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Glycerolipids as Selective Thrombin Inhibitors from the Fungus *Stereum Hirsutum*

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ABSTRACT A dry extract of *Stereum hirsutum* exhibited a significant inhibitory activity on thrombin (34%). A bioassay oriented fractionation of the extract of *Stereum hirsutum* has led to the isolation of two active fractions. On the basis of spectroscopic data, chemical reactions, and GC-MS analysis, complex mixtures of diacylglycerophospholipids (DAGPs) and diacylglycerols (DGs) have been isolated and identified. Docking studies suggest that some isolated compounds could bind to the thrombin active site in a similar manner as previously reported phosphonate thrombin inhibitors.

KEYWORDS Thrombin, Inhibition, Mushrooms, Extract, Screening, Docking

INTRODUCTION

Stereum hirsutum belongs to the phylum of Basidiomycetes (Basidiomycota) and is one of several fungi involved in a grapevine disease called esca (Larignon & Dubos, 1997).

Greater scientific interest in Basidiomycetes has been elicited as a result of the isolation of substances with antibiotic, cytostatic, immunostimulatory, antiviral, anti-allergic, hypoglycaemic, antilipemic, hypotensive, and central nervous system effects (Lindequist et al., 1990). Basidiomycetes as a source of thrombin inhibitors have been scarcely investigated until now, although their inhibitory activity against serine proteinases including trypsin has been reported (Pilgrim & Darwaza, 1985; Pilgrim et al., 1992; Odani et al., 1999). Thrombin is a multifunctional serine protease that plays a primary role in the pathogenic pathway of thrombosis as a consequence of its actions on the two principal components of blood clots, fibrin, and activated platelets and, therefore, a suitable target for inhibition of blood coagulation (Ries & Wienen, 2003).

In a preliminary study, 95 selected mushroom species have been screened in order to find novel specific non-peptidic thrombin inhibitors (Doljak et al., 2001). The extract of *Stereum hirsutum* considerably inhibited thrombin (34%) but it showed no inhibitory activity on trypsin.

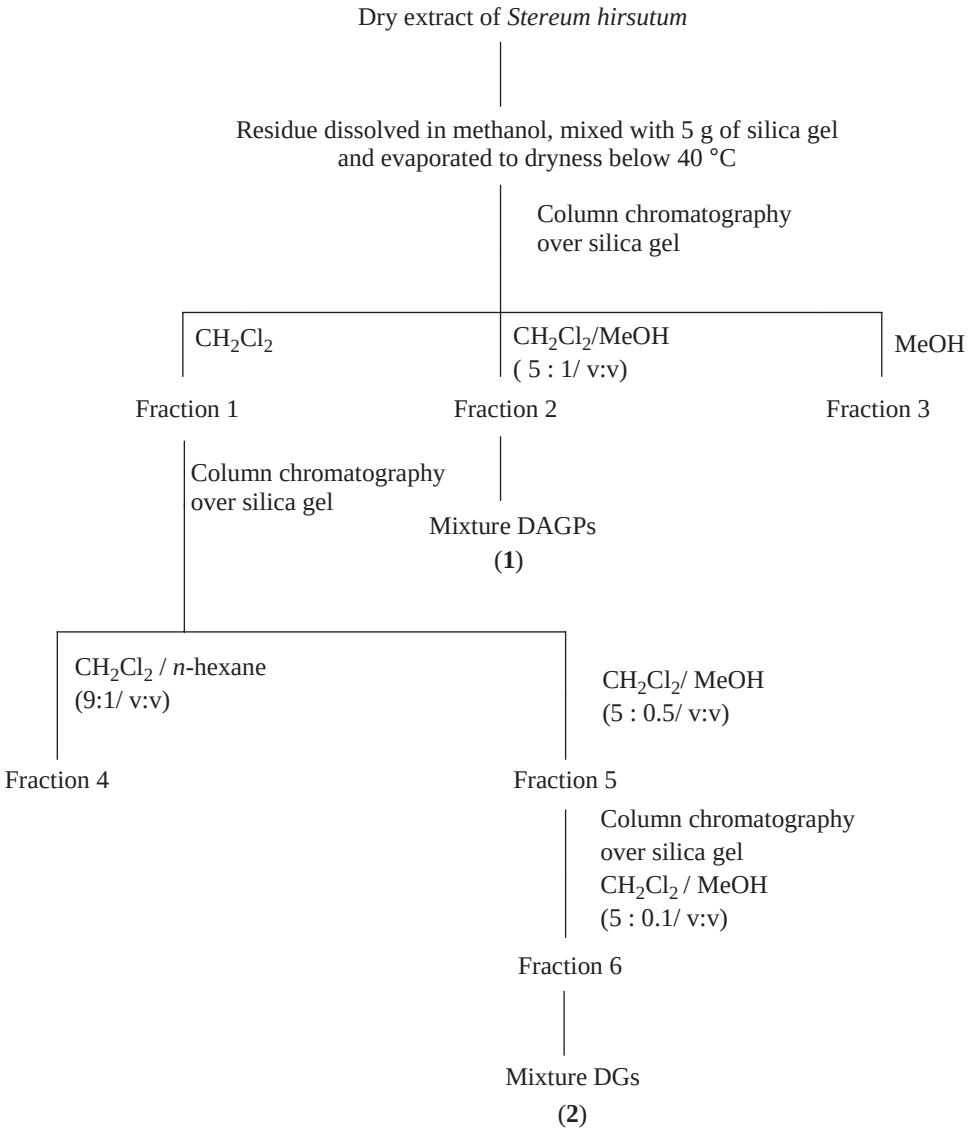
The genus Stereum is known to produce various sesquiterpenes, benzaldehydes, and benzofurans (Nair & Anchel, 1977; Xie et al., 1992; Bu' Lock et al., 1971).

