Sterol composition in field-grown and cultured mycelia of *Inonotus obliquus*

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Abstract: Sterols are one of the active classes of compounds in *Inonotus obliquus* for their effective therapy of many diseases. In field environment, this fungus accumulates large amount of sterols. In cultured mycelia, however, this class of compounds is less accumulated. For analyzing the factors responsible for differing sterol composition, the field-grown and cultured mycelia were extracted with 80% ethanol at room temperature and total sterols were prepared using silicon gel column chromatography followed by identification using either GC-MS or spectroscopic methods. For culturing *Inonotus obliquus*, the seed culture was grown either in basic medium consisting of glucose (2%), yeast extract (0.5%), KH_2PO_4 (0.01%), $MgSO_4 \cdot 7H_2O$ (0.05%) and distilled water at pH 6.5, or the basic medium supplemented with serial concentrations of $AgNO_3$. The results indicated that field-grown mycelia contained lanosterol and inotodiol (comprised 45.47% and 25.36% of the total sterols, respectively) and other 10 sterols (comprising the remaining 30.17%) including ergosterol biosynthetic intermediates such as 24-methylene dihydrolanosterol, 4,4-dimethylfecosterol, 4-methyl fecosterol, fecosterol and episterol. Column chromatography also led to the isolation of lanosterol, Inotodiol, trametenolic acid, foscoparianol B and a new triterpenoid foscoparianol D in field-grown mycelia. In comparison, the cultured mycelia only contained three sterols with ergosterol as the predominant one (82.20%). Lanosterol only accounted for 3.68%. Supplementing Ag^+ into the culture at 0.28 $\mu mol \cdot L^{-1}$ greatly enhanced content of lanosterol (accounting for 56.81%) and decreased the content of ergosterol (18.5%) together with the presence of intermediates for ergosterol biosynthesis. These results suggested that the sterol composition in mycelia of the fungus can be diversified by supplementing substances inhibiting enzymatic process towards the synthesis of ergosterol. Harsh growth conditions in field environment (i.e. temperature variation, UV irradiation etc.) can delay the synthesis of ergosterol and hereby diversify the sterol composition in the mycelia of *Inonotus obliquus*.

Key words: *Inonotus obliquus*; sterol composition; field-grown mycelia; cultured mycelia

CLC number: R284　　**Document code**: A　　**Article ID**: 0513 – 4870(2007)07 – 0750 – 07

桦褐孔菌野生菌丝体和培养菌丝体的甾体类化合物组成

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摘要: 甾体类化合物是桦褐孔菌治疗疾病的有效成分之一。该菌的野生菌丝体中有含量很高的多种甾体类化合物。然而人工培养的桦褐孔菌菌丝体中很少积累甾体类化合物。为了分析导致野生菌丝体和培养菌丝体甾体类

Received 2006-12-11.

Project supported by Natural Science Foundation of Jiangsu Province (grant No BK2006034), key project of Natural Science Foundation of Education Department, Jiangsu Province (grant No. 05KJA36012).

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成分差异的原因，本研究采用80%乙醇在室温下对菌丝体进行提取，用硅胶柱色谱制备总甾体类化合物，并以GC-MS和波谱学方法进行鉴定。与此同时，桦褐孔菌用基本培养基(葡萄糖2%，酵母膏0.5%，KH_2PO_4 0.01%，$MgSO_4 \cdot 7H_2O$ 0.05%，pH 6.5)或在基本培养基中加入不同浓度的$AgNO_3$进行培养。结果显示桦褐孔菌野生菌丝体甾体类化合物以羊毛甾醇和桦褐孔菌醇为主要成分，分别占45.47%和25.36%。另有10种次要成分，共占总甾体类化合物的30.17%，其中包括24-甲烯基二氢羊毛甾醇、4,4-二甲基粪甾醇、4-甲基粪甾醇、粪甾醇以及表甾醇。同时，从柱色谱分离还得到了羊毛甾醇、桦褐孔菌醇、木栓酸、桦褐孔菌醇B和1种新的甾醇类化合物桦褐孔菌醇D。相比之下，桦褐孔菌的培养菌丝体中仅含有3种甾醇类化合物，其中麦角甾醇占82.20%，桦褐孔菌醇占14.12%，而羊毛甾醇仅有3.68%。在基本培养基中加入0.28 μmol·L^{-1}的Ag^+可将羊毛甾醇的含量提高到56.81%，使麦角甾醇的含量下降到18.5%。与此同时，还检测到麦角甾醇合成途径中的中间体。这些结果表明，野生菌丝体甾醇类化合物种类多样性的原因可能与麦角甾醇生物合成受到抑制有关。同时苛刻的野生生长环境如温差变化和紫外线照射是造成野生菌丝体甾醇类化合物组成多样性的重要原因。

