m	122.1, C	
25	72.6, C		149.0, C	
26	26.3, CH_3	1.44, s	13.6, CH_3	0.98[a]
27	30.7, CH_3	1.40, s	12.5, CH_3	1.88, s
28	16.3, CH_3	1.11, s	15.4, CH_3	1.00, s
29	28.2, CH_3	1.10, s	28.0, CH_3	0.81, s
30	19.5, CH_3	1.65, s	24.4, CH_3	0.89, s
31			20.5, CH_3	1.94, s

Data from ^{13}C nuclear magnetic resonance (NMR) (δ) were measured in pyridine-d_5 at 125 MHz for compound 1 and in $CDCl_3$ at 125 MHz for compound 2. ^1H NMR data (δ) were measured in pyridine-d_5 at 500 MHz for compound 1 and in $CDCl_3$ at 500 MHz for compound 2. The assignments were based on ^1H-^1H correlative spectroscopy, distortionless enhancement by polarization transfer, heteronuclear single-bond quantum coherence spectroscopy, heteronuclear multiple-bond connectivity spectroscopy, and rotating frame Overhauser effect spectroscopy experiments.

[a]Overlapping signals.

TABLE 2: Nuclear Magnetic Resonance Spectroscopy Data for Compounds 3 and 4

Position	Compound 3		Compound 4	
	δ_C, Type	δ_H (*J* in Hz)	δ_C, Type	δ_H (*J* in Hz)
1	35.5, CH_2	1.74, m, 1.24[b]	37.9, CH_2	2.26, br d (13.2), 1.53, dd (3.6, 13.2)
2	27.8, CH_2	1.68 (2H)[b]	18.9, CH_2	1.76, m, 1.65, m
3	78.9, CH	3.23, dd (4.4, 11.2)	37.6, CH_2	2.03, dt (3.6, 13.2), 1.87, br d (13.2)
4	38.8, C		47.0, C	
5	50.3, CH	1.05, m	38.9, CH	2.94, dd (3.0, 16.8)
6	18.2, CH_2	1.70,[b] 1.53, m	44.9, CH_2	3.06, dd (3.0, 13.2), 2.98, m
7	27.2, CH_2	1.70,[b] 1.45, m	198.6, C	
8	134.5, C		131.7, C	
9	134.1, C		154.2, C	
10	37.0, C		38.0, C	
11	20.9, CH_2	2.03 (2H)[b]	124.7, CH	7.36, d (8.4)
12	26.4, CH_2	2.03 (2H)[b]	133.1, CH	7.46, dd (1.8, 8.4)
13	44.8, C		147.2, C	
14	49.4, C		125.4, CH	8.15, d (1.8)
15	30.9, CH_2[b]	1.68,[b] 1.24[b]	34.1, CH	2.84, m
16	30.9, CH_2[b]	1.68,[b] 1.24[b]	24.21, CH_3	1.17, d (7.0)
17	47.2, CH	1.40, m	24.17, CH_3	1.17, d (7.0)
18	15.7, CH_3	0.74, s	24.0, CH_3	1.22, s
19	19.1, CH_3	0.98, s	17.3, CH_3	1.50, s
20	41.1, CH	1.83, m	180.6, C	
21	12.9, CH_3	0.96, d (6.8)		
22	69.3, CH	4.17, m		
23	37.2, CH2	2.78, d (17.2), 2.66, dd (10.4, 17.2)		
24	203.4, C			
25	144.7, C			
26	125.8, CH_2	6.02, s, 5.85, s		
27	17.4, CH_3	1.89, s		
28	15.4, CH_3	0.99, s		
29	27.9, CH_3	0.81, s		
30	24.3, CH_3	0.88, s		

Data from ^{13}C nuclear magnetic resonance (NMR) (δ) were measured in $CDCl_3$ at 100 MHz for compound 3 and in pyridine-d_5 at 150 MHz for compound 4. ^1H NMR data (δ) were measured in $CDCl_3$ at 400 MHz for compound 3 and in pyridine-d_5 at 600 MHz for compound 4. The assignments were based on ^1H-^1H correlative spectroscopy, distortionless enhancement by polarization transfer, heteronuclear single-bond quantum coherence spectroscopy, heteronuclear multiple-bond connectivity spectroscopy, and rotating frame Overhauser effect spectroscopy experiments.

[a]Overlapping signals.