Among Stereum genus, *Stereum hirsutum* has recently been under phytochemical investigation. The discovery of new biologically active compounds from Basidiomycetes has centred upon the isolation and structure elucidation of new tricyclic sesquiterpenes (Yun et al., 2002), acetylenic aromatic compounds (Dubin et al., 2000), chromene, and aromatic aldehydes derivatives (Yun et al., 2002) from the fermentation broth of *Stereum hirsutum*.

In this paper, we report the isolation and biological investigation of active principles from *Stereum hirsutum* on the basis of inhibitory potency on thrombin. A bioassay oriented fractionation of the extract of *Stereum hirsutum* has led to the isolation of two active fractions (Scheme 1). On the basis of spectroscopic data, chemical reactions and GC-MS analysis, complex mixtures of diacylglycerophospholipids (DAGPs) (**1**) and diacylglycerols (DGs) (**2**) have been isolated and identified. Moreover, docking studies on thrombin were performed in order to clarify the binding mode of some isolated compounds.

RESULTS AND DISCUSSION

In a preceding paper (Doljak et al., 2001), we have reported a preliminary screening on 95 selected mushroom species, and since the extract of *Stereum hirsutum* considerably inhibited thrombin (34%), we wish to describe herein the isolation and structure elucidations from this fungus of DAGPs (**1**) and DGs (**2**) which are responsible for the activity of the extract.



SCHEME 1 **General Isolation Procedure for DAGPs (1) and DGs (2) from Dry Extract of *Stereum Hirsutum*.**

The dry extract of *Stereum hirsutum* collected in September 2002 in northeastern Slovenia was treated as shown in Scheme 1. Mixtures **1** and **2** were obtained by fractionation and silica gel column chromatography. Bioassay oriented fractionation of the extract of *Stereum hirsutum* afforded three fractions 1, 2, and 3, respectively, which were tested for inhibitory activity on thrombin (Table 1). Thrombin was potently inhibited by fraction 2 (52%) and fraction 1 (44%) while fraction 3 showed considerably lower inhibition (12%).

The fraction 2 was eluted with CH_2Cl_2/MeOH (5:1/v:v) to afford the complex mixture of **1**.

Diacylglycerophospholipids (**1**) show strong ammonium and ester absorptions at 3100 cm^{-1} and 1742 cm^{-1} in its FT-IR (fourier transform infrared) spectrum. The ^{1}H- and ^{13}C-NMR (^{13}C-Nuclear Magnetic Resonance) data of **1** are reported in Table 2. The ^{1}H-NMR spectrum of **1** reveals characteristic signals due to lipids, namely, a triplet at $\delta = 0.89$ ppm (terminal methyl group), and a strong broad signal at $\delta = 1.27$ ppm (methylene chain in fatty acid moiety). It also shows a signal due to a methine group bearing ester oxygens at $\delta = 5.21$ ppm, while the magnetically inequivalent glycerol Sn1 methylene protons resonated at $\delta = 4.41$ ppm (dd; 1H, J = 11.8, 6.5 Hz) and 4.15 ppm (dd; 1H, J = 11.0, 5.3 Hz). Both glycerol Sn3 methylene protons resonances were observed overlapped at $\delta =$ 3.98 ppm. Coupling between these glycerol backbone protons was confirmed by cross peaks in the 2D COSY spectrum that unequivocally confirmed their assignments. The ethanolamine group was identified by its characteristic head group $-C\underline{H}_2NH_3^{(+)}$ methylene protons resonance at about $\delta = 3.14$ ppm. The 2D COSY spectrum showed the cross peak ($\delta = 3.14$, $\delta = 4.02$) which identified the $-OCH_2$ head group methylene protons at $\delta = 4.02$ ppm. Furthermore, the signals due to methylene protons attached to the ester carbonyl of **1** are observed at $\delta = 2.32$ ppm (triplet-like m, 2H).

