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Squarrosidine and Pinillidine: 3,3′-Fused Bis(styrylpyrones) from *Pholiota squarrosa* and *Phellinus pini*

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Two novel fungal phenylpropanoid-derived polyketides, squarrosidine (**8**) and pinillidine (**9**), with potent xanthine oxidase (XO) inhibitory activities were isolated from the fruiting bodies of the mushroom *Pholiota squarrosa* and from the mycelium of *Phellinus pini* by bioassay-guided isolation. Structural analyses revealed that both metabolites from the tree fungi represent unprecedented 3,3′-fused bis(styrylpyrones).

Introduction

Phenylpropanoids are well known as highly abundant phenylalanine-derived plant metabolites, many of which play important ecological and physiological roles as dyes, antioxidants, radical scavengers, or metal chelators.[1] In contrast, much less is known about phenylpropanoids in fungi. The most abundant compounds are the styrylpyrones bisnoryangonine (**1**)[2] and hispidin (**2**),[3] which may polymerize during maturation of tree fungi, giving rise to "fungal lignins". Several phenylpropanoid-derived polyketides from tree fungi are potent antiinflammatory agents, such as inotilone (**3**), which exhibits an excellent COX-2 selectivity.[4] Steglich and coworkers reported the structure of fasciculine B (**4**), a blue fluorescent pigment of the fruiting

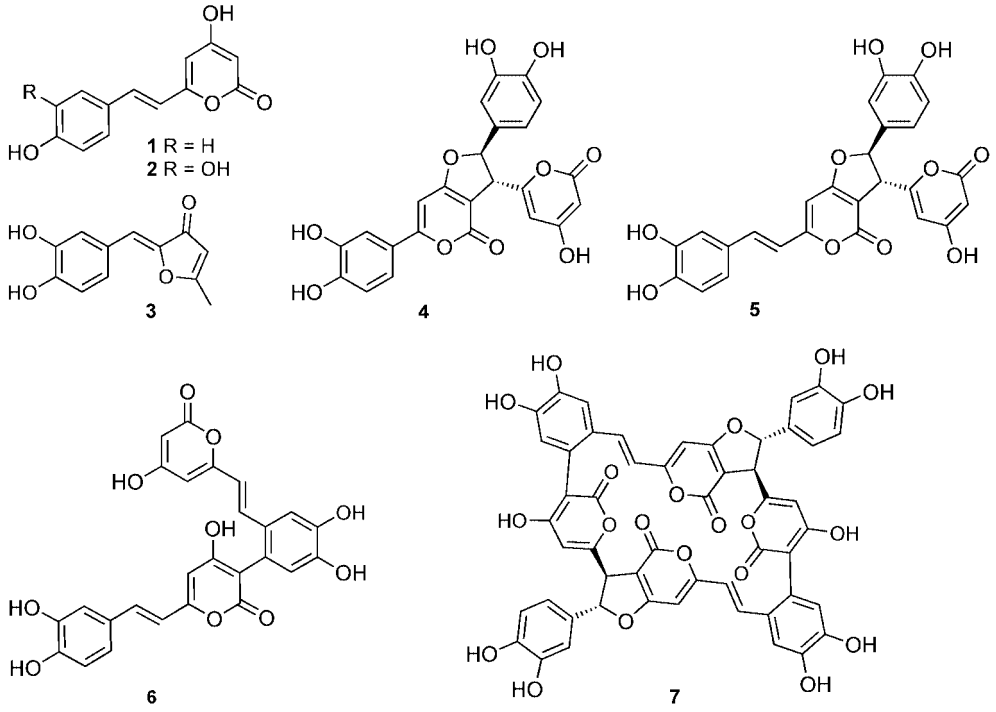

Figure 1. Structures of fungal phenylpropanoids

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body *Hypholoma fasciculare* that obviously results from intermolecular condensation of **2** with a lower homologue.[5] It is remarkable that various related metabolites result from di- or oligomerization of the styrylpyrone building blocks **1** and **2**. Important examples include hypholomine B (**5**),[5] which occurs in *Hypholoma* and *Pholiota* sp., as well as in the tree fungi *Inonotus* and *Phellinus* sp., 3,14′-bihispinidyl (**6**) from *Phellinus pomaceus*,[6] and the structurally intriguing phelligridimer A (**7**) from *Phellinus igniarius* (Figure 1).[7] Here we report the isolation and characterization of two unprecedented 3,3′-fused bis(styrylpyrones) from the tree fungi *Pholiota squarrosa* and *Phellinus pini*, named squarrosidine (**8**) and pinillidine (**9**) (Figure 2).

