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Structural Characterisation of Metabolites from *Pholiota spumosa* (Basidiomycetes)*

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Three compounds of different biosynthetic origin were isolated from the fruiting bodies of the gilled mushroom *Pholiota spumosa* (Basidiomycetes, Strophariaceae). Fasciculol E, a lanostane triterpenoid conjugated to a depsipeptide unit, was isolated for the first time from genus *Pholiota*. In addition, the first isolation of putrescine-1,4-dicinnamamide as a natural compound is reported: its structure was established by single crystal X-ray diffraction, and by spectroscopic methods. Crystallographic analysis indicated the presence of a co-crystallised related dicinnamamide, namely the new compound (*E*)-2,3-dehydroputrescine-1,4-dicinnamamide, whose occurrence was confirmed by LC-MS analysis. An interesting evolutionary issue arises, following the observation that the cinnamamides produced by *Pholiota spumosa* bear an unsubstituted benzene ring, contrarily to those found in plants, which have always phenolic functionalities, and as such perform a variety of biological roles.

Key words
Pholiota spumosa
Strophariaceae
fungi
terpenes
cinnamic acid amides
X-ray crystal structure

INTRODUCTION

Pholiota Kummer, a cosmopolitan genus of macrofungi, often endowed with attractive colours, belongs to the family Strophariaceae, order Agaricales, class Basidiomycetes. More than 30 species are known, distributed world wide. These mushrooms are frequently active parasites and wood destroyers, attacking forest trees as well as park trees, and their mycelium continues the destruction after the tree is cut. A few *Pholiotas* are edible and no species has been reported as toxic so far.[1]

Phytochemical studies of the genus are limited to the isolation of some stiryl-pyrone pigments, of the hispidine group, from *Ph. flammans*.[2,3]

In Chile, the genus is represented by 15 species, geographically distributed from Valparaiso (V Region) to Puerto Montt (X Region).[4,5] Continuing our studies on natural occurring compounds[6] (in particular on Chilean fungi[7]), and in search for compounds with biological activity, in this paper we report some secondary metabolites of *Ph. spumosa* (Fr.) Sing., whose carpophores grow

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abundantly in Central and Southern Chile. The mushroom is found on wood residues in *Pinus radiata* forests, where it has possibly been introduced from Northern America together with the tree.

EXPERIMENTAL

General Experimental Procedures

The melting point was determined on a Stuart Scientific SMP3 apparatus; optical rotation was recorded in CH_3OH solution with a Schmidt-Haensch polarimeter; IR spectra were recorded on a FT-IR Nicolet Impact 420. ESI and APCI mass spectra were recorded on a LCQ DECA Thermo-Finnigan ion trap mass spectrometer, controlled by Xcalibur software (version 1.1); EI mass spectra were recorded on a TSQ-70 Finnigan-Mat triple quadrupole spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 Digital Apparatus, operating at 400 and 100 MHz, respectively. TLC analysis was carried out on silica gel plates GF_{254} and RP-18 F_{254S} (Merck). Liquid chromatography was performed on silica gel 60 GF_{254}, or Lichroprep RP-18, or lipophilic Sephadex LH-20.

Fungal Material, Extraction and Isolation

The fruiting bodies of *Ph. spumosa* (800 g) were collected in the Reserva Nacional Forestal Lago Peñuelas (Valparaíso, Chile, V Region) in June 2001. A voucher specimen has been deposited in the Herbarium of Santa Maria University, Valparaiso, Chile. Immediately after collection, the mushrooms were minced at r.t. and extracted with acetone or, alternatively, with a mixture of ethyl acetate : methanol (3:1), yielding in both cases the same set of metabolites. The extracts were concentrated under reduced pressure below 35 °C, and the residue (2 g) was separated by column chromatography on silica gel ($CHCl_3$–MeOH from 9.5:0.5 to 0:1), RP-18 reversed phase (H_2O–MeOH from 3:1 to 0:1), and Sephadex LH-20 gel-permeation (CH_2Cl_2–MeOH from 1:1 to 0:1). By these procedures, compounds **1** (18 mg), and **2** (11 mg) could be isolated.

