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CERAMIDES FROM THE FUNGUS *PHELLINUS PINI*

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Key Word Index—*Phellinus pini*; Hymenochaetaceae; identification; ceramides; phytosphingosines; lignans; steroids.

Abstract—Two new ceramides were identified among the chemical constituents of the fungus *Phellinus pini*. The structures of these compounds, *N*-(2′-hydroxynonacosanoyl)-D-*erythro*-1,3,4-trihydroxy-2-amino-octadecane and *N*-(2′-hydroxytriacontanoyl)-D-*erythro*-1,3,4-trihydroxy-2-amino-octadecane, were established by spectroscopic and chemical means. In addition, the lignan (+)-pinoresinol and the steroids episterol and ergosterol peroxide have been isolated from the same source. Copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

Phellinus pini is a white-rot fungus that frutifies over the stem of *Pinus pinaster*. This species damages lignin from wood inducing its decay and giving a red colour over the surface attacked. This process reduces drastically the mechanical properties of wood and consequently its economic value [1].

In this paper we report our results related to the chemical constituents of *P. pini*. From a dichloromethane extract we have isolated a mixture of two ceramides, *N*-(2′-hydroxynonacosanoyl)-D-*erythro*-1,3,4-trihydroxy-2-amino-octadecane and *N*-(2′-hydroxytriacontanoyl)-D-*erythro*-1,3,4-trihydroxy-2-amino-octadecane, whose structures were established by spectroscopic and chemical means. This type of compound is known as an inducer of fungus frutification and thus plays an important role in the initial stage of their growth [2]. Together with these metabolites we have isolated the lignan (+)-pinoresinol and the steroids episterol and ergosterol peroxide.

RESULTS AND DISCUSSION

The two ceramides were isolated after extraction of the fungus with dichloromethane. The extract was chromatographed on a silica gel column, and the two ceramides were precipitated from the fraction collected with dichloromethane–methanol (99:1) as a white solid. Their IR spectrum presented hydroxyl bands at 3340 and 3220 cm^{-1}, and bands at 1620 and 1540 cm^{-1} due to the amide group. The ^1H NMR spectrum (Table 1) confirmed the presence of an amide with a proton signal at δ 8.58 (*d*, $J = 8.9$ Hz), It possessed five characteristic signals of geminal protons to hydroxyl groups at δ 4.28 (*m*), 4.36 (*m*), 4.42 (*dd*, $J_1 = 10.6$ Hz; $J_2 = 4.9$ hz), 4.51 (*dd*, $J_1 = 10.6$ Hz; $J_2 = 4.1$ Hz) and 4.62 (*m*). A sixth signal at low field was present at δ 5.12 (*m*) and was identified as a methyne proton vicinal to the nitrogen atom of the amide group. This assembly of ^1H NMR signals suggested the presence of a sphingosine type structure. To determine the number of hydroxyl groups the ceramides were acetylated with acetic anhydride–pyridine. The ^1H NMR spectrum of this derivative (Table 1) revealed four acetate groups which corresponded to four hydroxyl groups on the original structure. The absence of another hydroxyl group was confirmed by the IR spectrum of the acetyl derivative. The ^1H NMR spectrum of the two ceramides also presented methylene signals at δ 1.43 (*s*) and 1.58 (*s*) of two long carbon chains. One of them should be part of the sphingosine long chain base (LCB) structure and the other one was attributed to a variable second chain linked through the amide function. The ^1H and ^{13}C NMR (Table 2) spectra of the *N*-acetylphytosphingosine were practically superimposable with the part of the spectrum of the natural product attributed to the LCB. The sphingosine base possessed 18 carbon atoms, three hydroxyl groups and an amine group. This suggested the presence of the fourth hydroxyl group at the other carbon chain linked to the amide. In order to confirm the basic phytosphingosine structure, the mixture of the two ceramides was hydrolysed with potassium hydroxide in ethanol–water (9:1). The resulting amine triol was acetylated and its ^1H NMR spectrum was found to be identical to that of an authentic sample of tetraacetylphytosphingosine

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Table 1. ^{1}H NMR spectral data for compounds **1** and **2**, the acetyl derivative, *N*-acetylphytosphingosine and tetraacetylphytosphingosine (*d* values, TMS as internal standard)