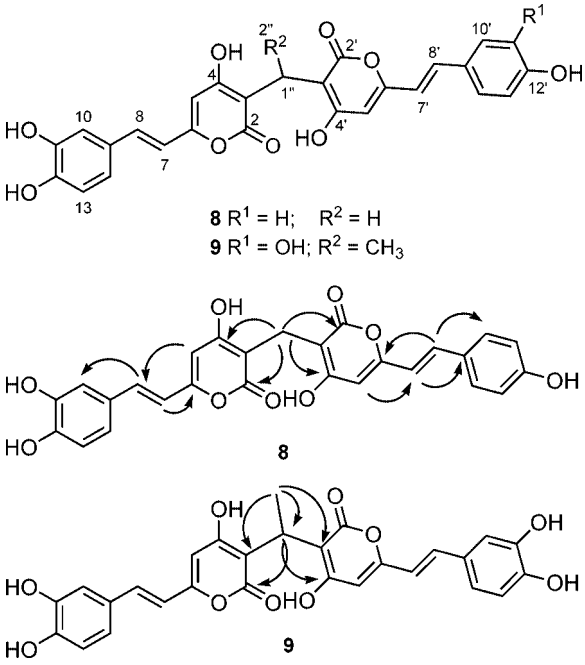


Figure 2. Structures of squarrosidine (**8**) and pinillidine (**9**), and selected HMBC correlations.

In the course of our search for new antiinfective and antiinflammatory compounds from fungal sources[4,8–10] we noted that the mushroom *Pholiota squarrosa* and *Phellinus pini* produce very similar, yet obscure compounds. According to preliminary physicochemical characterization, both compounds appeared to be dimers of metabolites with caffeoyl-derived moieties. To obtain sufficient quantities for full structural elucidation, crude ethyl acetate extracts of the fruiting body of the mushroom *Pholiota squarrosa* (500 g) and of the mycelium of the fungus *Phellinus pini* (20 L of fermentation broth) were individually subjected to open column chromatography on silica gel. Fractions containing phenylpropanoids were further purified by a variety of chromatographic techniques, including size selection on Sephadex LH-20 and preparative HPLC to yield **8** (4 mg) from the extract of *Pholiota squarrosa*. **5** (10 mg) and **9** (4 mg) were isolated from a culture of *Phellinus pini*. All physicochemical and spectrometric data obtained for compound **5** indicated its identity with hypholomine B (**5**, as a mixture of diastereomers).