Compound 4 was a white amorphous solid. The following data were obtained: $[\alpha]^{25}_{D}$, −19.3 (concentration, 0.1; MeOH); IR ν_{max}, 610, 703, 835, 908, 1082, 1127, 1194, 1252, 1383, 1460, 1489, 1562, 1607, 1681, 2869, 2948, and 3350 cm^{-1}; UV (MeOH) λ_{max}, 258 nm (log ε, 4.17); ECD (MeOH), 328.5 ($\Delta\varepsilon$ +2.04), 209.5 ($\Delta\varepsilon$ +6.22), 295.5 ($\Delta\varepsilon$ −1.60), and 253.5 ($\Delta\varepsilon$ −1.60); positive-ion ESIMS *m/z*, 315 $[M + H]^+$ and 337 $[M + Na]^+$; positive-ion HRESIMS *m/z*, 315.1959 $[M + H]^+$ (calculated for $C_{20}H_{27}O_3$, 315.1955). See Table 2 for ^1H NMR (pyridine-d_5, 600 MHz) and ^{13}C NMR (pyridine-d_5, 150 MHz) data.

D. Hepatoprotective Bioassay

Hepatoprotective effects of all compounds against D-galactosamine–induced damage to WB-F344 cells were determined with the MTT colorimetric assay, as described in the literature.[20] Bicyclol was used as the positive control. The optical density (OD) of the formazan solution was measured at 490 nm on a microplate reader. Inhibition was calculated with the following formula:

$$\text{Inhibition (\%)} = ([OD_{sample} - OD_{control}]/[OD_{normal} - OD_{control}]) \times 100.$$

E. Cytotoxicity Bioassay

Cytotoxicity of all compounds was evaluated against BEL7402, A-549, and KB human cancer cell lines with the MTT method, as described in the literature.[21] 5-Fluorouracil was used as the positive control.[21]

F. PTK Inhibitor Bioassay

The inhibitory activities of all compounds against PTKs were evaluated with an enzyme-linked immunosorbent assay, as described in the literature.[22]

III. RESULTS AND DISCUSSION

Compound 1 was obtained as a white amorphous powder with an $[\alpha]^{25}_{D}$ value of −114.4. The molecular formula of compound 1 was assigned as $C_{30}H_{48}O_6$ ($[M + Na]^+$, *m/z* 527.3350; calculated for 527.3343) using HREIMS. The IR spectrum showed absorption bands at 3386 and 1644 cm^{-1}, indicating the presence of hydroxyl and carbonyl groups. The ^1H and ^{13}C NMR spectra of compound 1 (Table 1) exhibited 7 methyl groups, 7 methylenes, 8 methines including 4 oxymethines (δ_H 3.47 [m], δ_C 77.7; δ_H 4.09]t; J = 8.0 Hz], δ_C 79.5; δ_H 4.64 [dd, J = 6.0, 9.0 Hz], δ_C 74.0; δ_H 4.82 [dd, J = 4.5, 9.5 Hz], δ_C 64.9), 8 quaternary carbons including 1 oxycarbon (δ_C 72.6), and 1 *α,β*-unsaturated ketone group (δ_C 204.7, 167.7, 141.6). The spectral data of compound 1 exhibited signals characteristic of a lanostane-type triterpene and were similar to those reported for 21,24-cyclopentalanosta-3*β*,21,25-triol-8-ene.[23] The evident differences were that compound 1 had a 21,24-cyclopentanol moiety in the side chain and 2 additional hydroxyl groups on the lanostane skeleton. The 21,24-cyclopentanol moiety in the side chain was proven by heteronuclear multiple-bond connectivity spectroscopy (HBMC) through long-range correlations from H-20 to C-21; from H-24 to C-21, C-22, and C-25; from H_3-26 to C-24, C-25, and Me-27; and from H_3-27 to C-24, C-25, and Me-26, and by ^1H-^1H correlative spectroscopy correlations between H-17/H-20/H-21/H-24 and H-22b (δ_H 1.48)/H-23b (δ_H 1.42). The positions of the 3 hydroxyl groups at C-3, C-11, and C-15 were determined by HMBC spectroscopy through correlations from H_3-28 to C-3, from H-11 to C-8 and C-9, from H-16a (δ_H 2.21) to C-15, and from H_3-30 to C-15. The carbonyl group at C-7 was proven by HMBC spectroscopy through correlations from H-6 to C-7. The relative configuration of compound 1 was proven by rotating frame Overhauser effect spectroscopy (ROESY). The correlations between H-17/H_3-30, H-24/

H_3-30, H-20/H_3-18, and H-21/H-12β/H_3-18 confirmed that H-17 and H-24 were α-oriented and H-20 and H-21 were β-oriented. The correlations between H-3/H_3-29, H-11/H_3-18, and H-15/H_3-18 indicated that H-3, H-11, and H-15 were all β-oriented. The absolute configuration was assigned as 5S,10S from positive and negative Cotton effects at 335 nm ($\Delta\varepsilon$ +4.65) and 254 nm ($\Delta\varepsilon$ −12.87), respectively, in the ECD spectrum.[24] Therefore, compound 1 was assigned the structure (−)-(3R,5S,10S,11R,15S,17R,20R,21S,24S)-21,24-cyclopenta-3,11,15,21,25-pentahydroxylanosta-8-en-7-one.