X-ray Analysis

Single crystal diffraction data were collected at r.t. on a Bruker SMART CCD area detector diffractometer, using graphite monochromatised Mo-Kα (λ = 0.71073Å) radiation. Absorption correction was performed using SADABS.[8] The structure was solved by direct methods[9] and refined by full-matrix least-squares.[10] All the hydrogen atoms were located in the electron density map, and then refined with geometric constraints.

Computational Details and Graphical Manipulations

Ab initio molecular orbital calculations were performed employing the DFT method based on Becke's[11] three parameters hybrid functional and Lee-Yang-Parr's[12] gradient-corrected correlation functional (B3LYP), using the 6-31G(d,p) basis sets, [13] as implemented in the Jaguar software.[14] Calculations of magnetic shielding σ were performed at the B3LYP/6-311+G(2d,2p) level of theory, employing the GIAO method, as implemented in Gaussian 98.[15] Graphical manipulations and conformational searches were performed employing the XP,[16] MOLDRAW[17] and MAESTRO[18] software.

Fasciculol E (***1***): 15 mg, white oil, $[\alpha]_D^{22}$ = +29.5 (MeOH; *c* = 0.45); lit.:[19] +30.6 (MeOH); ^{13}C NMR (CD_3OD): δ/ppm 174.1 (s, C-8'), 173.2 (s, C-1'), 171.9 (s, C-6'), 136.6 (s, C-9), 134.1 (s, C-8), 85.9 (d, C-3), 79.5 (d, C-24), 74.4 (d, C-12), 73.9 (s, C-25), 71.6 (s, C-3'), 68.0 (d, C-2), 62.0 (t, C-21), 52.6 (q, C-9'), 51.9 (d, C-5), 51.3 (s, C-14), 51.1 (s, C-13), 47.3 (t, C-5'), 46.9 (t, C-2'), 44.8 (t, C-1), 44.3 (d, C-17), 41.8 (t, C-7'), 40.0 (s, C-4), 39.3 (d, C-20), 39.2 (s, C-10), 33.2 (t, C-15), 32.8 (t, C-23), 29.4 (t, C-16), 29.0 (q, C-4'), 28.9 (t, C-22), 28.1 (t, C-7), 28.0 (q, C-29), 27.5 (t, C-11), 25.7 (q, C-26 or C-27), 24.9 (q, C-27 or C-26), 24.2 (q, C-28), 20.3 (q, C-19), 19.2 (t, C-6), 18.0 (q, C-30), 17.4 (q, C-18); MS ESI$^+$ *m/z*: 746 (M+Na)$^+$.

Putrescin-1,4-dicinnamamide (*dicinnamide*) (**2**): 12 mg, colorless crystals (EtOAc-MeOH); m.p. 247–248 °C, lit.:[21] 249–253 °C; IR (KBr) ν_{max}/cm^{-1}: 3296, 3084, 2960, 2945, 2922, 1655, 1618, 1578, 1556, 1342, 1227. ^{1}H NMR (DMSO-d_6) as by Niwa *et al.*[22]. ^{13}C NMR (DMSO-d_6, neglecting the symmetry related atoms): δ/ppm 164.8 (s, C=O), 138.3 (d, C-2'), 134.9 (s, Ar), 129.2 (d, Ar), 128.8 (2C, d, Ar), 127.3 (2C, d, Ar), 122.4 (d, C-1'), 38.3 (t, C-1), 26.6 (t, C-2); MS ESI$^+$, *m/z*: 371 (M+Na$^+$, 100), 349 (M+H$^+$, 13); MS APCI, *m/z*: 349 (M+H$^+$); MS EI, *m/z*: 348 (M$^+$, 0.4), 257 (1), 201 (0.5), 131 (92), 103 (100), 77 (57).