TABLE 1 **Inhibitory Activities of Fractions Isolated from *Stereum Hirsutum* on Thrombin**

Fraction	Mean inhibitory activity on thrombin (%)*
1	44
2	52
3	12
4	14
5	34
6	41

*Each dry fungal fraction 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 fraction, the thrombin activity in the reaction mixture with fungal fraction was subtracted from the thrombin activity in the reaction mixture without fungal fraction (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%.

TABLE 2 **^{1}H- and ^{13}C-NMR Chemical Shifts (δ Values) of 1 (in $CDCl_3$)**

C, H	^{1}H-NMR δ (m, J in Hz)	^{13}C-NMR δ
Sn 1a	4.41 (dd; 1H, J = 11.8, 6.5)	63.4
Sn 1b	4.15 (dd; 1H, J = 11.0, 5.3)	—
Sn 2	5.21 (m; 1H)	71.3
Sn 3	3.98 (m; 2H)	64.4
1″	4.02 (m)	66.0
2″	3.14 (m)	68.3
$\underline{C}$=O	—	174.1, 173.1
$\underline{CH}$=$\underline{CH}$	5.14–5.40 (m)	127.9–132.5
=–$\underline{CH_2}$–=	2.77 (t)	25.8
OCO$\underline{CH_2}$	2.30 (m)	34.1, 34.2
COCH$_2\underline{CH_2}$	1.61 (m)	25.0
=–$\underline{CH_2}CH_2$	2.05 (m)	27.3
$\underline{CH_2}$	1.20–1.32 (br s)	29.0–32.0
$\underline{CH_3}$	0.89–0.96 (t)	14.3

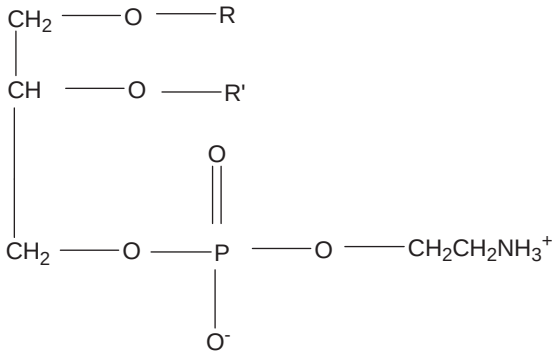
The ^{13}C-NMR spectrum of **1** exhibits the signals of two carbonyl carbons at $\delta = 174.1$, 173.1 ppm, of olefinic carbons at $\delta = 127.9$–132.5 ppm and of the methylene groups at $\delta = 25.0$–32.0 ppm, together with the signals due to the glycerol moiety and to the ethanolamine group (Table 2). These data strongly suggest that **1** is a di-fatty acid ester of glycerophosphoethanolamine.

To confirm this observation, **1** was treated with 0.5 N NaOH in MeOH and **3** was obtained (Fig. 1). Afterwards, **1** was heated with BF_3 in MeOH to give a mixture of fatty acid methyl esters.

The GC-MS analysis of the methyl esters indicated the presence of six kinds of esters as reported in Table 4. Consequently, **1** is thought to be the mixture of the di-fatty acid esters of glycerophosphoethanolamines. Six fatty acids were identified among which linoleic acid predominated. Fatty acids with 16 and 18 carbons are most abundant with linoleic and oleic acid as the principal unsaturated fatty acids, and palmitic acid as the major saturated one.

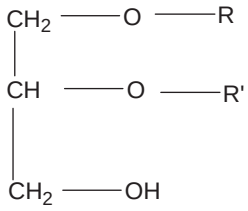
The fraction **1**, which showed a significant inhibitory activity on thrombin (41%) (Table 1), was eluted with CH_2Cl_2/MeOH (5:0.5 and 5:0.1/v:v) to afford the complex mixture of **2** (Scheme 1).