Compound 2 was obtained as a white amorphous powder with an $[\alpha]^{25}_D$ value of +102.1. The molecular formula of compound 2 was assigned as $C_{31}H_{48}O_3$ ($[M + Na]^+$, *m/z* 491.3507; calculated for 491.3496) by HREIMS. The IR spectrum showed absorption bands for hydroxyl (3482 cm^{-1}) and carbonyl (1701 cm^{-1}) groups. The ^1H and ^{13}C NMR spectra of compound 2 (Table 1) exhibited 8 methyl groups, 9 methylenes, 5 methines including 2 oxymethines (δ_H 4.39 [dt, J = 3.5, 15.0 Hz], δ_C 78.6; δ_H 3.23 [dd, J = 4.5, 11.5 Hz], δ_C 79.0), 9 quaternary carbons including 4 olefinic carbons (δ_C 122.1, 134.0, 134.8, and 149.0), and a carbonyl carbon (δ_C 167.1). The spectral data of compound 2 also exhibited characteristic signals for a lanostane-type triterpene and were very similar to those reported for 3-hydroxy-8,24-diene-lanosta-21,23-lactone.[25] The evident difference was that compound 2 had 1 additional methyl (δ_H 1.94, 3H, s; δ_C 20.5) at C-24, which was proven by the tetra-substituted olefinic bond (δ_C 122.1 [C] and 149.0 [C]) and the long-range correlations from H_3-31 to C-22, C-24, and C-25 on HMBC spectroscopy. The relative configuration of compound 2 was determined through analysis of the ROESY spectrum. The correlations between H-3/H_3-28, H-17/H_3-30, and H-17/H-21/H-23 indicated that H-3, H-17, H-21, and H-23 were all α-oriented. Therefore, compound 2 was characterized as (17α,21α,23α)-24-methyl-3β-hydroxy-5α-lanosta-8,24-diene-21,23-lactone.

Compound 3 was obtained as a white amorphous solid with an $[\alpha]^{25}_D$ value of +76.2. The molecular formula of compound 3 was determined to be $C_{30}H_{48}O_3$ ($[M + H]^+$, *m/z* 457.3662; calculated for 457.3676) by HREIMS. The IR spectrum showed absorption bands at 3409 cm^{-1}, indicating the presence of a hydroxyl group. Comparing the spectral data of compound 3 (Table 2) with those of compound 2 suggests that compounds 3 and 2 had the same lanostane skeleton but a different side chain. The NMR data for the side chain exhibited 2 methyl groups (δ_H 0.96 [3H, d, J = 6.8 Hz], δ_C 12.9; δ_H 1.89 [3H, s], δ_C 17.4), 2 methylenes including 1 olefinic methylene (δ_H 6.02 [1H, s], 5.85 [1H, s]; δ_C 125.8), 2 methines including 1 oxymethine (δ_H 4.17 [1H, m]; δ_C 69.3), 2 quaternary carbons including 1 olefinic carbon (δ_C 144.7), and 1 carbonyl carbon (δ_C 203.4), which were similar to those of inonotsutriols D and E,[26] indicating that compound 3 was the 24-oxo derivative of inonotsutriol D or E. This was proven by HMBC through long-range correlations from H-22 to C-24; from H_2-23 to C-24; from H-26b (δ_H 5.85) to C-24, C-25, and Me-27; and from H_3-27 to C-24, C-25, and C-26. The relative configuration of compound 3 was established by ROESY. The correlations between H-3/H_3-28 indicated that H-3 was α-oriented.[26] Therefore, compound 3 was designated as 3β,22-dihydroxy-5α-lanosta-8,25-dien-24-one.

Compound 4 was obtained as a white amorphous powder with a $[\alpha]^{25}_D$ value of −19.3. The molecular formula of compound 4 was assigned as $C_{20}H_{26}O_3$ ($[M + H]^+$, *m/z* 315.1959; calculated for 315.1955) by HREIMS. The ^1H NMR spectra of compound 4 (Table 2) exhibited 2 methyl groups (δ_H 1.22, 1.50, each 3H, s), an isopropyl group (δ_H 1.07 [6H, d, J = 7.0 Hz], 2.84 [1H, m]), an aliphatic methine (δ_H 2.94 [1H, dd, J = 3.0, 16.8 Hz, H-5]), signals for 3 aromatic proton (δ_H 7.36 [1H, d, J = 8.4 Hz], 7.46 [1H, dd, J = 1.8, 8.4 Hz], 8.15 [1H, d, J = 1.8 Hz]), and 8 proton signals due to 4 methylenes. In addition to proton-bearing carbon resonances corresponding to the aforementioned units, the ^{13}C NMR spectra exhibited an aliphatic carbon (δ_C 47.0), 6 quaternary carbons consisting of signals for 3 aromatic carbon (δ_C 131.7, 147.2, 154.2), a ketone group (δ_C 198.6), and a carboxyl group (δ_C 180.6). The spectral data of compound 4 were similar to those reported for 12-hydroxy-13-isopropyl-7-oxo-20-oic acid[27] and suggest that compound 4 is a 12-dehydroxy analogue. This was supported by HMBC correlations from H-1β to C-20; from H_3-16 and H_3-17 to C-13 and C-15; from H-11 to C-8, C-9, C-10, and C-13; from H-12 to