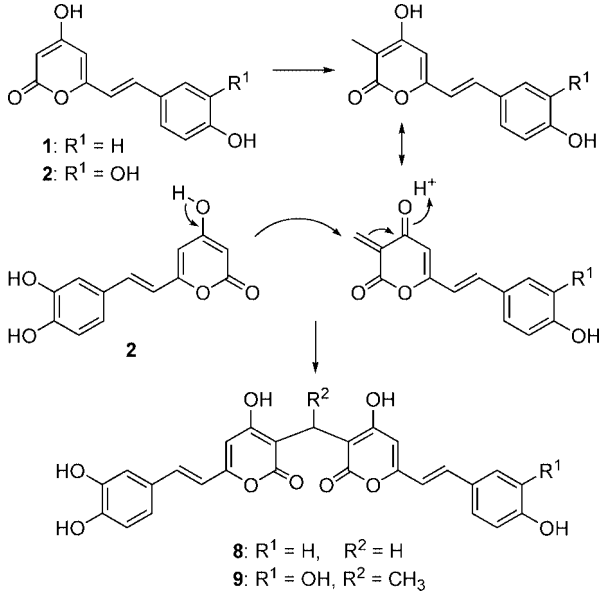
The molecular formula of **8** was determined as $C_{27}H_{20}O_9$ based on HR-ESIMS and ^{13}C NMR spectroscopic data. The ^1H NMR spectrum shows three signals attributable to the characteristic ABX spin coupling system at δ = 6.76 (1 H, d, J = 8.1 Hz, H-13), 6.88 (1 H, dd, J = 1.8, 8.1 Hz, H-14), and 6.96 (1 H, d, J = 1.8 Hz, H-10). In addition, two signals can be assigned to an AA′BB′ spin coupling system of a *para*-disubstituted phenyl moiety at δ = 6.76 (2 H, d, J = 8.7 Hz, H-11′ and H-13′), 7.43 (2 H, d, J = 8.7 Hz, H-10′ and H-14′). Furthermore, the signals at δ = 6.52 (1 H, d, J = 15.9 Hz, H-7), 6.97 (1 H, d, J = 15.9 Hz, H-8), 6.62 (1 H, d, J = 15.9 Hz, H-7′) and 7.06 (1 H, d, J = 15.9 Hz, H-8′) can be attributed to two *trans*-disubstituted double bonds. The ^{13}C NMR and DEPT spectra show one methylene sp^3 and 26 sp^2-C signals including 11 methines, 13 quaternary C atoms (7 of which are oxygenated) and two carboxyl C atoms. The carboxyl functions were confirmed IR spectroscopically (band for conjugated carboxyl C=O at 1661 cm^{-1}). HMQC and HMBC experiments helped resolving the structure. In the HMBC spectrum the correlation of H-10 with C-8, C-9, C-11, C-12, and C-14, the correlation of H-13 with C-9, C-11, C-12 and C-14 and the correlation of H-14 with C-8, C-9, C-10, C-12 and C-13 confirmed the presence of the 11,12-dihydroxystyryl moiety. Moreover, the correlation of H-8 with C-6, C-9, C10, and C-14, that of H-7 with C-5, and the three-bond correlations of H-5 with C-3 revealed hispidin (**2**) as a substructure of **8**. This observation was confirmed by comparison of the ^{13}C NMR spectroscopic data with those of an authentic hispidin reference. In the second part of **8** a noticeable feature attributable to a *para*-hydroxystyryl moiety was the appearance of four sp^2 methine C signals as two chemically equivalent pairs and the correlation of the two *trans* olefinic protons (H-7′ and H-8′) with C-9′. Furthermore, the correlation of H-8′ with C-6′; the correlation of H-7′ with C-5′, C-6′ and that of H-5′ with C-3′, C-4′ and C-6′ helped identifying bisnoryangonin (**1**) as the second part of the molecule. Notably, bisnoryangonin is a known metabolite of *Pholiota squarrosa-adiposa*.[2] HMBC data (correlation of H-1′ with C-2, C-2′, C-3, C-3′ C-4 and C-4′) finally revealed that the two parts are connected through the C-1′ methylene. Thus, **8** represents an unprecedented methylene-bridged bis(styrylpyrone) derivative and was named squarrosidine (Figure 2).

IR and UV spectra of compound **9** proved to be almost identical with the data obtained for **8**, suggesting that it is a homologue of **8**. Its molecular formula was deduced as $C_{28}H_{22}O_{10}$ from HR-MS and ^{13}C NMR data. Furthermore the ^{13}C NMR spectrum of **9** showed a great similarity to that of an authentic hispidin reference except for the presence of additional methyl and methine signals. Based on HMBC correlations **9** was unequivocally identified as a derivative of **8**, bearing additional methyl and hydroxyl groups, and was named pinillidine (Figure 2).

Given that the crude extracts of *Pholiota squarrosa* and *Phellinus pini* exhibited a notable xanthine oxidase inhibitory effect, hypholomine B (**5**), squarrosidine (**8**) and pinillidine (**9**) were evaluated in the XO assay. Compared to the standard allopurinol (IC_{50} 4.4 μM) **5**, **8** and **9** proved to be

potent inhibitors of xanthine oxidase with IC_{50} values of 6.7, 8.1, and 5.8 μM, respectively. It should be noted that xanthine oxidase catalyses the crystallisation of uric acid in the joints, which is the main cause of gouty arthritis.[11] The search for new inhibitors of this enzyme is of great importance since allopurinol, the only compound used clinically for the treatment of gouty arthritis, is now associated with infrequent but severe side effect.[12]

Squarrosidine and pinillidine represent new members of fungal phenylpropanoids with an unprecedented 3,3′-fusion. The only known, albeit plant-derived, 3,3′-bipyrone compounds are helipyrone,[13] dicoumarol,[14] gerberinol,[15] and 1,13-dimethyl-6*H*,7*H*,8*H*-chromeno[3′,4′:5,6]pyrano-[3,2-*c*]chromene-6,8-dione.[16] The formation of squarrosidine can be rationalized by the nucleophilic vinylogous addition of the enol of **1** to a tautomeric form of a methylated bisnoryangonin derivative (or vice versa). For the formation of pinillidine (**9**), two alternative pathways are conceivable, both of which would involve the fusion of bisnoryangonin derivatives. The additional methyl group could be derived from subsequent methylation of the methylene bridge. An ethyl substituent at the pyrone ring would be less likely (Scheme 1).