Diacylglycerols (**2**) exhibited strong ester absorptions at 1745 cm^{-1} and a hydroxyl (3360 cm^{-1}) in its FT-IR spectrum. The ^{1}H- and ^{13}C-NMR data of **2** are



1

	R, R'
a	-CO- $(CH_2)_{14}\,CH_3$
b	-CO-$(CH_2)_7CH{=}CH(CH_2)_5CH_3$
c	-CO-$(CH_2)_{16}CH_3$
d	-CO-$(CH_2)_7CH{=}CH(CH_2)_7CH_3$
e	-CO-$(CH_2)_7(CH{=}CHCH_2)_2(CH_2)_3CH_3$
f	-CO-$(CH_2)_7(CH{=}CHCH_2)_3CH_3$



2

3: R = R' = H

	R, R'
a	-CO- $(CH_2)_{10}\,CH_3$
b	-CO-$(CH_2)_{12}CH_3$
c	-CO-$(CH_2)_{13}CH_3$
d	-CO-$(CH_2)_{14}CH_3$
e	-CO-$(CH_2)_7CH{=}CH(CH_2)_5CH_3$
f	-CO-$(CH_2)_7CH{=}CH(CH_2)_7CH_3$
g	-CO-$(CH_2)_{15}CH_3$
h	-CO-$(CH_2)_{16}CH_3$
i	-CO-$(CH_2)_7CH{=}CH(CH_2)_7CH_3$
l	-CO-$(CH_2)_7(CH{=}CHCH_2)_2(CH_2)_3CH_3$
m	-CO-$(CH_2)_{18}CH_3$
n	-CO-$(CH_2)_7(CH{=}CHCH_2)_3CH_3$
o	-CO-$(CH_2)_9CH{=}CH(CH_2)_7CH_3$
p	-CO-$(CH_2)_{20}CH_3$

FIGURE 1 **Chemical Structures of 1, 2–O-diacyl–*sn*–glycerol–3–phosphoethanol-amines (1) and DGs (2).**

reported in Table 3. The ^{1}H-NMR spectrum of **2** reveals characteristic signals due to lipids, namely, a triplet at $\delta = 0.89$ ppm (terminal methyl group), and a strong broad signal at $\delta = 1.27$ ppm (methylene chain in fatty acid moiety). It also shows a signal due to a methine group bearing ester oxygens at $\delta = 5.15$ ppm, while the glycerol Sn1 methylene protons, resonated at $\delta = 4.35$ ppm (dd; 1H, J = 11.9, 5.2 Hz) and 4.15 (dd; 1H, J = 11.0, 5.3 Hz), respectively. The multiplet at $\delta = 4.10$ ppm is due to the Sn3 methylene protons. The absence of the two double doublets at $\delta = 4.46$ and 4.58 ppm in the ^{1}H-NMR spectrum of 2 confirm that the position Sn3 in the glycerol moiety is free. Coupling between these glycerol backbone protons was confirmed by cross peaks in the 2D COSY spectrum that unequivocally gave their assignments.

TABLE 3 ^{1}H- and ^{13}C-NMR Chemical Shifts (δ Values) of 2 (in $CDCl_3$)

C, H	^{1}H-NMR δ (m, J in Hz)	^{13}C-NMR δ
Sn 1a	4.35 (dd; 1H, J = 11.9, 5.2)	60.8
Sn 1b	4.15 (dd; 1H, J = 11.7, 3.1)	—
Sn 2	5.15 (m; 1H)	69.5
Sn 3	4.10 (m)	57.5
C=O	—	178.1, 178.1
CH=CH	5.39–5.57 (m)	127.6–132.9
=–CH_2–=	2.80 (t)	25.4
OCOCH_2	2.30 (t)	33.6, 33.9
COCH_2CH_2	1.65 (m)	24.9
=–CH_2CH_2	2.05 (m)	27.2
CH_2	1.20–1.32 (br s)	28.7–31.3
CH_3	0.89–0.96 (t)	13.9

Furthermore, the signals due to methylene protons attached to the ester carbonyl of 2 are observed at δ = 2.30 ppm (triplet-like m, 2H).

The ^{13}C-NMR spectrum of 2 exhibits the signals of two carbonyl carbons at δ = 178.1, 178.4 ppm, of olefinic carbons at δ = 127.6–132.9 ppm, and of the methylene groups at δ = 24.9–31.3 ppm, together with the signals due to the glycerol moiety (Table 3). These data suggest that 2 is a di-fatty acid ester of glycerol.

To confirm this observation, 2 was treated with 0.5 N NaOH in MeOH and 3 was obtained. Additionally, 2 was heated with BF_3 in MeOH to give a mixture of fatty acid methyl esters.

The GC-MS analysis of the methyl esters indicated the presence of fourteen kinds of esters as reported in Table 4. Consequently, **2** was confirmed to be a mixture of the di-fatty acid esters of **3** (Fig. 1). Fourteen fatty acids were identified among which palmitic acid predominated. Fatty acids with 16 and 18 carbons are most abundant, with linoleic and oleic acid as the principal unsaturated fatty acids.