关键词：桦褐孔菌；甾醇类化合物组成；野生菌丝体；培养菌丝体

Introduction

The medicinal fungus *Inonotus obliquus* (Fr.) Pilat (Hymenochaetaceae) usually grows on the living trunks of birch in Far East of Russia, northeast China and other adjacent countries at latitudes of 45° N – 50°N. Over the periods, this fungus has been used as an effective agent to treat patients suffering breast cancer, hepatoma and gastrointestinal cancers without incurring any unacceptable toxicity[1,2]. Studies disclosed that *n*-hexane extract of the fungus mainly contained lanosterol analogues, ergosterol and its peroxides[3,4], and possessed many pharmacological activities including inhibiting cholesterol biosynthesis[5], antiproliferation of tumor cells[6,7], immunomodulation[8,9] and antioxidation[10]. Because of the pharmaceutical importance and extremely slow growth rate of the fungus, natural reserves have nearly been exhausted[11]. Previous works on the culture of *I. obliquus* mainly focused on the accumulation of melanins[12,13] and polysaccharides[14]. The factors affecting sterol accumulation have not yet been reported. In our previous study, ergosterol was the main component, and lanosterol only presented as a minor constituent. Other lanosterol analogues were hardly detectable in cultured mycelia of *I. obliquus* and its pharmacological actions were hereby significantly reduced[4]. To the best of our knowledge, the reasons leading to the difference in sterol composition have not yet been well elucidated between the field-grown and cultured mycelia. In this study, we reported the sterol composition of field-grown and cultured mycelia and tried to incubate *I. obliquus* with supplementation of Ag^+ in order to find the factors leading to the difference in sterol composition between field-grown and cultured mycelia of *I. obliquus*.

Results and Discussion

1 Sterol composition analyzed by spectroscopic method

Repeated chromatography of the total sterol from field-grown mycelia gave lanosterol (**1**), inotodiol (**2**), trametenolic acid (**3**), foscoparianol B (**4**) and a new triterpenoid foscoparianol D (**5**).

Compound **5** gave a quasi-molecular ion peak at m/z 497 $[M+Na]^+$ in its positive ESIMS spectrum and was assigned a molecular formula of $C_{30}H_{50}O_4$, which was confirmed by HRESIMS (found $[M+Na]^+$ 497.702 2, calcd 497.701 6), ^1H and ^{13}C NMR data.

The ^1H NMR spectrum of **5** showed characteristic signals for five inotodiol typical tertiary methyls (δ_H 0.74 – 1.06) and a secondary methyl at δ_H 1.05. The ^1H NMR spectrum of **5** also contained two double doublets at δ_H 3.44 (J = 8.8, 4.4 Hz) and δ_H 4.52 (J = 10, 6 Hz), corresponding to two protons attached to hydroxyl carbons. Comparing the ^1H NMR spectrum of **5** with that of inotodiol showed the spectra to be very similar in the molecular framework, symbolizing that compound **5** is also an inotodiol analogue. The only differences were the two olefinic protons at δ_H 5.07 (br s) and δ_H 5.31 (br s), one vinylic methyl at δ_H 1.61 and one proton at δ_H 5.34 (dd, J = 8, 0.8 Hz). The ^{13}C NMR spectrum also showed compound **5** to be an inotodiol analogue [3]. The presence of a double bond between C-8 and C-9 as in inotodiol is obvious. The other characteristic signals for lanostane-type triterpenes are the singlets of quaternary carbons δ_C: 49.1, 44.5, 42.9 and 36.8 caused by C-14, C-13, C-4 and C-10, respectively. The signal at δ_C 77.5 can be assigned to C-3, compared to δ_C 78.7 in inotodiol[15]. The doublet at δ_C 77.4 (C-22) is close to