Scheme 1. Biosynthetic model for the formation bis(styrylpyrones) **8**.

In conclusion, we have isolated two novel bis(styrylpyrones) from *Pholiota squarrosa* and *Phellinus pini* and identified them as potent XO inhibitors. **8** and **9** represent new members of fungal phenylpropanoids that feature an unprecedented mode of bis(styrylpyrone) fusion. The occurrence of the closely related metabolites in two unrelated saprotrophic tree fungi could imply similar ecological roles, such as quenching reactive oxygen species in plant hypersensitive response.[8,17,18]

Experimental Section

General: UV spectra were measured with a Spericord 200 Carl Zeiss technology spectrometer. IR spectra (film) were recorded on a JASCO FT/IR-4100 spectrometer. High-resolution electron impact mass spectra (EI-MS) were recorded on an AMD 402 double-focusing mass spectrometer with BE geometry (AMD, Intestra, Harpstedt, Germany). NMR spectra were recorded on a Bruker Avance 500 DRX spectrometer (Bruker, Karlsruhe, Germany) at 500 MHz for ^1H and 125 MHz for ^{13}C; chemical shifts are given in ppm relative TMS as internal standard. Open column chromatography was performed on silica gel (60, Merck; 0.063-0.2 μm) and sephadex LH-20. HPLC was conducted using a Gilson binary gradient HPLC system equipped with a UV detector (UV/Vis-151) monitoring at 300 nm, using a preparative column packed with Nucleosil 100-7 C_{18}. Analytical HPLC was conducted using a Nucleosil 100 C_{18} column (5 μm, 125 × 4.6 mm) with acetonitrile/H_2O (83:17, v/v) as eluent (flow rate 2 mL/min, 95:5 to 5:95 in 22 min) and UV detection at 370 nm. TLC was carried out with silica gel 60 F_{254} plates. Spots were visualized by spraying with vanilline/H_2SO_4, followed by heating. All solvent used were spectral grade or distilled prior to use.

Xanthine Oxidase Assay: The xanthine oxidase activity was measured using lucigenin as the chemiluminescence substrate. It was conducted according to the method described by Pierce et al.[19] The inhibitory effects of the test compounds are indicated in terms of IC_{50}. Allopurinol was used as a reference.

Fungal Material: The fruiting body of the mushroom *Pholiota squarrosa* was collected in Jena (Germany) and its taxonomic identification was verified by Dr. Martin Roth (HKI, Pilot Plant for Natural Products), where a specimen was deposited. *Phellinus pini* was retrieved from the fungal collection of HKI. Fifteen days old malt agar-plate cultures were used to inoculate malt liquid medium. The surface cultivation was carried out under sterile conditions in 500 mL Erlenmeyer flasks containing 300 mL medium.

Isolation Procedures

***Pholiota squarrosa*:** 500 g of fresh fruiting bodies of was cut in small pieces, dried and crushed. The resulting powder was extracted three times with ethyl acetate and chloroform-MeOH (3 × 2 L each). The ethyl acetate extract (3 g of a brown solid) was first pre-screened and then applied to open column chromatography on silica gel (0.063 ca. 0.1 mm, 4 × 60 cm column). Final purification was achieved by preparative HLPC on a reverse-phase column (Phenomenex Hydro-Rp 80 column, Synergi 10 μm, 250 × 21 mm, and acetonitrile/H_2O (83:17, v/v) as eluent (flow rate 15 mL/ min, 95:5 to 5:95; 30 min, UV detection at 370 nm), yielding **8** (4 mg).

