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NEW TRITERPENES FROM THE FUNGUS *Gloeophyllum odoratum*

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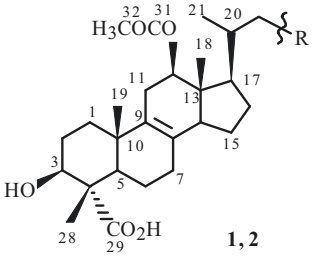
*Two new triterpene acids (**1**, **2**), together with a complex mixture of lecithins (**3**), were isolated from the fungus* Gloeophyllum odoratum. *Their structures were elucidated on the basis of extensive spectroscopic analyses. The extract of* Gloeophyllum odoratum *considerably inhibited thrombin (72% at a concentration of 120 μg/mL); the less polar fraction of the methanolic extract from the mushroom exhibited interesting activity (47% at a concentration of 120 μg/mL) against thrombin. Compounds **1** and **2** were responsible for this activity.*

Keywords: *Gloeophyllum odoratum*, triterpene acids, thrombin, lecithins.

Extensive investigations on Basidiomycetes have shown that fungi are nearly inexhaustible sources of new secondary metabolites and of biologically active components [1]. Our previous chemical study on the fungus *Gloeophyllum odoratum* led to the isolation and characterization of a new tetracyclic triterpene together with two known ones [2]. A number of volatiles have been identified from fungus [3], together with lipid metabolites such as ergosterol, ergosterol peroxide, and trametenolic and eburicoic acids as minor compounds [4, 5]. In a preliminary study, 95 selected mushroom species have been screened in order to find novel specific nonpeptidic thrombin inhibitors [6]. The extract of *Gloeophyllum odoratum* considerably inhibited thrombin (72% at a concentration of 120 μg/mL).

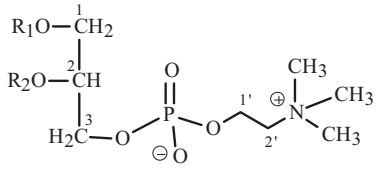
Thrombin is a multifunctional serine protease that plays a primary role in the pathogenic pathway of thrombosis as a consequence of fibrinogen cleavage to fibrin and protease-activated receptor (PAR) activation. Therefore, it represents a suitable and validated target for inhibition of blood coagulation [7]. There is currently only one orally active direct thrombin inhibitor available on the market: dabigatran, which was introduced in 2008 in the prodrug form of dabigatran etexilate [8, 9].

The need for new orally active, safe, selective, and potent thrombin inhibitors, especially with new molecular scaffolds, is significant [10]. By attaching pharmacophore groups required for high-affinity binding to thrombin, these scaffolds may potentially be transformed into potent thrombin inhibitors [11].



1, 2

1: R = CH_2-C(=CH_2)-$CH(CH_3)_2$
2: R = CH_2-$C(CH_3)$=$C(CH_3)_2$



3

3: R_1,R_2 = acyl chains
C_{16}, $C_{16:1\omega7}$, $C_{16:1\omega9}$,
$C_{18:1\omega9}$, $C_{18:2\omega6}$, $C_{18:3\omega3}$

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TABLE 1. ^{1}H (400 MHz) and ^{13}C NMR (125 MHz) Data for Compounds **1** and **2** ($CDCl_3$, δ, ppm, J/Hz)