that of inotodiol corresponding to the ^{13}C resonance of C-22 on the side chain that is attached by a hydroxyl group[16]. ^{13}C NMR spectrum of **5** also contains one doublet at δ_C 85.8, one singlet at δ_C 144.6, one triplet at δ_C 111.1. In ^1H-^1H COSY spectrum, the signal at δ_H 4.52 (H-22) coupled with the multiplet δ_H 2.15, and multiplet δ_H 2.15 (H-23) coupled with the broadened doublet δ_H 5.34 (H-24). In HMBC spectrum, long range correlations were observed between the signal at δ_H 4.52 (H-22) and δ_C 85.8, the signal at δ_C 85.8 with broadened singlet δ_H 5.31 and δ_H 5.07, which demonstrates that the two typical methylene protons are bounded to C-27, and the methyl signal (δ_C 12.9) is C-26 (Figure 1); the doublet at δ_C 85.08 is C-24, on which a highly oxygenated moiety is bonded. According to the mass spectrum, this moiety is a hydroperoxy (-OOH). All the assignments of the NMR signals of **5** were confirmed by X-ray crystallography (Figure 2). The atomic coordinates and thermal displacement parameters are shown in Table 2, as wall as selected bond length and angles in Table 2 and 3, respectively. Based on the signal assignments, **5** is 3β, 22-dihydroxyl-24-hydroperoxyl-lanosta-8, 25 (27)-dien (foscoparianol D).

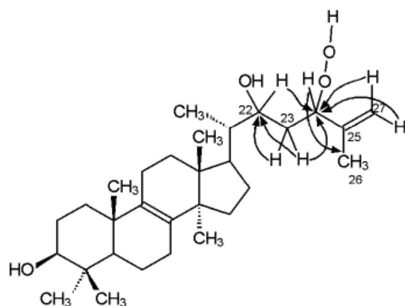

Figure 1　Key HMBC correlations of **5**

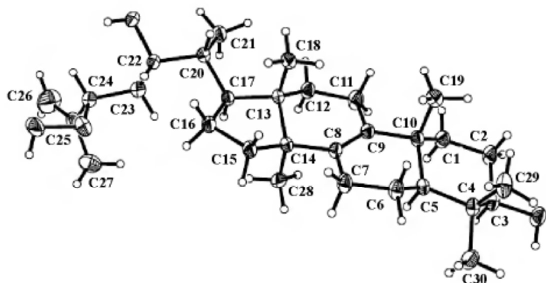

Figure 2　ORTEP diagram of **5**, showing 30% probability displacement ellipsoids and the atom-numbering scheme

2　Sterol composition analyzed by GC-MS

GC-MS analysis showed that field-grown mycelia lanosterol and inotodiol, comprising 45.47% and 25.36% of the total sterols at concentrations of 0.389% and 0.217% dry wt mycelia, respectively (Table 1). In addition, 10 minor components of sterol were also present in the field-grown mycelia: 24-methylene dihydrolanosterol (**6**), 4, 4-dimethyl-fecosterol (**7**), 4-methylfecosterol (**8**), fecosterol (**9**), ergosta-5, 7, 9 (11), 22-tetraenol, episterol (**10**), ergosta-7,22-dien-3-ol (**11**), ergosterol (**12**), ergosta-5,7,9(11),22-tetraenol (**13**), ergosta-5,7,9 (11), 22-tetraenol benzoate (**14**) and ergosterol peroxide (**15**). Sterols **6** – **11** are typical intermediates of the ergosterol biosynthetic pathway of filamentous fungi[17]. In contrast, the sterol composition in cultured mycelia only comprised of 3 sterols with predominant one being ergosterol, accounting for 82.20% of the total at a concentration of 0.178% dry wt mycelia. The sterol with second majority was inotodiol, comprising 14.28% of the total at a concentration of 0.034% dry wt mycelia whereas lanosterol was only found to be 0.38% of the total at a concentration of 0.008 dry wt mycelia. The intermediate fecosterol only presented in trace element (Table 1).