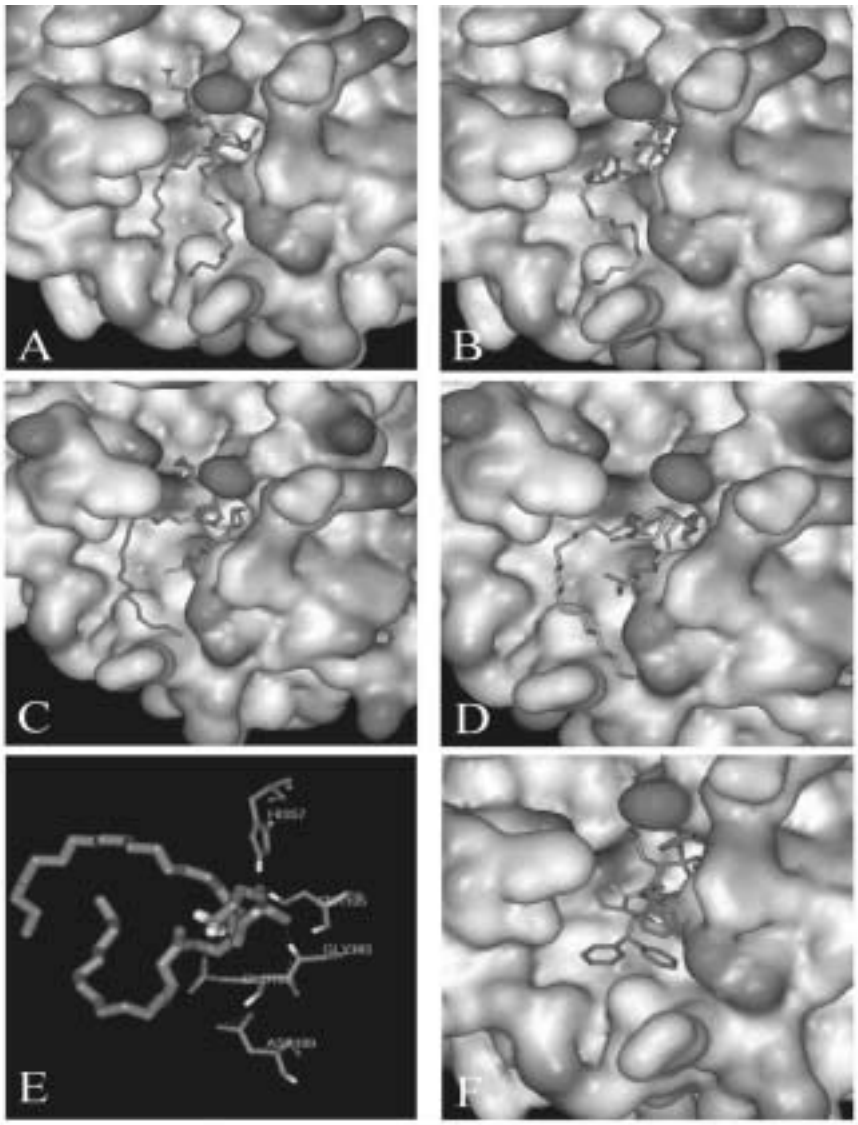
The boronate inhibitors of thrombin were reported to bound potently to thrombin active site as "transition state analogues" and the same is true for phosphonate inhibitors (Coburn, 2001; Skordalakes et al., 2001). The main structural feature of phosphonates responsible for high binding affinity to thrombin is the phosphorus moiety which is tetracoordinated (or pentacoordinated-metastable state) in the thrombin active site (Skordalakes et al., 2001). Since DAGPs share almost the same structural feature with the reported inhibitor (phosphate/phosphonate moiety), we have presumed that the isolated DAGPs bind to the thrombin active site in a similar manner.

TABLE 4 Fatty Acid Composition of DAGPs (1) and DGs (2) Isolated from *Stereum Hirsutum*

			1	**2**
Rt	Fatty acid	m/z $[M]^+$	% of total fatty acids	
3.66	C_{12}	214	—	0.77 ± 0.20
5.01	C_{14}	242	—	1.12 ± 0.16
5.71	C_{15}	256	—	2.92 ± 0.03
6.46	C_{16}	270	19.77 ± 0.54	33.60 ± 0.74
6.66	$C_{16:1\omega9}$	268	—	2.23 ± 0.18
6.82	$C_{16:1\omega7}$	268	1.03 ± 0.05	—
7.15	C_{17}	284	—	0.67 ± 0.10
7.84	C_{18}	298	3.20 ± 0.08	7.33 ± 0.16
8.16	$C_{18:1\omega9}$	296	13.29 ± 0.95	11.65 ± 0.45
8.71	$C_{18:2\omega6}$	294	58.00 ± 1.98	32.55 ± 1.45
9.17	C_{20}	326	—	0.56 ± 0.05
9.32	$C_{18:3\omega3}$	292	4.70 ± 0.08	0.57 ± 0.02
9.55	$C_{20:1\omega9}$	324	—	0.88 ± 0.09
10.41	C_{22}	354	—	3.59 ± 0.05

Data are expressed as weight percentage of total fatty acids. Measurements were carried out in triplicates and expressed as the mean value ± SD.