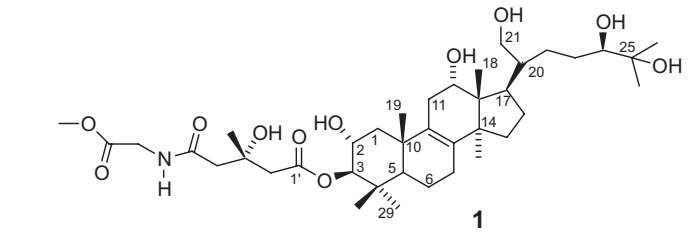
(E)-2,3-Dehydroputrescine-1,4-dicinnamamide (***3***): LC-MS ESI$^+$, *m/z*: 369 (M+Na$^+$, 100), 347 (M+H$^+$, 24). Conditions employed in the LC-MS run: A (water + 0.1% HCOOH), B (2.5:1 MeOH : CH_3CN): linear gradient from 65 % A to 35 % A in 45 min. Flow rate: 1.1 ml min^{-1}. Column: Hypersil BDS C-18 250 × 4.6 mm. Detection: UV at 243 nm.

RESULTS AND DISCUSSION

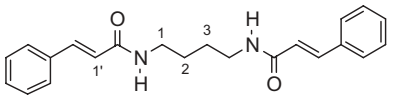
The crude extract of *Ph. spumosa* was partitioned employing various different liquid chromatography techniques, such as direct phase silicagel, C-18 reversed phase, and Sephadex LH-20 (for details see Experimental section). Among the several fractions obtained, the less polar ones contained sterols ubiquitous in Basidiomycetes, *i.e.* ergosterol, its corresponding 5–6 endoperoxide, and 5,6-dehydroergosterol, whereas the more polar fractions contained compounds **1**–**3**.

Inspection of the NMR spectra of compound **1** revealed a lanostane triterpene moiety linked to a depsipeptide unit. Comparison of its NMR, MS, optical rotation, and TLC mobility data, with literature data, confirmed the identity of **1** with fasciculol E, a rare metabolite previously isolated from the fruiting bodies of *Naematoloma fasciculare* (Strophariaceae, Basidiomycetes).[19] The present finding confirms the close relationship of the ge-

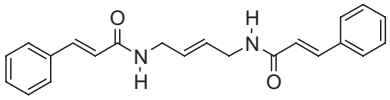
Scheme 1.



1



2



3

nera *Pholiota* and *Naematoloma* inside the family Strophariaceae, as already suggested previously, on the basis of similar stiryl-pyrone pigments.[3] A complete ^{13}C NMR characterization of **1** is here reported (see experimental section), as it was not provided in the original paper.[19] Fasciculols, and related fasciculic acids, are a family of lanostane triterpenoids that have shown important biological activities, such as the inhibitor ability towards calmodulin.[20]

Compound **2** was obtained as a colourless powder, poorly soluble in most organic solvents, except in DMSO-d_6, where complete NMR analysis was carried out. This, along with IR and MS spectra (EI, APCI and ESI ionization modes), indicated two structural units: a cinnamic moiety (^{1}H NMR multiplets at δ 7.6 and 7.4 ppm, and two doublets at 7.5 and 6.6 ppm, along with the EI-MS ions at *m/z* 77, 103 and 131), and an amidic or urea-like C(=O)N-H group (an exchangeable proton at 8.1 ppm in the ^{1}H NMR spectrum, a quaternary carbon resonance at 165 ppm in the ^{13}C NMR spectrum, and IR peaks at 3296, 1655 and 1618 cm^{-1}). In addition, the APCI and ESI mass spectra were clearly indicative of M_r = 348, consistent with the molecular formula $C_{22}H_{24}N_2O_2$.

In order to carry out the crystallographic analysis of **2**, suitable crystals were obtained by slow evaporation of a warm (50 °C) ethyl acetate : methanol solution (*ca.* 2:1). Actually, the crystals, when observed at the optical microscope, appeared to be a mixture of two morphologically different types: thin transparent white plates (crystals A), and yellow needle-like crystals (crystals B). X-ray analysis of both types of crystals was performed.