Lanosterol is the precursor of ergosterol biosynthesis[15]. The presence of 24-methylene dihydrolanosterol, a 4,4,14-trimethyl sterol which is unsaturated at C-24, indicates that alkylation at C-24 to form a $\Delta^{24(28)}$ methylene precedes all C-4 and C-14 demethylations. Sequential demethylations at C-14 and C-4 are represented by 4, 4-dimethyl fecosterol, 4-fecosterol and fecosterol, while $\Delta^8 \rightarrow \Delta^7$ isomerisation is represented by the presence of fecosterol and episterol. The presence of ergosta-7,22-dienol suggests that C-24(28) saturation and C-22(23) desaturation precede the $\Delta^7 \rightarrow \Delta^{5,7}$ transformations in *I. obliquus*[17]. Each step of biotransformation towards ergosterol biosynthesis is driven by specific enzymes. In field environment, however, these enzymatic reactions can easily be ceased or inhibited by oxidative stress, leading to the accumulation of lanosterol and the presence of a number of intermediates in ergosterol biosynthetic pathway. In comparison, such biotransformation can easily be achieved in culture conditions, resulting in mass accumulation of ergosterol with little accumulation of lanosterol and ergosterol biosynthetic intermediates.

Supplementing Ag^+ into the culture resulted in a significant change in sterol composition of cultured mycelia of *I. obliquus*. A slight increase in the content of lanosterol and ergosterol was detected in the culture with the addition of 0.7 $\mu mol \cdot L^{-1}$ Ag^+ (final concentration), and further enhanced in response to the increase of Ag^+ concentration at 0.14 and 0.21 $\mu mol \cdot L^{-1}$. Further increase of Ag^+ concentration to 0.28 $\mu mol \cdot L^{-1}$ modified the content of lanosterol and ergosterol to 58.61% and 18.05% of the total, respectively. Concomitant with the change in the accumulation of the two sterols, the intermediates like 24-methylene dihydrolanosterol, 4,4-fecosterol, 4-methylfecosterol, fecosterol and episterol were also

Table 1 Sterol compositions of field-grown and cultured mycelia analyzed by GC-MS

Sterol	Field-grown mycelia	Mycelia from the culture with different concentration of Ag^+ / $\mu mol \cdot L^{-1}$					
		0	0.7	0.14	0.21	0.28	0.35
Lanosterol (**1**)	3.89(45.47)	0.08(3.68)	0.11(7.53)	0.19(8.26)	0.21 (7.00)	2.11(58.61)	1.42(71.33)
Inotodiol (**2**)	2.17(25.36)	0.31(14.28)	Tr	–	–	–	–
24-Methylene dihydrolanosterol (**6**)	Tr	–	–	–	–	0.21	0.16
4,4-Dimethylfecosterol (**7**)	0.34(3.97)	–	–	–	–	0.09(2.50)	0.27(13.56)
4-Methylfecosterol (**8**)	0.98(11.45)	–	–	–	Tr	Tr	0.06(3.01)
Fecosterol (**9**)	Tr	Tr	–	Tr	–	0.38(10.55)	Tr
Episterol (**10**)	Tr	–	–	Tr	–	0.23(6.38)	Tr
Ergosta-7,22-dien-3-ol (**11**)	0.21(2.45)	–	–	–	0.05 (1.68)	0.14(3.88)	Tr
Ergosterol (**12**)	0.37(4.32)	1.78(82.2)	2.05(92.46)	2.11(91.73)	2.69 (90.57)	0.65(18.05)	0.24(12.06)
Ergosta-5,7,9(11),22-tetraenol (**13**)	0.56(6.54)	–	–	–	Tr	–	Tr
Ergosta-5,7,9(11),22-tetraenol benzoate (**14**)	Tr	–	–	–	–	–	Tr
Ergosterol peroxide (**15**)	0.035(0.40)	–	–	–	–	–	–