C-9, C-11, and C-15; from H-14 to C-7, C-8, and C-12; and from H-15 to C-13, Me-16, and Me-17. The relative configuration was proven by ROESY. The correlations between H-5 and H_3-19 indicated that H-5 was α-oriented. Moreover, the ECD spectrum of compound 4 displayed a positive Cotton effect at 328.5 nm ($\Delta\varepsilon$ +2.04) and a negative Cotton effect at 295.5 nm ($\Delta\varepsilon$ −1.60), which corresponded to the n→π* and π→π* transition of the conjugated 8-en-7-one chromophore. On the basis of the octant rule for the α,β-unsaturated ketone group,[28–30] the 5S,10S configurations were assigned to compound 4.

The isolated known compounds were identified by comparing their spectroscopic data with values reported in the literature: inotodiol (compound 5),[25] lanosterol (compound 6),[25] 3β,22-dihydroxylanosta-8,24-dien-11one (compound 7),[5] trametenolic acid (compound 8),[25] ergosta-7,22-dien-3β-ol (compound 9),[31] 24β-ethylcholest-4-en-3β-ol (compound 10),[32] ergosta-7-en-3β-ol (compound 11),[33] vanillic acid (compound 12),[34] protocatechuic aldehyde (compound 13),[35] and 4-(3,4dihydroxyphenyl)but-3-en-2-one (compound 14).[36]

IV. CONCLUSIONS

Compounds 1–14 isolated from *I. obliquus* sclerotia were bioassayed for their hepatoprotective effects against D-galactosamine–induced toxicity in WB-F344 cells, using bicyclol as the positive control. All compounds (10 μmol/L) showed hepatoprotective activity (Table 3). Compounds 2 and 6–8 exhibited moderate hepatoprotective effects, each with inhibition > 70%. They were also tested for cytotoxicity against 3 human tumor cell lines (Bel-7402, A-549, and KB), using 5-fluorouracil as the positive control (Table 4). Compound 3 exhibited cytotoxicity against the KB cell line, compound 13 exhibited cytotoxicity

TABLE 3: Hepatoprotective Effects of 10 Compounds (10 μmol/L) against D-Galactosamine–Induced Toxicity in WB-F344 Cells

Compound	Cell Survival Rate (% of Normal)	Inhibition (% of Control)[a]
Normal	100 ± 3.3	—
Control	69 ± 3.6	—
Bicyclol	80 ± 1.1*	—
2	92 ± 6.9†	74.1
4	80 ± 3.2†	35.4
5	87 ± 3.2*	58.1
6	93 ± 6.3†	77.4
7	95 ± 2.5*	83.8
8	93 ± 0.6‡	77.4
9	83 ± 0.5*	45.2
10	82 ± 5.6†	41.9
11	81 ± 1.2†	38.4
12	87 ± 4.6*	58.1

Results are expressed as the mean ± standard deviation (n = 3; n = 6 for normal and control). Bicyclol was used as the positive control (10 μmol/L).
[a]Inhibition (%) = ([OD_{sample} − $OD_{control}$]/[OD_{normal} − $OD_{control}$]) × 100, where OD is the optical density.
*P < 0.01; †P < 0.05; ‡P < 0.001.

TABLE 4: Cytotoxicity of 3 Compounds Against Human BEL-7402, A-549, or KB Cell Lines

Compound	IC_{50} (μmol/L) in Cells		
	BEL-7402	A-549	KB
3	> 10.0	> 10.0	9.6
13	3.5	2.9	> 10.0
14	4.5	> 10.0	> 10.0
5-Fluorouracil	5.5	3.1	8.1

IC^{50}, half-maximal inhibitory concentration.

against the Bel-7402 and A549 cell lines, and compound 14 exhibited cytotoxicity against the Bel-7402 cell line; each compound had a half-maximal inhibitory concentration < 10 μmol/L. Moreover, compounds 13 and 14 showed inhibitory activity against PTKs, with a half-maximal inhibitory concentration of 23.8 and 7.4 μmol/L, respectively.

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