Crystallographic analysis of crystals A showed that **2** had the molecular structure of putrescine-1,4-dicinnamamide (Figure 1). This compound (often referred to as dicinnamide) has already been reported in the literature as a synthetic product,[21,22] but never, to our knowledge, as a natural product. Phenylpropanoid derivatives, such as **2**, belong to a family of metabolites widely occurring in plants, in particular in the Angiospermae, while their presence in fungi has never been reported so far. In the various different monocinnamamides and dicinnamamides isolated from plants, the amino unit can be part of an aliphatic polyamine, as putrescine[23] and spermidine,[24] or of an aromatic amino acid as tryptophane.[25] In the Cambridge Structural Database (CSD)[26] only the crystal structure of a cyclic dicinnamamide of natural origin can be found, namely cadabicine, derived from spermidine.[24] It has been demonstrated that the above compounds play an important role in regulating the flowering of Angiospermae, being typically absent in sterile plants.[27] Other bioactivities of cinnamamide-like compounds are also notable, for instance, their high α-glucosidase inhibitory effects: thanks to this property, they seem to be useful suppressors of the postprandial glucose level, thus acting as potential hypo-cholesterolemic agents.[22]

The main crystallographic data of **2** are reported in Table I. Compound **2** shows in the solid state a rather elongated conformation (Figure 1), and sits on an inversion centre, located in the middle of the C2-C3 bond.

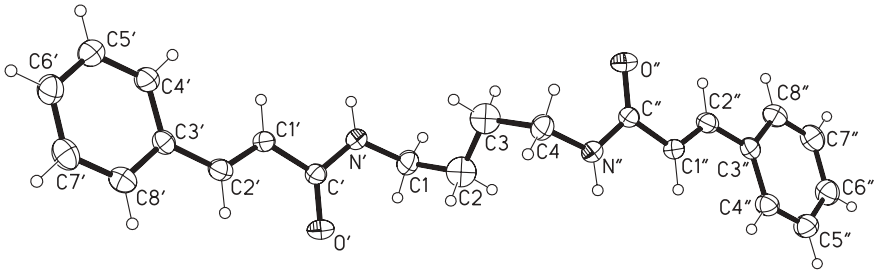


Figure 1. Drawing of the molecular structure of compound **2**, showing the adopted labelling scheme, with displacement ellipsoids drawn at the 25 % probability level. A crystallographic inversion centre is present in the middle of the C2-C3 bond.

TABLE I. Crystal Data for crystal A (compound **2**) and crystal B (Co-crystal **2** + **3**)

	Compound **2** »Crystal A«	Co-crystal **2** + **3** »Crystal B«
Empirical formula	$C_{22}H_{24}N_2O_2$	$C_{22}H_{24}N_2O_2$ + $C_{22}H_{22}N_2O_2$
M_r	348.43	–
Temperature	293(2) K	
Wavelength / Å	0.71073	
Crystal system	Orthorhombic	Orthorhombic
Space group	*P*bca	*P*bca
Unit cell dimensions / Å:		
a	12.530(8)	12.577(9)
b	9.652(2)	9.689(4)
c	15.427(6)	15.519(7)
Volume / Å^3	1865.7(1)	1891.1(2)
Z	4	4
Density (calculated) / Mg m^{-3})	1.240	–
Absorption coefficient / mm^{-1}	0.080	0.074
F(000)	744	696
Crystal size / mm^3	0.39 × 0.34 × 0.06	0.41 × 0.12 × 0.10
Crystal appearance	Transparent plates	Yellow needles
Theta range for data collection	2.64 to 23.34°	2.62 to 23.23°
Index ranges	$-13\le h\le 13$ $-10\le k\le 10$ $-17\le l\le 17$	$-11\le h\le 12$ $-10\le k\le 2$ $-15\le l\le 8$
Reflections collected	8093	13558
Independent reflections	1343 [*R*(int) = 0.0273]	1116 [*R*(int) = 0.0107]
Completeness to theta = 23.34°	99.0 %	82.2 %
Refinement method	Full-matrix least-squares on F^2	
Data / restraints / parameters	1343 / 0 / 119	1116 / 0 / 118
Goodness-of-fit on F^2	1.256	1.227
Final *R* indices [$I > 2\sigma(I)$]	R_1 = 0.0673 wR_2 = 0.1984	R_1 = 0.0602 wR_2 = 0.1667
R indices (all data)	R_1 = 0.0949 wR_2 = 0.2184	R_1 = 0.0862 wR_2 = 0.1786
Largest diff. peak and hole / eÅ^{-3}	0.771 and –0.433	0.553 and –0.103