The content of each sterol was represented by ‰ of the dry mycelia. The figure in the brackets stands for the relative content of each sterol among the total sterols (%)

Table 2 Ion species in the mass spectral data of sterols (as TMSi ethers) isolated from cultured and field-grown mycelia of *I. obliquus* (Intensities as a percent of the base peak are in parentheses)

Sterol number	**1**	**2**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**[c]	**15**
RR_t[a]/min	1.17	1.46	1.45	1.34	1.27	1.09	1.13	1.05	1.02	1.21	1.89	1.34
Fragmentation												
$[M]^+$	498(35.6)	514(20.5)	512(33)	498(60)	484(100)	470(49)	470(23)	470(67)	468(33)	466(10)	498(2.4)	500(2.4)
$[M]^+ - 15(-CH_3)$	483(100)	499(18.0)	497(33)	483(13)	469(47)	455(46)	455(30)	455(32)	453(2)	451(2)	–	485(9.2)
$[M]^+ - 90$(-TMSiOH)	408(5.6)	424(21.3)	–	408(22)	394(35)	380(18)	–	380(20)	37819)	376(44)	–	410(21.2)
$[M]^+ - 105(-CH_3$-TMSiOH)	393(21.1)	409(25.4)	407(100)	393(54)	379(19)	365(23)	365(19)	–	363(100)	361(11)	–	385(19)
$[M]^+ - 105$(-benzoate)	–	–	–	–	–	–	–	–	–	–	392(2)	–
$[M]^+ - 131$(-TMSiOH = $CH-CH_2-CH_3$)	–	383(15.4)	–	–	353(11)	–	–	–	337(60)	–	–	–
$[M]^+ - 129(-C_{20}-C_{28})$	–	–	–	–	–	343(100)	–	–	–	–	–	–
$[M]^+ - 127(-C_{20}-C_{28}-2H)$	–	–	383(2)	–	357(20)	–	343(100)	343(87)	–	–	–	–
$[M]^+ - 215(-C_{20}-C_{28}$-TMSiOH)	–		–	–	269(27)	355(29)	255(23)	255(100)	253(23)	251(100)	–	285(100)
$[M]^+ - 217(-C_{20}-C_{28}$-TMSiOH-2H)	–	–	297(3)	–	–	253(34)	253(25)	253(12)	251(3)	249(25)	–	–
$[M]^+ - 241(-C_{20}-C_{28}$-TMSiOH-$C_2H_2$)	–	–	–	–	243(20)	229(17)	–	229(33)	227(10)	225(12)	–	–
$[M]^+ - 242[-(-C_{20}-C_{28}+H)$-TMSiOH-$C_2H_2$]	–	–	–	257(26)	–	–	–	–	226(9)	224(5)	–	–
$[M]^+ - 243[-(-C_{20}-C_{28}+2H)$-TMSiOH-$C_2H_2$]	–	–	–	–	241(24)	–227(35)	227(10)	213 (22)	225(4)	223(16)	–	–
$[M]^+ - 257(-C_{20}-C_{28}$-TMSiOH-42[b])	255(30.1)	257(12.7)	255(15)	255(22)	227(45)	227(29)	213(25)	153(100)	201(15)	209(24)	–	243(52)
Additional ions		171(100)	241(23)		213(32)	–	–	–	–		376(82) 69(100)	–

The sterol number indicates the compounds in Table 1. [a]Retention time relative to lanosterol; [b]Loss of C_3H_3 from C_{15} to C_{17}; [c]Determined as real ion peak (not the TMSi ether)

detected (Table 1).

It has been reported that lower concentration of Ag^+ is the elicitor of HMGCoAR, the enzyme representing rate-limiting reaction for lanosterol biosynthesis[18]. The presence of Ag^+ at higher concentration inhibits DNA synthesis[19] and hence slows down the synthesis of the enzymes driving for the biotransformation of lanosterol to ergosterol, leading to an increased accumulation of lanosterol, a less accumulation of ergosterol and the presence of intermediates towards ergosterol biosynthesis.