***Phellinus pini*:** The whole culture broth (20 L) was extracted twice with two volume equivalents of ethyl acetate. The residue (10 g) of the evaporated ethyl acetate extract (from 20 L of culture broth) was first pre-screened and then applied to a silica gel column in chloroform. Elution was performed with 500 mL portions of $CHCl_3$, $CHCl_3$/MeOH (9:1), and $CHCl_3$/MeOH (7:3). The fractions obtained were separated using Sephadex LH 20 and HLPC on reverse-phase column (Phenomenex Hydro-Rp 80 column, C-[18], 250 × 21 mm, 10 μm, and acetonitrile/H_2O (83:17, v/v) as eluent (flow rate 15 mL/ min, 95:5 to 5:95; 30 min, UV detection at 370 nm). The individual peaks were collected and purified by the same preparative procedure, yielding **5** (10 mg) and **9** (4 mg).

Squarrosidine (8): Yellow oil, R_t = 11.79 min (analytical HPLC). UV (MeOH): λ_{max} = 219, 258, 369 nm. IR (film): $\tilde{\nu}$ = 3204, 1661, 1601, 1556, 1513, 1414, 1273, 1022, 987 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz): see Table 1. ^{13}C NMR ($CDCl_3$, 125 MHz): see Table 1. ESI-MS (neg. ion mode): *m*/*z* 487.0 $[M - H]^-$. MS/MS (neg. ion mode): *m*/*z* 257.0, 245.0, 213.1, 211.2, 185.1, 159.1. HR-ESIMS: *m*/*z* 487.1065 $[M - H]^-$ (calcd. *m*/*z* 487.1029 $[M - H]^-$ for $C_{27}H_{19}O_9$).

Table 1. ^{1}H- and ^{13}C-NMR spectroscopic data of **8** (in) and **9** (chemical shifts δ in ppm, coupling constants J [Hz] in parentheses, TMS as internal standard)

	8, $CDCl_3$ solution		**9**, $[D_6]$DMSO solution	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1	–	–	–	–
2	–	164.2	–	164.2
3	–	101.2	–	105.2
4	–	172.5	–	166.5
5	5.85 s	105.2	6.13 s	101.4
6	–	155.4	–	156.6
7	6.52 d (15.9)	117.3	6.64 d (16.0)	116.1
8	6.97 d (15.9)	131.5	7.16 d (16.0)	133.7
9	–	127.2	–	126.8
10	6.96 d (1.8)	113.6	7.01 d (1.9)	114.0
11	–	146.5	–	145.5
12	–	145.4	–	147.1
13	6.76 d (8.1)	115.6	6.74 d (8.3)	115.7
14	6.88 dd 1.8, 8.1	119.5	6.93 dd (1.9, 8.3)	120.1
1′	–	–	–	–
2′	–	164.2	–	164.2
3′	–	101.2	–	105.2
4′	–	172.5	–	166.5
5′	5.84 s	105.2	6.13 s	101.4
6′	–	155.4	–	156.6
7′	6.62 d (15.9)	117.4	6.64 d (16.0)	116.1
8′	7.06 d (15.9)	131.1	7.16 d (16.0)	133.7
9′	–	126.7	–	126.8
10′	7.43 d (8.7)	128.5	7.01 d (1.9)	114.0
11′	6.76 d (8.7)	115.6	–	145.5
12′	–	158.1	–	147.1
13′	6.76 d (8.7)	115.6	6.74 d (8.3)	115.7
14′	7.43 d (8.7)	128.5	6.93 dd (1.9, 8.3)	120.1
1′′	3.33 s	18.9	4.55 q (7.4, 15.0)	25.5
2′′			1.45 d (7.5)	16.5

Pinillidine (9): Yellow powder, R_t = 11.2 min (analytical HPLC). $[\alpha]_D^{20}$ = –17.2 (c = 0.07, MeOH). UV (MeOH): λ_{max} = 256, 388 nm. IR (film): $\tilde{\nu}$ = 3189, 2921, 1649, 1584, 1557, 1494, 1375 cm^{-1}. ^{1}H NMR ($[D_6]$DMSO, 300 MHz): see Table 1. ^{13}C NMR ($CDCl_3$, 125 MHz): see Table 1. ESI-MS (neg. ion mode) m/z 517.8 $[M - H]^-$. MS/MS (neg. ion mode): m/z 270.9, 244.9. HR-ESIMS: m/z 517.1135 $[M - H]^-$ (calcd. m/z 517.1135 $[M - H]^-$ for $C_{28}H_{21}O_{10}$).

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