Starting from the crystal structure of human α-thrombin complex with a tripeptide phosphonate inhibitor (Ki = 20 nM) (Skordalakes et al., 2001; www.rcsb.org), four DAGPs with randomly chosen fatty acids were docked to the thrombin active site in order to clarify the binding mechanism of isolated substances. Docking results reveal two binding modes of model compounds: the one in which the amino group of DAGPs is pointed towards Asp 189 (at the bottom of S1 specificity pocket) and the phosphate moiety is near Ser 195 (Fig. 2B and D) and the second, in which the amine is in the vicinity of Glu 192 (at the entrance of S1 pocket) and the phosphate is between His 57 and Ser 195 (Fig. 2A and C). As expected in both modes, lipid alkyl chains are embedded through S1, S2, and S3 pocket area in order to achieve optimum hydrophobic interactions. The most favorable free binding energy was predicted for model compound 3 (Fig. 2E, ΔG_{bind} = −17,37 kcal/mol) which corresponds to the second binding mode: phosphate moiety forms ionic bond with His 57 (proton from phosphate has migrated to imidazole nitrogen of His 57) and is additionally coordinated with –OH (Ser 195) and –NH– group (Gly 193), while amino group forms ionic bond with –COOH group (Glu 192). One of the alkyl chains is embedded in S2 and S3 pocket area while the second is set in the beginning of the



COMPOUND	R (Figure 1)	R'(Figure 1)	ΔG_{bind} [kcal/mol]
Model compound 1 (**A**)	a	a	- 17,31
Model compound 2 (**B**)	a	b	-13,47
Model compound 3 (**C**, **E**)	b	c	-17,37
Model compound 4 (**D**)	c	d	-16,08
Tripeptide phosphonate inhibitor (**F**)	/	/	-18,79

FIGURE 2 **Docked Conformations in Thrombin Active Site with the Most Favorable Free Binding Energy as a Result of Docking Studies. Protein is Presented as Surface While Model Compounds are Presented in "Stick" Rendering (A, B, C, D, and F). E: Model Compound 3 ("Ball and Stick" Rendering) with Amino Acids Involved in Crucial Interactions ("Stick" Rendering), as Calculated with AutoDock. For the Sake of Clarity, Only "Polar" Hydrogens Are Shown.**

S1 pocket. The lack of phosphate tetracoordinated (or pentacoordinated) state could be due to the lack of solvent (water molecules) in the thrombin active site as docking studies were performed with non-solvated protein. The calculated free binding energy of this DAGP is comparable with the free binding energy calculated with AutoDock for reported tripeptide phosphonate inhibitor (Fig. 2F). Additionally, the reported x-ray crystallographic studies of the later in complex with thrombin [data not shown (Skordalakes et al., 2001; www.rcsb.org)] reveal that the same amino acids in thrombin active site complex phosphonate moiety as docking studies predicted for phosphate moiety in DAGPs. This leads us to presume that the isolated DAGPs share the thrombin-binding mode with phosphonate thrombin inhibitors.

MATERIALS AND METHODS

NMR-spectroscopy: nuclear magnetic resonance spectra were recorded with a Varian Unity 400 spectrometer and a Varian Gemini 200 MHz spectrometer (Trieste, Italy). ^{13}C-NMR: 100.4 MHz, Unity 400 spectrometer. NMR spectra were obtained by using $CDCl_3$ as solvent; chemical shifts are expressed as δ units (ppm) relative to tetramethylsilane (TMS) as internal standard. The abbreviations s, d, dd, t, q, m, and br s refer to singlet, doublet, doublet of doublet, triplet, quartet, multiplet, and broad singlet, respectively. Electrospray analysis: API Perkin Elmer (voltage + 5600 with orifice 90 and/or 120). Silica gel column chromatography: Kieselgel 60 (230–400 Mesh, 60Å Merck). FT-IR spectra: Jasco IR-700 infrared spectrophotometer. All solvents were distilled before use. TLC: Kieselgel 60 F_{254} (20 × 20 cm; 0.2 mm, Merck).

The fatty acid composition of **1**, **2** was released as methyl esters by the Association of Official Analytical Chemists (AOAC) methylation procedure, and analyzed by GC-MS (Helrich, 1990). A Carlo Erba GC 8000 instrument is coupled directly to a MD 800 mass spectrometer (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. The chromatographic conditions were the following: 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. A mass spectrometer scanned from *m/z* 100 to *m/z* 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.