The crystal packing is characterised by stacking interactions between the cinnamamide planar moieties, as depicted in Figure 2. Taking the amide group as the tail and the cinnamic group as the head of the planar cinnamamide moiety, head-tail stacking interactions are observed (Figure 2), with a distance between the cinnamamide groups of 3.535(5) Å. The planes depicted in Figure 2 are connected along the *b* axis mainly by intermolecular NH·····O hydrogen bonds, which show geometric features corresponding to medium force hydrogen bonds NH·····O and N·····O distances equal to 2.07(1) and 2.897(5) Å, respectively and NH·····O angle equal to 159.8(2)°.

The crystal data of crystal B are also shown in Table I, where it can be seen that it is isostructural with crystal A, with small, but significant differences in the cell parameters and volume (see Table I). The structure was affected by some degree of structural disorder, since the electron density corresponding to the atom C2 (and to the symmetry related C3) is split into two positions, with a 3:1 ratio, as sketched in Figure 3. It is worth noting that the refined C2-C3 bond length [1.40(1) Å] is shorter than a single bond, whereas in the residual electron density in the difference Fourier map, two peaks can be located, corresponding to the two hydrogen atoms of a typical methylene group. This kind of disorder can be attributed either to a conformational disorder, with two possible positions of the C2 atom, due to the flexibility of the C2-C3 single bond, or to a co-crystallisation of two compounds, the major one being **2**, and the minor one being a related dicinnamamide bearing a double bond between C2 and C3 in *E* configuration [(*E*)-2,3-dehydroputrescine-1,4-dicinnamamide (**3**)].

To evaluate these two hypotheses, some theoretical calculations, at the B3LYP/6-31G(d,p) level, were carried out. The mobility of the N-C1-C2-C3 torsion angle in **2** and **3** was investigated to find the conformations best

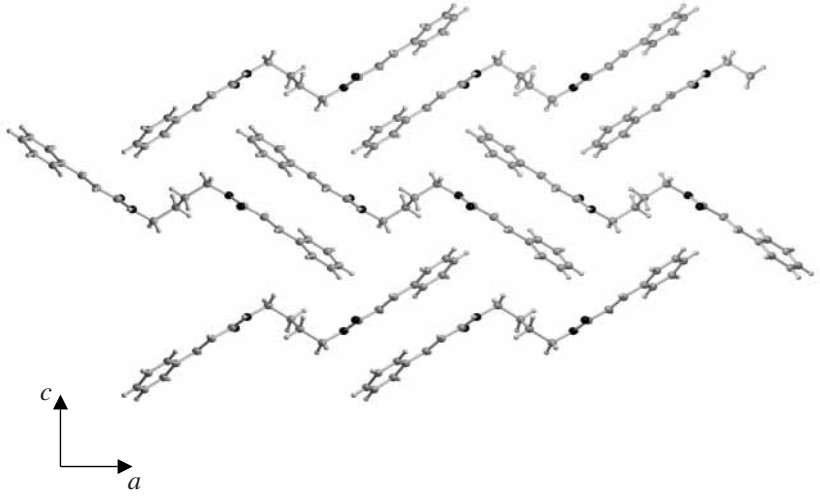


Figure 2. Crystal packing of compound **2**, looking along the *b* axis (C and H atoms in grey, N and O atoms in black).