Experimental

1 General experimental procedures

Ergosterol, lanosterol and cholesterol standards were purchased from Sigma, USA. The derivatising reagent, BSTFA (+1% TMCS)[*N,O-bis*(trimethylsilyl) trifluoroacetimide + trimethylchlorosilane], was purchased from Pierce, USA. GC-MS was performed using a Hewlett-Packard HP 6890 gas chromatography equipped with a Hewlett-Packard 5973 mass selective detector. The GC column was WCOT (CP-SIL5: 30 m by 0.25 mm internal diameter with a film thickness of 0.25 mm) and was obtained from Chrompak, Varian, Australia. A Wiley 275 Mass Spectral database was used to analyze mass spectral data. All the NMR spectra (including ^1H, ^{13}C, HSQC, NOESY and HMBC) were determined on a Bruker AV-400 spectrometer with tetramethylsilane (TMS) as internal standard. Single crystal was determined using X-ray diffraction spectrometer (Bruker SMART CCD 1000). Silica gel (200 – 300 mesh, Qingdao Oceanic Chemical Plant, China), macroporous resin (ADS-17, Tianjing Nankai University, China) columns were used for the isolation of sterols from field-grown mycelia, and the eluants were detected with Dragendorff's reagent.

2 Fungi materials and PDA culture

I. obliquus was collected in Changbai Mountains of Mudanjiang Region, northeast China. The vouch specimen (KLBMP04005) identified by Russian mycologist Prof. Margarita A Bondartseva was preserved in the Herbarium of Key Laboratory for Biotechnology on Medicinal Plants of Jiangsu Province. The mycelia of the fungus were successfully isolated from the field-grown mycelial tissue using potato dextrose agar (PDA) slant and subcultured each three months. The slants were incubated at 25 ℃ for 7 days and then stored in refrigerator (4 ℃).

3 Inoculation preparation

I. obliquus was initially grown on PDA medium in a Petri dish for 7 days and then transferred to the seed culture medium by punching out 15 mm^2 of the agar plate culture for 10 peaces with a sterilized self-designed cutter. The seed culture was grown in a 500 mL-conical flask with 200 mL conventional medium consisting of glucose (2%), yeast extract (0.5%), KH_2PO_4 (0.01%), $MgSO_4 \cdot 7H_2O$ (0.05%) and distilled water at pH 6.5. The culture was implemented on a rotary shake incubator (Shen Neng Bao Cai, Shanghai China) at 26 ℃ and 140 rpm for 8 days.

4 Shake flask culture

The medium used for the shake flask culture was identical to that of the seed culture. $AgNO_3$ (AR, Shanghai chemical reagent factory No 4, China) was supplemented into the medium at final concentrations of 0, 0.07, 0.14, 0.21, 0.28 and 0.35 $\mu mol \cdot L^{-1}$. The seed culture (ca 10%, *v/v*) was inoculated into 1 L-conical flask containing 400 mL autoclaved medium supplemented with different concentrations of Ag^+ and cultivated at 26 ℃ on a rotary shake incubator at 140 $r \cdot min^{-1}$ in darkness. All the cultures in this experiment were performed in triplicate. At the end of the culture (day 14 post-inoculation), the mycelia were sampled by centrifugation at 20 kg for 10 min, washed three times and lyophilized for the extraction of sterols.