C atom	**1**		C atom	**2**	
	δ_H	δ_C		δ_H	δ_C
1	1.36, 1.72 (2H, m)	35.22	1	1.38, 1.70 (2H, m)	35.24
2	1.62, 1.76 (2H, m)	26.91	2	1.52 (2H, m)	26.90
3	4.00 (1H, dd, J = 11.5, 4.2)	75.17	3	4.04 (1H, dd, J = 11.3, 4.0)	75.32
4	–	53.44	4	–	53.46
5	1.85 (1H, m)	45.76	5	1.85 (1H, m)	45.80
6 (16)	1.35, 1.68 (2H, m)	20.79	6	1.63, 1.77 (2H, m)	25.40
7	2.01 (2H, m)	27.48	7	2.00 (2H, m)	27.51
8	–	127.76	8	–	127.83
9	–	135.16	9	–	135.10
10	–	36.15	10	–	36.17
11	2.55 (2H, br.dd, J = 16.2, 10.2)	29.89	11	2.58 (2H, br.dd, J = 17.2, 6.4)	29.69
12	4.88 (1H, dd, 8.5, 7.8)	79.63	12	4.90 (1H, dd, 8.4, 7.7)	79.85
13	–	45.78	13	–	45.80
14	2.14 (1H, m)	51.36	14	2.15 (1H, m)	51.33
15	1.38, 1.64 (2H, m)	23.18	15	1.36 (2H, m)	23.26
16 (6)	1.50, 1.84 (2H, m)	25.67	16	1.35, 1.62 (2H, m)	20.89
17	1.48 (1H, m)	54.91	17	1.48 (1H, m)	54.87
18	0.70 (3H, s)	7.95	18	0.74 (3H, s)	8.00
19	1.00 (3H, s)	19.83	19	1.03 (3H, s)	19.89
20	1.54 (1H, m)	34.10	20	1.54 (1H, m)	34.34
21	0.90 (3H, d, J = 6.6)	20.69	21	0.86 (3H, d, J = 6.6)	20.55
22	1.10, 1.61 (2H, m)	33.46	22	1.00, 1.52 (2H, m)	35.22
23	1.87, 2.12 (2H, m)	32.40	23	2.15 (2H, m)	32.65
24	–	156.57	24	–	128.20
25	2.22 (1H, septet d, J = 6.8, 0.9)	33.66	25	–	123.52
26 (27)	1.01 (3H, d, J = 6.8)	21.76	26 (27)	1.65 (3H, s)	20.01
27 (26)	1.01 (3H, d, J = 6.8)	21.93	27 (26)	1.64 (3H, s)	18.52
28	1.15 (3H, s)	10.40	28	1.19 (3H, s)	10.45
29	–	182.23	29	–	182.20
30	4.65 (2H, q, J = 1.4),	106.17	30	1.71 (3H, s)	20.90
	4.71 (2H, dd, 1.5, 1.2)		31	–	170.99
31	–	170.73	32	2.05 (3H, s)	21.55
32	2.03 (3H, s)	21.52			

It was reported that compounds with the triterpene core significantly inhibit thrombin [12].

Our continuing investigation of this fungus aimed at discovering potential new drug leads has resulted in the isolation and characterization of two new triterpene acids (**1**, **2**), together with a complex mixture of diacylglycerophosphatidylcholines (**3**).

In this paper, we describe their structural elucidation based on the physicochemical and chemical evidence.

The fungus *Gloeophyllum odoratum* (50 g, wet) was soaked in MeOH to provide a crude extract, which was subjected to repeated column chromatography to provide a fraction (70.6 mg), followed by purification on RP-18 HPLC to give compounds **1** (12 mg), **2** (15 mg), and **3** (20 mg).

Compound **1** was obtained as a yellow amorphous powder. The molecular formula of **1** was elucidated as $C_{32}H_{50}O_5$ by HR-MS.

The ^{1}H NMR spectrum (Table 1) shows the presence of four quaternary methyls (δ 0.70, 1.00, 1.15, 2.03), three secondary methyls [0.90 (d, J = 6.6 Hz); 1.01 (d, J = 6.8 Hz), 1.01 (d, J = 6.8 Hz)], five methines [1.85, 2.14, 1.48, 1.54, 2.22 (septet d, J = 6.8, 0.9)], two oxygen-bearing methines [4.00 (dd, J = 11.5, 4.2 Hz), 4.88 (dd, J = 8.5, 7.8 Hz)], and two exomethylene protons at δ 4.65 (q, J = 1.4 Hz) and 4.71 (dd, J = 1.5, 1.2 Hz). The ^{13}C NMR spectrum displayed 32 carbon resonances due to four olefinic carbons [δ 127.76 (s), 135.16 (s), 156.57 (s), 106.17 (t)], two oxygen-bearing methine carbons (75.17, 79.63), three sp^3 quaternary carbons (53.44, 36.15, 45.78), five sp^3 methine carbons (45.76, 51.36, 54.91, 34.10, 33.66), nine sp^3 methylene carbons (35.22, 26.91, 20.79, 27.48, 29.89, 23.18, 25.67, 33.46, 32.40), seven methyl carbons (7.95, 10.40, 19.83, 21.52, 21.76, 21.93, 20.69), one carboxylic carbon (182.23) and one acetoxylic carbon (170.73) (Table 1).