H	(**1**) + (**2**)*†	Acetyl†‡ (**1**) + (**2**) derivatives	*N*-*† Acetylphyto-sphingosine	Tetraacetyl‡§ phytosphingosine
1A	4.42 *dd*	4.01 *m*	4.45 *t*	4.00 *dd*
1B	4.51 *dd*	4.34 *m*		4.29 *dd*
2	5.12 *m*	4.43 *m*	5.07 *m*	4.47 *dddd*
3	4.36 *m*	5.11 *m*	4.33 *m*	5.10 *dd*
4	4.28 *m*	4.94 *m*	4.24 *m*	4.94 *ddd*
5(2H)	2.10 *m*	1.82 *m*	2.03 *m*	1.67 *m*
6(2H)	1.80 *m*	1.65 *m*	1.75 *m*	—
$\underline{CH}_2$	1.43 *s*	1.25 *s*	1.30 *s*	1.25 *s*
Me-18 and Me-n′	0.85 *t*	0.88 *t*	0.84 *t*	0.88 *t*
N-$\underline{H}$	8.58 *d*	6.58 *d*	8.59 *d*	5.94 *d*
2′	4.62 *m*	5.11 *m*	—	—
3′(2H)	2.05 *m*	1.65 *m*	—	—
$\underline{CH}_2'$	1.58 *s*	1.25 *s*	—	—
OH	6.22 *br s*	—	6.51 *br s*	—
	6.72 *br s*	—	6.59 *br s*	—
	6.74 *br s*	—	6.59 *br s*	—
	7.64 *br s*	—	—	—
OAc	—	2.18 *s*	—	2.05 *s*
	—	2.08 *s*	—	2.05 *s*
	—	2.05 *s*	—	2.02 *s*
	—	2.02 *s*	—	—
$NHCO_2Me$	—	—	2.09 *s*	2.07 *s*
J(Hz)				
1A, 1B	10.6	—	4.9	11.6
1A, 2	4.9	—	4.9	3.1
1B, 2	4.1	—	4.9	4.8
2, 3	—	—	—	8.1
3, 4	—	—	—	3.1
4, 5A	—	—	—	4.2
4, 5B	—	—	—	8.9
18, 17	6.0	5.7	6.6	6.3
Hn′, Hn′-1	6.0	—	—	—
N-$\underline{H}$, 2	8.9	9.0	8.2	9.4

*In pyridine-d_5 solution.
†300 MHz.
‡In $CDCl_3$ solution.
§200 MHz.

(Table 1). The absolute configuration of the chiral centres of the phytosphingosine was confirmed to be (2*S*, 3*S*, 4*R*) [3] by comparing the value of the optical rotation obtained for the acetylated phytosphingosine with that of an authentic sample.

The FAB-mass spectrum of the initial mixture showed the presence of two molecular ions of *m/z* 767 and 753. Considering the mass of phythosphyngosine, one of the ceramides had a C_{30} acyl chain and the other a C_{29} acyl chain. The location of the hydroxyl group of the carbon chain linked to the amide group must be α to the carbonyl, as the geminal proton δ value was characteristic of a proton in this position [4]. This is indeed the most usual position of that group in known ceramides [5–7] that possess one hydroxyl group on the carbon chain.

From the above data we propose the structures of the ceramides to be *N*-(2′-hydroxynonacosanoyl)-D-*erythro*-1,3,4-trihydroxy-2-amino-octadecane (**1**) and *N*-(2′-hydroxytriacontanoyl)-D-*erythro*-1,3,4-trihydroxy-2-amino-octadecane(**2**).

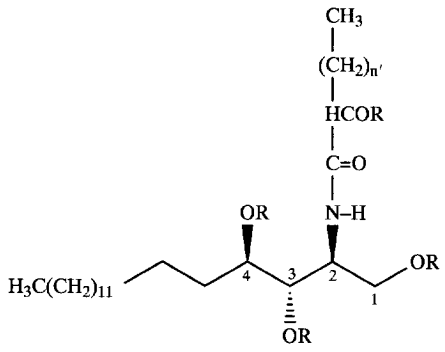
The structures of the lignan (+)-pinoresinol [8, 9] and of the steroids episterol [10] and esgosterol peroxide [11, 12] were confirmed by comparison of their physical and spectroscopic data with those already published for these compounds.

It is important to note that (+)-pinoresinol is a constituent of *Pinus* species [8, 9] and its presence in the material of the fungus is surprising. This could be due to contamination of the fungus material from the host wood, although we made a careful separation of the fungal and host materials.

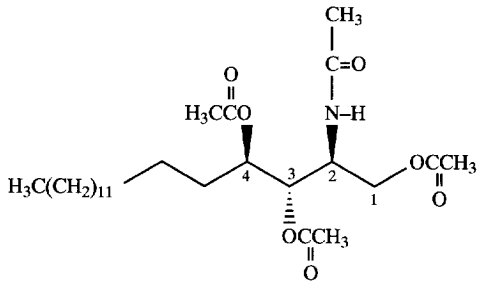
Table 2. ^{13}C NMR spectral data for compounds **1** and **2** and *N*-acetylphytosphingosine*

C	(1) + (2)	*N*-Acetylphyto-sphingosine*
1	62.0 *t*	61.9 *t*
2	53.0 *d*	53.3 *d*
3	76.8 *d*	76.7 *d*
4	73.0 *d*	73.0 *d*
5	34.1 *t*	34.1 *t*
6	26.7 *t*	26.6 *t*
7–15	30.2 *t*–29.7 *t*	30.3 *t*–29.6 *t*
16	30.4 *t*	30.3 *t*
17	23.0 *t*	23.0 *t*
18	14.3 *q*	14.3 *q*
C=O	175.3 *s*	170.2 *s*
2′	72.5 *d*	—
3′	35.7 *t*	—
4′	25.8 *t*	—
5′-n′-3	30.2 *t*–29.7 *t*	—
n′-2	32.2 *t*	—
n′-1	23.0 *t*	—
n′	14.3 *q*	—
$NHCOCH_3$	—	23.3 *q*

*In pyridine-d_5 solution (75.4 MHz; TMS as int. standard).