5 Sterol isolation and spectroscopic identification

Well powdered field-grown mycelia of *I. obliquus* (mycelia, 3 kg) were extracted five times with 80% ethanol at room temperature for 24 h. The ethanol extracts were combined and concentrated under reduced pressure, which afforded 191 g crude extract. The concentrated extracts were successively separated on the macroporous resin (ADS-17) under the elution of $EtOH-H_2O$ (70:1, *v/v*), leading to the isolation of total sterols (13 g). For further isolation, 3.8 g of the total sterols was applied to a silica gel column chromatography under the elution of CH_3Cl-MeOH (60:1, *v/v*), which resulted in the isolation of lanosterol (**1**, 1 150.6 mg), inotodiol (**2**, 597.8 mg), trametenolic acid (**3**, 379.3 mg), and a mixture with typical triterpenoid sterol spots under the spray of Dragendorff's reagent (R_f 0.35 – 0.37). The mixture (590 mg) was further isolated using CC under the elution of $CHCl_3$-MeOH (99:1, *v/v*), which afforded several long chain fatty acids and a mixture

(129 mg) containing two sterols with R_f values of 0.35 and 0.37 ($CHCl_3$-MeOH, 70 : 1, *v/v*). For the purification of the two sterols, 60 mg of the mixture was applied on a high resolution TLC plate (18 cm × 15 cm, Merck, Germany) and was given foscoparianol B (25 mg) and a new triterpenoid (**5**, 24 mg). Compound **5** was crystallized in 95% ethanol at room temperature, leading to the formation of a colorless crystal with $[\alpha]_D^{15}$ +63.7° (c, 1.0, in CH_3Cl). IR of **5**: 3 570, 3 440, 1 614, 1 467 cm^{-1}. ^1H NMR data of **5** (400 MHz, in pyridine-d_6) δ: 5.34 (dd, J = 8, 0.8 Hz, H-24), 5.31 (br s, H-27), 5.07 (br s, H-27), 4.52 (dd, J = 10, 6 Hz, H-22), 3.44 (dd, J = 8.8, 4.4 Hz, H-3), 2.02 (dd, J = 8, 6 Hz, H-23), 1.75 (m, H-20), 1.61 (s, H-26), 1.58 (m, H-17), 1.16 (m, H-5), 1.06 (s, H-19), 1.05 (d, J = 5.6 Hz, H-21), 1.04 (s, H-28), 0.97 (s, H-29), 0.88 (s, H-30), 0.74 (s, H-18). ^{13}C NMR data of **5** (100 MHz, in pyridine-d_6) δ: 33.6 (C-1), 27.1 (C-2), 77.4 (C-3), 42.9 (C-4), 50.3 (C-5), 17.6 (C-6), 26.2 (C-7), 133.8 (C-8), 133.8 (C-9), 36.8 (C-10), 20.8 (C-11), 28.0 (C-12), 44.5 (C-13), 49.1 (C-14), 30.8 (C-15), 30.7 (C-16), 47.2 (C-17), 15.8 (C-18), 18.9 (C-19), 43.0 (C-20), 12.4 (C-21), 68.7 (C-22), 39.0 (C-23), 85.8 (C-24), 146.9 (C-25), 12.9 (C-26), 111.6 (C-27), 20.8 (C-28), 15.5 (C-29), 24.1 (C-30). X-ray crystal data of **5**: A colorless crystal of the title compound with dimensions of 0.20 mm × 0.18 mm × 0.16 mm was mounted on a SMART CCD 1 000 area diffractometer for data collection with a graphite-monochromated MoKα (λ = 0.710 73Å) radiation at 294 (2) K by using ω-2θ scan technique in the range of 1.35° < θ < 26.41°. A total of 18 258 reflections were collected, of which 4 804 were independent with R_{int} 0.027 4 and 3 289 were considered as observed [$I > 2\sigma(I)$]. The corrections for *Lp* factors were applied. The structure was solved by direct methods with SHELXS-97 and expanded by using the Fourier technique. The non-hydrogen atoms were refined anisotropically. The hydrogen atoms were determined with theoretical calculations and refined isotropically. A full-matrix least-squares refinement gave the final R = 0.051 4 and wR = 0.098 8 ($w = 1/[\sigma^2(F_o^2) + (0.051\ 8P)^2 + 0.457\ 4P]$, where $P = (F_o^2 + 2F_c^2)/3$). S = 1.069. The maximum peak in the final difference Fourier map was 0.183 e/Å^3 and the minimum peak 0.174 e/Å^3. In the final circle refinement the largest parameter shift $(\Delta/\sigma)_{max}$ is 0.000. Triclinic, P1, a = 7.323 9(17) Å, b = 12.905(3) Å, c = 15.489(4) Å, α = 90.618