Reagents and Standards

Sodium hydroxide was obtained from BDH (Poole, UK). Methanol, *n*-hexane, *n*-heptane, methylene chloride, chloroform, anhydrous sodium sulphate, and sodium chloride were purchased from Merck (Darmstadt, Germany).

Lauric acid methyl ester (C12-ME, ~99% purity), myristic acid methyl ester (C14-ME, ~99% purity), pentadecanoic acid methyl ester (C15-ME, ~99% purity), palmitic acid methyl ester (C16-ME, >99% purity), palmitoleic acid methyl ester (C16:1ω9-ME, >99% purity), heptadecanoic acid methyl ester (C17-ME, ~99% purity), stearic acid methyl ester (C18-ME, ~99% purity), oleic acid methyl ester (C18:1ω9-ME, ~99% purity), linoleic acid methyl ester (C18:2ω6-ME, ~99% purity), linolenic acid methyl ester (C18:3ω3-ME, ~99% purity), arachidic acid methyl ester (C20-ME, ~99% purity), *cis*-11-eicosenoic acid methyl ester (C20:1ω9-ME, ~99% purity), and behenic acid methyl ester (C22-ME, ~99% purity) were purchased from Sigma-Aldrich (Milano, Italy).

Preparation of Stereum Hirsutum Extract

Stereum hirsutum was collected in September 2002 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. The resulting homogenate was then centrifuged at 1100 g for 10 min.

Subfractionation of Extract from Stereum Hirsutum

Dry extract (20 g) was redissolved in methanol, mixed with 5 g of silica gel, and evaporated in vacuo. The dry extract was chromatographed on silica gel using CH_2Cl_2, CH_2Cl_2/MeOH (5:1/v:v), and MeOH to give three fractions, denominated 1, 2, and 3, respectively. 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/v:v) and CH_2Cl_2: MeOH (5:0.5/v:v) as eluents to afford fractions 4 and 5, respectively. Fraction 5 was concentrated in vacuo and submitted to column chromatography over silica gel using CH_2Cl_2: MeOH (5:0.1/v:v) as eluent to afford fraction 6 (Scheme 1). The fractions obtained by subfractionation of extract from *Stereum hirsutum* were tested for inhibitory activity.

Determination of Inhibitory Activity

Inhibitory activity of fungal fractions on human thrombin (Sigma, St. Louis, Missouri, USA) was

determined using 96-well microtitre plates and the chromogenic substrate H-D-phenylalanine-L-pipecolyl-L-arginine-paranitroaniline dihydrochloride (S-2238; Chromogenix, Orangeburg, New York, USA). To determine thrombin inhibition, 40 µL HBSA buffer (pH 7.5) containing 10 mmol/L Hepes (Sigma), 150 mmol/L NaCl, and 0.1% bovine serum albumin (Sigma) was mixed with 50 µL thrombin solution (0.5 NIH units/mL) (all concentrations in parentheses are final concentrations in the mixture). Ten microliters of fungal fraction dissolved in dimethyl sulfoxide (DMSO) (120 µg/mL) was added to bring the total volume of the mixture to 100 µL. After incubation at room temperature for 15 min, 50 µL substrate (0.5 mmol/L) was added. A mixture without enzyme (blank sample) and a mixture without the fungal fraction (positive control) were introduced by analogy for every fungal fraction. Thrombin inhibitor argatroban (Daiichi, Tokyo, Japan) at the concentration 0.1 µg/mL substituted the fungal fraction (negative control). After addition of chromogenic substrate, absorbance was measured with a Multiscan MCC/340 spectrophotometer (Titertek, Huntsville, Alabama, USA) at 405 nm every 30 s for a total duration of 20 min. Change in absorbance directly reflected 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 fraction, the thrombin activity in the reaction mixture with fungal fraction was subtracted from the thrombin activity of the positive. The difference was then expressed as a percentage of the thrombin activity of the positive control and defined as the inhibitory activity. Inhibitory activity was tested in duplicate for each fungal fraction and the results averaged. It was observed that DMSO at the concentrations employed did not interfere with the reaction conditions.

Isolation of 1 and 2

In continuation of our previous study (Doljak et al., 2001), dry extract of *Stereum hirsutum* was treated as shown in Scheme 1. The R_f values (TLC) of each mixture were as follows: **1**, $R_f = 0.48$ [solvent: CH_2Cl_2/MeOH (5:1)] – **2**, $R_f = 0.77$ [solvent: CH_2Cl_2/MeOH (5: 0.1)].