1 (R = H; n′=26)
2 (R = H; n′=27)
Ceramide tetraacetate derivative (R = Ac)



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EXPERIMENTAL

Plant materials were collected in February 1985, in a forest of *Pinus pinae* at Fonte da Telha, Portugal.

Mps: uncorr. IR spectra: Perkin Elmer 157 G apparatus; ^1H and ^{13}C NMR spectra: Varian XL-300 equilibrium, under standard conditions. EIMS: Shimadzu VG 12-250 and QP-1000 spectrometers; FAB-MS: VG Auto Spec spectrometer.

Extraction and isolation of the compounds. Dried and finely powdered *P. pini* (2.5 kg) were successively extracted in a Soxhlet with CH_2Cl_2 and MeOH. The extract obtained with CH_2Cl_2 (45 g) was subjected to CC on silica gel (Merck No. 7734), with CH_2Cl_2 and CH_2Cl_2–MeOH (99:1) as eluent.

The fr. collected with CH_2Cl_2 was rechromatographed on a silica gel column, eluted with petrol–EtOAc mixt (4:1, 2:1 and 1:1), yielding the following compounds in order of increasing chromatographic polarity: 5α-ergosta-7,24(24′)-dien-3β-ol (episterol), isolated as the acetate derivative (12 mg), 5α,8α-epidioxyergosta-6,22-dien-3β-ol (ergosterol peroxide) (84 mg) and 2:6-bis-(4-hydroxy-3-methoxyphenyl)-3:7-dioxyabicyclo[3.3.0]octane, [(+)-pinoresinol] (960 mg).

A solid (60 mg) was sepd directly by initial filtration from the fr. eluted with CH_2Cl_2–MeOH (99:1). This material was identified as a mixt. of two ceramides: *N*-(2′-D-hydroxynonacosanoyl)-D-*erythro*-1,3,4-trihydroxy-2-amino-octadecane (**1**) and *N*-(2′-D-hydroxytriacontanoyl)-D-*erythro*-1,3,4-trihydroxy-2-amino-octadecane (**2**).

The previously known compounds [episteryl acetate, ergosterol peroxide and (+)-pinoresinol] were identified from their physical (mp, $[\alpha]_D$) and spectroscopic (IR, ^1H NMR, EIMS) data as compared with those already published for these compounds [6–8].

Ceramides (**1**) *and* (**2**). Aggregate, mp 145° (MeOH); $[\alpha]_D^{20}$ +11.5° (pyridine; *c* 1.128). IR ν_{max}^{KBr} cm^{-1}: 3340 (OH), 3220 (OH), 2920, 2850, 1620 (amide). ^1H NMR: Table 1. ^{13}C NMR: Table 2. EIMS (TMSi derivative) (direct inlet) 70 eV, *m/z* (rel. int.): $[M]^+$ absent, 412(2), 372(2), 335(1), 299(13), 245(3), 218(17), 217(15), 205(18), 191(6), 147(19), 129(19), 103(27), 93(33), 88(21), 81(12), 77(13), 75(26), 73(100), 69(16), 57(29), 55(18), 44(34). Positive FAB-MS: *m/z* 767 $[M]^+$, 753 $[M']^+$.

Ceramide tetraacetates. The ceramides were acetylated with Ac_2O–pyridine at room temp. for 48 hr. After work-up the ceramide tetraacetate obtained was an aggregate, mp 53–54° (MeOH); $[\alpha]_D^{20}$ +19.2° ($CHCl_3$; *c* 0.729). IR ν_{max}^{KBr} cm^{-1}: 2940, 2870, 1740 (C=O), 1660 (amide), 1255. ^1H NMR: Table 1. EI-MS (direct inlet) 70 eV, *m/z* (rel. int.): $[M]^+$ absent, 792(5), 733(4), 712(11), 625(12), 612(11), 544(33), 543(100), 510(21), 464(35), 451(33), 450(87), 440(22), 422(18), 390(11), 310(22), 265(25), 264(52), 167(11), 153(14).

Basic hydrolysis of ceramides (**1**) *and* (**2**). A soln (9 ml) of 1 N KOH in MeOH–H_2O (9:1) was added to the ceramide mixt. (9.2 mg), and the reaction was maintained at 80° under Ar. After 36 hr the mixt. was

diluted with H_2O and extracted with CH_2Cl_2. The aq. phase was then acidified with 2% H_2SO_4 and extracted with CH_2Cl_2. Both organic phases were dried with Na_2SO_4 and the solvents evapd at red. pres. The residue obtained from the first organic extract was acetylated with Ac_2O–pyridine to yield the tetra-acetylphytosphingosine (**3**) $[\alpha]_D^{20}$ +26.2° ($CHCl_3$; *c* 0.367) $[\alpha]_D^{20}$ +23.1° ($CHCl_3$; *c* 1.236) [13]. ^1H NMR: Table 1.

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