Table 3 Atomic coordinates (×10^4) and thermal displacement parameters (Å^2 ×10^3)

Atom	*x*	*y*	*z*	U_{eq}	Atom	*x*	*y*	*z*	U_{eq}
O(1)	8 377(3)	6 373(2)	9 478(1)	53(1)	C(14)	3 076(4)	6 837(2)	4 792(2)	34(1)
O(2)	−2 308(3)	5 229(2)	969(1)	45(1)	C(15)	3 076(4)	6 837(2)	4 792(2)	42(1)
O(3)	−1 926(3)	8 030(2)	998(2)	55(1)	C(16)	3 076(4)	6 837(2)	4 792(2)	41(1)
O(4)	−2 192(5)	8 452(2)	122(2)	82(1)	C(17)	3 076(4)	6 837(2)	4 792(2)	33(1)
C(1)	4 614(4)	5 338(2)	7 457(2)	39(1)	C(18)	2 896(4)	5 040(2)	3 776(2)	43(1)
C(2)	6 005(4)	5 325(2)	8 351(2)	34(1)	C(19)	6 535(5)	4 752(2)	6 449(2)	55(1)
C(3)	7 197(4)	6 403(2)	8 604(2)	45(1)	C(20)	−7 06(4)	5 433(2)	2 520(2)	36(1)
C(4)	10 027(5)	6 199(3)	3 267(2)	59(1)	C(21)	−2 271(4)	4 677(2)	2 822(2)	49(1)
C(5)	6 974(4)	6 725(2)	7 000(2)	34(1)	C(22)	−1 518(4)	6 005(2)	1 694(2)	36(1)
C(6)	7 941(4)	7 941(4)	7 457(2)	36(1)	C(23)	−3 007(4)	6 653(2)	1 817(2)	41(1)
C(7)	6 476(4)	7 334(2)	8 351(2)	36(1)	C(24)	−3 629(4)	7 294(2)	1 020(2)	43(1)
C(8)	4 586(4)	6 610(2)	8 604(2)	51(1)	C(25)	−5 248(4)	7 294(2)	1 079(2)	52(1)
C(9)	4 148(4)	5 867(2)	7 928(2)	36(1)	C(26)	−4 916(5)	8 683(3)	1 781(3)	66(1)
C(10)	5 595(4)	5 664(2)	7 000(2)	36(1)	C(27)	−6 931(7)	7 560(4)	526(4)	103(2)
C(11)	2 235(4)	5 141(3)	5 855(2)	51(1)	C(28)	1 994(4)	7 610(2)	4 890(2)	47(1)
C(12)	790(4)	5 282(2)	6 698(2)	41(1)	C(29)	8 374(4)	6 795(2)	6 698(2)	45(1)
C(13)	1 745(4)	5 799(2)	5 649(2)	32(1)	C(30)	9 237(5)	7 959(3)	2 949(2)	58(1)

U_{eq} is defined as one third of the trace of the orthogonalized U_{ij} tensor

(4)°, β = 102.834(4)°, γ = 99.759(3)°, V = 1 404.9(6) Å^3. Z = 2, Dc = 1.122 Mg/m^3, μ = 0.072 mm^{-1}. F(000) = 524. For atomic coordinaters and thermal displacement parameters, see Table 3.

6 Sterol extraction and analysis

Well-powdered mycelia (5 mg) in triplicate were sampled and ground in a mortar and pestle, separately. The internal standard cholesterol (50 μg) was added and the mixture was saponified followed by GC-MS according to the protocol described previously[17] Sterol components were identified by mass spectral fragmentation and retention time comparison with an authentic standard (for ergosterol and lanosterol), or by mass spectral matching with database (other sterols) (see Table 2). Lanosterol and ergosterol were quantified in mycelial extracts using the pre-determined response factor 0.56 + 0.19 (mean of five replicates) with respect to cholesterol internal standard. The response factor was linear in sterol concentration range present in mycelia.

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