1: FT-IR (film): 3300 cm^{-1} (N-H), 1742 (C=O), 1415 (C-O). ^{1}H-NMR and ^{13}C-NMR data are reported in Table 2. ESI-MS: m/z = 803 (M, 100%)$^+$, 691 (M, 48%)$^+$, 630 (691-C_2H_7NO, 45%)$^+$, 282 ($C_{18}H_{35}O_2$, 15%)$^+$.

2: FT-IR (film): 3400 cm^{-1} (O-H), 1740 (C=O). ^{1}H-NMR and ^{13}C-NMR data are reported in Table 3. ESI-MS: m/z = 760 (M + Na, 6%)$^+$, 725 (760 – H_2O-15, 24%)$^+$, 612, 279 ($C_{18}H_{31}O_2$, 10%)$^+$.

Methanolysis of 1, 2

Fifty milligram of **1** and **2** were heated with 4 mL of a 0.5 N solution of NaOH in MeOH reflux for 20 min. The reaction mixture was employed in the next step without further purification. A solution of **1** and **2**, after alkaline treatment, was heated with 5 mL of a solution of BF_3/MeOH reflux for 2 min. The reaction mixture was extracted with *n*-heptane (2 mL) reflux for 1 min and quenched with a saturated NaCl solution; the *n*-heptane phase, dried over Na_2SO_4, was filtered and evaporated under reduced pressure to give a mixture of fatty acid methyl esters analyzed directly by GC-MS.

The fatty acid methyl esters were identified by comparison with standards and the ratios were calculated from the total peak areas (Table 4).

Docking Studies

Thrombin Setup

Skordalakes et al. have recently reported the crystal structure of human α-thrombin in complex with the phosphonate tripeptide inhibitor (Skordalakes et al., 2001). This crystal structure was downloaded from PDB (PDB ID: 1H8D) (www.rcsb.org) and set up using AutoDock Tools. All water molecules and the ligand were removed from the protein and polar hydrogens were added. The grid maps were calculated with AutoGrid and the grids were placed around thrombin active site (near catalytic triade) since docking to the active site was presumed to occurr. The grids were defined as follows: the grid center with coordinates 15,735; –13,485; 22,988, grid dimensions of 61 × 61 × 61 points (22,5 × 22,5 × 22,5 Å, grid-point spacing of 0,369 Å).

Ligand Setup

Four model DAGPs (**1**, R, R′ = a,a; a,b; b,c; c,d) and the phosphonate tripeptide thrombin inhibitor (Skordalakes et al., 2001) were used as ligands for

docking to thrombin. The starting conformations were generated by energy minimization with HyperChem 7.5 software package (Hypercube, Inc.) using Molecular mechanics method (MM^+, Steepest descent algorithm, max. 1000 steps) followed by semi-empirical MNDO method [Polak-Ribiere algorithm (Conjugate Gradient), RMS gradient of 0,01 kcal/(Åmol), max. 100 cycles]. The same tool was used to assign partial atomic charges. Since only 32 rotatable bonds were allowed for docking calculations, the geometries of some alkyl C–C bonds in fatty acid alkyl chains were fixed until the criterion of maximum 32 rotatable bonds was achieved. As for the protein, non-polar hydrogens were not included.

Docking

Docking calculations were carried out with AutoDock, version 3.05 (The Scripps Research Institute) using Lamarckian Genetic Algorithm method (LGA) as a search method. Fifty independent docking runs were performed for each ligand with 200 individuals, $2{,}5 \times 10^6$ energy evaluations, a mutation rate of 0, 20, a crossover rate of 0, 80, and an elitism value of 1. Results with less than 1.5 Å difference in positional root-mean-square deviation (RMSD) of the heavy atom coordinates between the docked ligands were clustered together. The described protocol was performed in triplicate for each model compound and the selected inhibitor. The cluster/docked conformation with the most favourable free binding energy is presented as the result.

CONCLUSIONS

On the basis of inhibitory potency on thrombin (inhibitory activity over 30%), we have shown that *Stereum hirsutum* constitutes a good starting material for isolation and structure elucidation of further compounds that are active against thrombin. A bioassay oriented fractionation of the extract of *Stereum hirsutum* has led to the isolation of lipid mixtures. Our findings suggest that DAGPs (**1**) together with DGs (**2**) are responsible for the inhibitory activity of the extract of *Stereum hirsutum* on thrombin. The results of the docking studies suggest that DAGPs (**1**) could bind potently to the thrombin active site which is consistent with previously reported binding mode of thrombin inhibitors based on phosphonate moiety. Proposed binding mode thus offers a possible explanation for molecular mechanism of inhibitory activity of isolated compounds on thrombin.

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