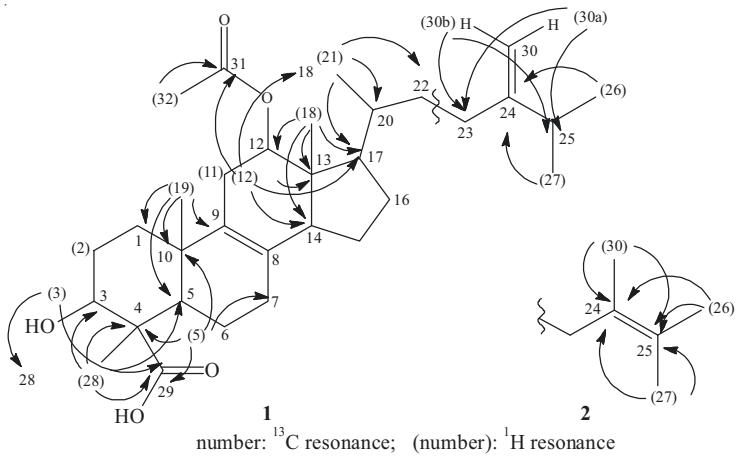

Fig. 1. Key HMBC correlations of compounds **1** and **2**.

The spectroscopic data suggested that **1** is a C_{32} steroidal compound. The structural assignment was achieved by ^{1}H–^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBC) experiments. The locations of the hydroxy groups were determined to be at C-3 and C-12 based on DQCOSY correlations of H_2-1–H_2-2–H-3 and H-12–H_2-11, coupled with the heteronuclear multiple bond correlations (HMBC) of Me-19 with C-1 and C-5 and of Me-18 with C-12 (Fig. 1). Of the seven methyl groups present in the compound, Me-21, 26, and 27 are in the form of doublets: the last two are isochronous, and thus Me-21 can be unambiguously assigned. It gives HMBC correlations, from low to high fields, with C-17, C-20, and C-22. The HMBC interactions of Me-18 are with C-12, C-13, C-14, and C-17, except C-13, all are CH carbons confirmed by the DEPT spectrum signals. It turns out that one resonance belongs to Cq 13 but is isochronous with C-5. The HMQC spectrum gives the deshielded double doublet of H-12, which in turn confirms C-13, C-14, C-17, and C-18, but also reveals the carboxylic C-31: the acetyl group is bound to C-12. Another HMBC correlation of C-31 reveals the Me-32. Although hidden under other resonances, H-14 unambigously interacts by DQCOSY with H_2-15, and H-12 interacts by DQCOSY with the multiplets of H_2-11; one of them is isolated and, although broadened, shows HMBC interactions with the vinylic C-8 and C-9. The vinylic C-9 also interacts with Me-19, so the other vinylic resonance C-8 is assigned by default. The fourth Me-19 also gives HMBC correlations with C-5, C-10, and C-1. The isochronicity of C-5 and C-13 is then corroborated. Although hidden under other frequencies, H-5 confirms C-10 and C-19 and reveals one acidic Cq resonance C-29 and one methylic resonance C-28. A last detected interaction is with H_2-7. The HMBC interaction of the fifth Me-28 confirms C-5 and the carboxylic C-29 and reveals one Cq and one deshielded CH, unambiguously assigned to C-4 and C-3: thus the acidic and methylic groups are geminal and bound at the 4 position. In the HMQC spectrum C-3 correlates with H-3 resonance; the HMBC interactions of H-3 confirm C-28 and C-29, and the DQCOSY interaction identifies H_2-2. Moreover, in the HMQC spectrum, another set of correlations reveals H-2/C-2, H-28/C-28, H-11/C-11, H-12/C-12, H-14/C-14, H-18/C-18, H-15/C-15, and H-30/C-30. The last two methyls are in the isopropylic group: the resonances Me-26 and Me-27 are diastereotopic, and because of the far reaching action of the stereo center 20, the Me-26 and Me-27 resonances are isochronous. They give HMBC interactions with vinylic C-24 and NOESY interaction with a vinylic proton resonance, which must be attributed to *cis* H-30a. Both vinylic H-30a and H-30b show HMBC interaction with the other vinylic resonance C-30 and HMBC interactions with H_2-23 and H-25.