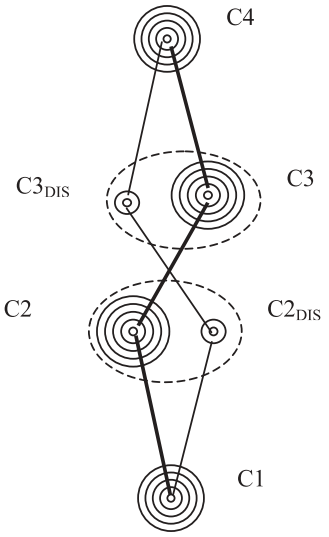


Figure 3. The interpretation of the disorder of the C2 and C3 atoms. Circles represent the isodensity surfaces, bold and normal lines represent the two disordered positions.

fitting the electron density of the peak $C2_{DIS}$ (see Figure 3). The conformational analysis indicated that a suitable conformation of **2** apt to fit the $C2_{DIS}$ peak could be found with $\Delta E = +1.1$ kJ mole^{-1} above the minimum energy X-ray conformation, indicating that the hypothesis of a conformational disorder is likely. Similarly, the more stable conformer of **3** is also capable of fitting the $C2_{DIS}$ peak and therefore the hypothesis of a co-crystallisation of **2** and **3** is also a reasonable one. Therefore, both effects (conformational disorder of **2** and co-crystallization of **2** and **3**) should contribute to explain the disorder observed in the »crystal B« structure ($C2_{DIS}$ and $C3_{DIS}$ peaks in Figure 3).

A proton NMR spectrum of the sample used for the X-ray analysis failed to reveal the ^{1}H chemical shifts calculated for **3** at about 5.9 (H-2) and 4.4 (H-1) ppm: this was surely due to the extreme paucity of the material employed (~ 1 mg), but was also indicative that the 3:1 molar ratio of compounds **2** and **3**, as suggested by the X-ray analysis of the single crystal, must be much poorer in **3** in the macroscopic sample.

We then carried out an LC-MS analysis, and a very small chromatographic peak, with a mass spectrum consistent with the 346 molecular mass of **3**, was actually detected at a retention time slightly longer than that of compound **2**. A more clarifying result was obtained from the LC-MS analysis of a different chromatographic fraction, equally consisting of sub-milligram amounts. The peaks of **2** and **3**, separated at baseline, could be integrated and yielded an approximate 96 : 4 molar ratio (Figure 4). This unfavourable ratio would explain the difficulty of having clear NMR signals of **3**

CONCLUSIONS

Isolation of metabolites from *Pholiota spumosa* (Basidiomycetes, Strophariaceae), provided both known compounds with remarkable biological activity (Fasciculol