Significant dipolar interactions are detected among the methynic resonances H-3 and H-5 and one H_2-1 methylenic resonance; no interaction is detected between the resonances H-5 and H_3-19. Because of the ubiquitous β-orientation of methyl 19, methynic protons H-3 and H-5 are α-oriented, the junction between A and B is *trans*, and the hydroxyl group geminal to 3 is β-oriented.

Another set of dipolar interactions is among the methynic resonances H-12, H-14, and H-17, while no interaction is detected between them and methylic CH_3-18. The orientation of methyl 18 makes protons 12, 14, and 17 tend toward the α-orientation; furthermore, the junction between rings C and D is *trans*, and the ester group 31 and the aliphatic chain, geminal to protons H-12 and H-17, respectively, are β-oriented.

TABLE 2. ^{1}H (400 MHz) and ^{13}C (125 MHz) NMR Data for **3** ($CDCl_3$, δ, ppm, J/Hz)

C atom	δ_H	δ_C
1a	4.40 (1H, dd, J = 11.8, 6.5)	62.0
1b	4.15 (1H, dd, J = 11.0, 5.3)	–
2	5.21 (m)	68.8
3	3.97 (m)	63.9
1′	4.23 (t)	66.0
2′	3.65 (m)	68.5
C=O	–	173.1, 172.7
CH=CH	5.23-5.40 (m)	124.1, 125.9, 127.8, 127.9, 129.6, 129.6, 130.1, 130.2
$CH{=}CH\underline{C}H_2CH{=}CH$	2.90 (t, J = 7.4)	25.6, 26.7
$OCO\underline{C}H_2$	2.30 (m, J = 7.4)	34.0, 34.1
$COCH_2\underline{C}H_2$	1.60 (m)	24.8
$=CH_2$	2.05 (m)	27.2
$N(Me)_3^+$	3.22, 3.23	55.1
CH_2	1.20–1.40 (br.s)	29.0-31.9
CH_3	0.90–0.99	14.0, 14.1

No dipolar interaction is found between CH_3-18 and CH_3-19, which are too far away; however, small correlations are detected with one H_2-11 resonance of methylenic 11, which is in intermediate position. The dipolar interaction between the signals H_3-18 and H-20 corroborates the *β*-orientation of the aliphatic chain. One important dipolar interaction is detected between the methyl signals H_3-19 and H_3-28: methyl 28 is therefore *β*-oriented, and the geminal acid group 29 is *α*-oriented.

On the basis of these findings, the structure of **1** was elucidated as 4-methyl-24-methylene-5*α*-cholest-8(9)-en-12*β*-acetoxy-3*β*-ol-29-oic acid.

Compound **2** was obtained as a pale yellow amorphous powder. The molecular formula of **2** was elucidated as $C_{32}H_{50}O_5$ by HR-MS. The ^{1}H NMR spectrum showed the presence of seven *tert*-methyl groups (δ 0.74, 1.03, 1.19, 1.64, 1.65, 1.71, 2.05), one secondary methyl [0.86 (d, J = 6.6 Hz)], four methines (1.48, 1.54, 1.85, 2.15), and two oxygen-bearing methines [4.04 (dd, J = 11.3, 4.0 Hz), 4.90 (dd, J = 8.4, 7.7 Hz)]. The ^{13}C NMR spectrum displayed 32 carbon resonances due to four olefinic carbons [δ 123.52 (s), 127.83 (s), 128.20 (s), 135.10 (s)], two oxygen-bearing methine carbons (75.32, 79.85), three sp^3quaternary carbons (53.46, 45.80, 36.17), four sp^3methine carbons (54.87, 51.33, 45.80, 34.34), nine sp^3 methylene carbons (35.24, 35.22, 32.65, 29.69, 27.51, 26.90, 25.40, 23.26, 20.90), eight methyl carbons (21.55, 20.90, 20.55, 20.01, 19.89, 18.52, 8.00, 10.45), one carboxylic carbon (182.20), and one acetoxylic carbon (170.99) (Table 1). The spectroscopic data suggested that **2** is a C_{32} steroidal compound. The ^{1}H and ^{13}C NMR spectra (Table 1) of **2** were very similar to those of **1**, indicating that **2** is also a cholestane-type steroid. However, signals for a vinylic methyl [δ_H 1.71, δ_C 20.9] were observed rather than signals for the terminal methylene group in **1**. The ^{1}H–^{1}H COSY and HMBC spectra of **2** were also similar to those in **1**, except for the ^{1}H–^{1}H COSY correlation of H_2-23 (δ 2.15) with Me-30 (δ 1.71), as well as the HMBC correlations of Me-30 with C-24 (δ 128.2) and C-25 (δ 123.5), together with Me-26 (δ 1.65) and Me-27 (δ 1.64) with C-24 and C-25. These data indicated that a tetrasubstituted olefin was present at C-24 (25) (Fig. 1). The orientations of the hydroxy group at C-3 and of the acetoxy group at C-12 in **2** were determined to be *β* from the J-values of H-3 and H-12 as well as from analysis of the NOESY spectrum, and were the same as those in **1**. HMBC correlations were observed between H-12/C-13, C-18, C-14, C-17, and C-31, H-11/C-9, C-8, Me-27/C-24, Me-26/C-25, and Me-28/C-29, providing evidence for the location of the acetoxy group at C-12, of the two double bonds at C-8 and C-24 as well as the carboxy group connected at C-4.