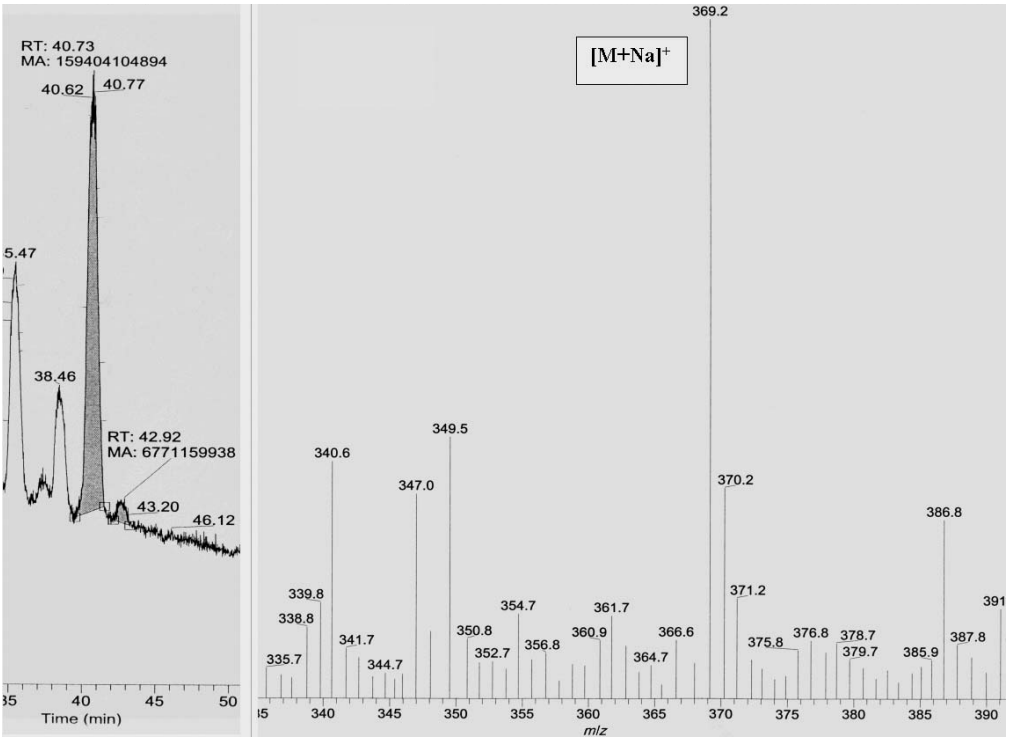


Figure 4. Left: Chromatogram of a fraction containing compounds **2** (R_T = 40.73 min.) and **3** (R_T = 42.92 min.). The Total Ionic Current is shown. Right: Mass spectrum (ESI$^+$) of the peak at 42.92 min. For details, see Experimental.

E), and new natural compounds (dicinnamides), belonging to a class which is novel to the fungal kingdom.

Some interesting biochemical implications may follow from this work. The isolation of Fasciculol E (**1**) from *Pholiota*, confirms the affinity of this genus with *Naematoloma*, where **1** was found for the first time. Such chemotaxonomic indications are becoming of increasing importance in the effort at arriving to a more natural classification of higher fungi.

As concerns the finding of dicinnamide **2** and its dehydro derivative **3** in a Basidiomycete mushroom, it is interesting to note that all cinnamamides occurring in Angiospermae (plants) have oxygen substituents on the aromatic rings, being derivatives of coumaric, caffeic or ferulic acids.[20,27] The biological activity of plant cinnamamides is strictly linked to their phenolic structure, since putrescine 1,4-dicinnamamide (**2**) is not an active inhibitor of α-glucosidase.[22] Indeed, differently from plants, fruiting bodies of *Ph. spumosa* produce unsubstituted aromatic cinnamamides: this finding raises interesting issues on the evolutionary selection of these molecules in a few Basidiomycetes, and therefore on their biological role.

Supplementary Materials. – The crystallographic information file has been deposited with the Cambridge Crystallographic Data center for compound **2**, with deposition number CCDC-221783. These data can be obtained free of charge *via* www.ccdc.cam.ac.uk/retrieving.html (or from Cambridge Crystallographic Data Centre, 12 Union road, Cambridge, CB2 1EZ, UK, +44 1223 336408: E-Mail: deposit@ccdc.cam.ac.uk).

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SAŽETAK

Strukturna karakterizacija metabolita iz gljive *Pholiota spumosa* (Basidiomycetes)

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Izolirana su tri spoja različitoga biosintetičkoga podrijetla iz gljive *Pholiota spumosa* (Basidiomycetes). Fascikulol E, lanostastanski triterpenoid konjugiran s peptidnom jedinicom, izoliran je po prvi puta iz roda *Pholiota*. Uz to prikazana je prva izolacija putrescin-1,4-dicinamamida kao prirodnoga spoja. Njegova je struktura ustanovljena pomoću rentgenske difrakcije i pomoću spektroskopskih metoda. Kristalografska je analiza ukazala na postojanje sukristaliziranoga novoga spoja (*E*)-2,3-dehidroputrescin-1,4-dicinamamida, čije je prisustvo potvrđeno LC-MS analizom. Zanimljivo pitanje vezano na evoluciju slijedi iz zapažanja da dicinamamidi proizvedeni gljivom *Pholiota spumosa* sadrže nesupstituirani benzenski prsten u suprotnosti s dicinamamidima koji su nađeni u biljkama i koji uvijek sadrže fenolnu skupinu i kao takovi imaju različito biologijsko djelovanje.