From the above evidence, the structure of **2** was elucidated as 4,24-dimethyl-5*α*-cholest-8(9),24(25)-dien-12*β*-acetoxy-3*β*-ol-29-oic acid.

The ^{1}H and ^{13}C NMR spectral data of **3** indicated the presence of a complex mixture of diacylglycerophospholipids. The ^{1}H and ^{13}C NMR spectral data of **3** indicated the presence of glycerol, long-chain unsaturated fatty acid ester moieties, and choline as head group. Analysis of the ^{1}H and ^{13}C NMR spectral data of **3**, together with ^{1}H–^{1}H COSY, HMQC, and HMBC experiments, led to the assignments of all the ^{1}H and ^{13}C NMR signals for glycerol moieties and the head group, as shown in Table 2. The multiplet at δ_H 5.21 was ascribed to the backbone glycerol Sn2 proton. The magnetically inequivalent glycerol Sn1 methylene protons resonated at δ_H 4.40 (dd, J = 11.8, 6.6 Hz) and 4.15 (dd, J = 11.0, 5.3 Hz), while both glycerol Sn3 methylene proton resonances overlapped at δ_H 3.97. Coupling between these glycerol backbone protons was confirmed by the cross peaks in the 2D COSY spectrum, which unequivocally gave their assignments.

TABLE 3. Fatty Acid Composition of **3**

Fatty acid methyl esters	t_R, min	%	Fatty acid methyl esters	t_R, min	%
16	6.27	6.1 ± 0.12	18:1ω9	8.02	22.95 ± 0.25
16:1ω7	6.59	0.93 ± 0.02	18:2ω6	8.58	66.13 ± 0.48
16:1ω9	6.66	2.74 ± 0.10	16:3ω3	9.14	1.16 ± 0.05

TABLE 4. Inhibitory Activities of Fraction 9 and Compounds **1–3** Isolated from *G. odoratum* towards Thrombin, %

Inhibitor	Mean inhibitory activity on Thrombin	Inhibitor	Mean inhibitory activity on Thrombin
Extract	72.1	**1**	33.1
Fraction 9	47.0	**2**	31.0
		3	23.4

Each dry fungal fraction and isolated compounds **1**–**3** was dissolved in DMSO and then added to the reaction mixture to a final concentration of 120 μg/mL. The thrombin activity was defined as the increase of absorbance per minute. For each sample, the thrombin activity in the reaction mixture with the fungal fraction or single compound was subtracted from the thrombin activity in the reaction mixture without the fungal fraction (compound) (positive control). The difference was then expressed as a percentage of the thrombin activity of the positive control and defined as the inhibitory activity on thrombin. The inhibitory activity of argatroban (0.1 μg/mL) on thrombin was 53%.

The choline head group was identified by the characteristic $-N(CH_3)_3^+$ proton singlet at δ 3.22, 3.23. The two choline head group methylene protons [$-CH_2CH_2N(CH_3)_2^+$] resonated at δ_H 3.65 (2H-2′) and δ_H 4.23 (2H-1′) and were confirmed by their cross peaks in the 2D COSY spectrum. Furthermore, the ^{13}C NMR spectrum of **3** showed two carbonyl carbon signals arising from the acyl groups at δ_C 173.1 and 172.7, and their locations in the molecule was similarly confirmed by the long-range coupling detected by the HMBC experiment. Thus, the carbonyl carbons at δ_C 173.1 and 172.7 were correlated with the proton signals of H-1 and H-2 of the glycerol part, respectively.

To characterize the structure of the fatty acid moiety, the complex mixture **3** was treated with NaOH–MeOH according to a reported method, and then with a solution of BF_3–MeOH, yielding a mixture of fatty acid methyl esters [13]. The fatty acid moiety was determined by GC-MS analyses of the above methyl esters and by comparison with a standard sample (Table 3). Consequently, the structure of **3** was determined as a complex mixture of 16:0, 16:1ω7, 16:1ω9, 18:1ω9, 18:2ω6, and 18:3ω3 diacylglycerophospholipids.

A bioassay-oriented fractionation of the extract of *Gloeophyllum odoratum* led to the isolation of an active fraction (Fr. 9) from which compounds **1–3** were isolated and identified. This active fraction showed an inhibitory activity of 47% on thrombin at a concentration of 25 μg/mL. At the concentration employed (up to 10%), DMSO did not interfere with the reaction conditions. The data are shown in Table 4. When tested alone, the complex mixture **3** did not show significant inhibitory activity against thrombin, while compounds **1** and **2** showed an inhibitory activity of 33.1 and 31.0% on thrombin at a concentration of 25 μg/ mL.

In comparison with the known thrombin inhibitor argatroban (53% of inhibitory activity on thrombin at 0.1 μg/mL), it becomes clear that compounds **1** and **2** are not potent thrombin inhibitors [7]). However, the triterpene core might be a useful fragment for the design of potent thrombin inhibitors.

Furthemore, these specific compounds provided further confirmation of the typical profile of secondary metabolites found in this family of mushrooms and might be useful for further chemotaxonomic studies.

EXPERIMENTAL

General Experimental Procedures. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. A JEOL JMS-GC mass spectrometer was used for high-resolution electron ionization MS (HR-MS). EI-MS analysis were performed with a VG-ZAB 2F spectrometer. The ionizing energy was 70 eV in all cases, and compounds were introduced by direct insertion. NMR spectra were measured on a Varian Unity-400 Fourier transform spectrometer (^1H NMR: 400 MHz;

^{13}C NMR: 100 MHz) using tetramethylsilane (TMS) as an internal standard. Silica gel (Merck, 230–400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F_{254}, 0.2 mm) were used for analytical TLC. Isolation by HPLC was performed by PerkinElmer Series 4 Liquid Chromatograph equipped with a reversed-phase column (Gemini 10 μ C_{18} 110 A, 250 mm × 10 mm). The fatty acid composition of **3** was released as methyl esters using the Association of Official Analytical Chemists (AOAC) methylation procedure and analyzed by GC-MS [13]). A Carlo Erba GC 8000 instrument coupled directly to an MD 800 mass spectrometer was used (Carlo Erba, Milan, Italy). A SP 2330 fused silica capillary column, 30 m × 0.32 mm I.D., 0.20 μm film thickness (Supelco, Inc., Bellefonte, PA) was employed. Chromatographic conditions: column temperature was programmed from 100°C (kept for 2 min) to 250°C at 10°C/min (maintained for 10 min), injector and detector temperature 270°C, carrier gas (helium) and flow rate 2.0 mL/min. Transfer line temperature was kept at 260°C. The mass spectrometer scanned from *m/z* 100 to 400 at 0.5 s cycle time. The ion source was set at 180°C, and spectra were obtained by electron impact (70 eV). Identification of compounds was carried out by comparison of retention times and mass spectra of standards.

Material. *G. odoratum* was collected in May 2010 in northeastern Slovenia, authenticated by A. Piltaver, and stored at –20°C. A representative voucher specimen was deposited at –80°C at the Faculty of Pharmacy, Ljubljana. Freshly frozen mushroom (450 g) was disrupted with an electric blender and homogenized with 2.5 L of 50% (v/v) MeOH. The homogenate was exposed to ultrasound for 10 min, macerated by rotation (125 rpm) at room temperature for 24 h, and again exposed to ultrasound for 10 min. The resulting homogenate was filtered through Whatman Grade 589 Black Ribbon quantitative filter paper, and the organic solvent was evaporated below 40°C and then air dried for 24 h at room temperature.

Extraction and Isolation. The dry extract (10 g) was redissolved in MeOH and subjected to silica gel column chromatography eluting with solvent of increasing polarity (CH_2Cl_2–MeOH) to give 20 fractions. Fraction 9 was fractionated by flash SiO_2 CC [$CHCl_3$–MeOH (10:0.5→10:1)] to yield 15 fractions (9.1–9.15). Fraction 9.5 was subjected to repeated CC [$CHCl_3$–MeOH (10:0.5)] and further purified by semipreparative HPLC over Gemini RP-18 [CH_3CN–MeOH–H_2O (70:15:15)], pH 5, with H_3PO_4 to afford compounds **1** (12 mg) and **2** (15 mg). Fraction 9.10 was purified by CC [$CHCl_3$–MeOH–H_2O (60:25:4)] to afford the complex mixture of diacylglycerophosphatidylcholines (**3**) (11 mg).

Compound 1. Amorphous powder; $[\alpha]_D$ –32.7° (*c* 0.85, MeOH). ^{1}H and ^{13}C NMR data, see Table 1. HR-MS (EI): *m/z* 514.3660 (calcd for $C_{32}H_{50}O_5$, 514.3658). EI-MS (70 eV; *m/z*, $I_{rel.}$, %): 514 (M, 21%)$^+$, 454 (M – CH_3CO_2H, 100%)$^+$, 439 (M – CH_3CO_2H – CH_3, 48%)$^+$, 421 (M – CH_3CO_2H – CH_3 – H_2O, 17%)$^+$, 329 (M – CH_3CO_2H – side chain, 82%)$^+$.

Compound 2. White amorphous powder; $[\alpha]_D$ –42.6° (*c* 0.65, MeOH). ^{1}H and ^{13}C NMR data, see Table 1. HR-MS (EI): *m/z* 514.3660 (calcd for $C_{32}H_{50}O_5$, 514.3658). EI-MS (70 eV; *m/z*, I_{rel}, %): 514 (M, 14%)$^+$, 454 (M – CH_3CO_2H, 100%)$^+$, 439 (M – CH_3CO_2H – CH_3, 39%)$^+$, 421 (M – CH_3CO_2H – CH_3 – H_2O, 13%)$^+$, 329 (M – CH_3CO_2H – side chain, 50%)$^+$.

Compound 3. Yellow oil. For ^{1}H and ^{13}C NMR data, see Table 2. The fatty acid composition of **3** is reported in Table 3.

Determination of Inhibitory Activity. Inhibitory activities of fungal fractions on human thrombin (Sigma, St. Louis, USA) were determined in 96-well microtiter plates using chromogenic substrates *H*-D-phenylalanine-L-pipecolyl-L-arginine-*p*-nitroaniline dihydrochloride (S-2238). The stock solutions of the enzyme and substrates were prepared according to the manufacturers' instructions. Thrombin and S-2238 were employed at final concentrations of 0.5 NIH units/mL and 0.5 mM, respectively, in a final volume of 200 μL. Half of the final volume of the mixture comprised HBSA buffer (pH 7.5) containing 10 mMHepes (Sigma), 150 mMNaCl, and 0.1% bovine serum albumin (Sigma), and 10% of the final volume was dimethyl sulfoxide (DMSO) with the tested compounds dissolved. The final concentrations of the tested inhibitors were 120 μg/mL for the fractions and 25 μg/mL for the purified compounds. All components except the substrate were first mixed and then incubated for 15 min at 37°C. Finally, the substrate was added and absorbance was measured with a Safire spectrophotometer (Tecan, Mannedorf, Switzerland) at 405 nm every 30 s for a total duration of 20 min. A mixture without enzyme (blank sample) and a mixture without the fungal fraction (solvent control) were introduced by analogy. The thrombin inhibitor argatroban (Daiichi, Tokyo, Japan) at the concentration 0.1 μg/mL replaced the fungal fraction and served as a positive control. Change in absorbance directly reflects cleavage of the chromogenic substrate with release of *p*-nitroaniline. Absorbances of samples were corrected by subtracting the absorbance of the blank samples. The increase of absorbance per minute in the linear part of the graph was defined as enzyme activity. For each tested inhibitor, the enzyme activity was subtracted from the enzyme activity of the positive control. The difference was then expressed as a percentage of the enzyme activity in the positive control and defined as the inhibitory activity. Inhibitory activity was tested in triplicate and the results averaged.

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