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Biotransformation of spirolaxine by *Absidia cuneospora* and *Trametes hirsuta*: Formation of β-glycosyl and β-xylosyl derivatives

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ABSTRACT

Incubation of spirolaxine **1a** with the fungus *Absidia cuneospora* afforded 5β-glucosyl-spirolaxine **2b**; the structure of the biotransformed product was deduced on the basis of MS, NMR data and chemical semi-synthesis. When using *Trametes hirsuta* as a bioagent **1a** afforded 5β-xylosyl-spirolaxine **3a**; the identification of the sugar moiety came from acidic hydrolysis of the new metabolite.

1. Introduction

(+)-Spirolaxine (**1a**) and (+)-sporotricale represent the major metabolites extracted from cultures of the white rot fungus *Sporotrichum laxum* (Basidiomycetae), now *Phanerochaete pruinosum*; they are structurally similar, both containing a 5-hydroxy-3-methoxyphthalide moiety linked through a polymethylene chain to a spiroacetal group in compound **1a** and to a hemiacetal group in sporotricale [1].

1a has been reported to possess cholesterol lowering activity [2]. More recently, we reported its activity on some endothelial cells and on a variety of tumor cell lines (e.g. LoVo and HL60) [3]. The interest of such type of compounds prompted us to determine the stereochemistry of the four stereocentres (all *R*) of **1a** on the basis of an X-ray crystallographic analysis and of a series of CD experiments [4].

Very recently, synthetic approaches to the 5-*O*-methylether of (+)-**1a** [5,6] and to the 5-*O*-methylether of sporotricale [7] were described. **1a** belongs to a small group of fungal metabolites, produced by *Phanerochaete* sp., that have received attention for their inhibitory activity against the micro-aerophilic Gram-negative bacterium *Helicobacter pylori* [8], where compound **1a** exhibited the best activity. The peculiarity of all these derivatives was the selectivity, in fact when tested against a panel of other microorganisms they did not show antibacterial activities [8].

Successively we have investigated the ability of some microorganisms to accomplish structural modifications of the spirolaxine ring and studied the biological activity of the modified compounds with respect to the parent compound.

Biotransformation of compound **1a** with *Bacillus megaterium* afforded three new metabolites: 7′R-hydroxyspirolaxine (**1b**), 8′S-hydroxyspirolaxine (**1c**) and 7′S, 8′R-dihydroxyspirolaxine (**1d**) (see Fig. 1), all hydroxylated in the six-membered ring, while with Cunninghamella echinulata **1a** produced the 12′R-hydroxy derivative (**1e**) in the five-membered ring; the biological activity of the metabolite **1b** was studied with respect to the parent compound [9].

The present paper deals with the isolation and the structural identification of two new metabolites, (**2b**) and (**3a**), obtained by incubation of **1a** with *Absidia cuneospora* and *Trametes hirsuta*, respectively.

2. Experimental

2.1. General experimental procedures

UV spectra were measured for solutions in 95% EtOH. Mass spectra were obtained with a Bruker Esquire 3000 spectrometer. The proton and carbon NMR spectra were carried out on a Bruker D*MX-500* instrument at the temperature of 23 °C in acetone-d_6. The assignment of the proton signals is based on the chemical shift correlation experiments (COSY) while the carbon nuclei were assigned from the heteronuclear correlation experiments via one-bond (HSQC) and long-range (HMBC) coupling constants. HPLC

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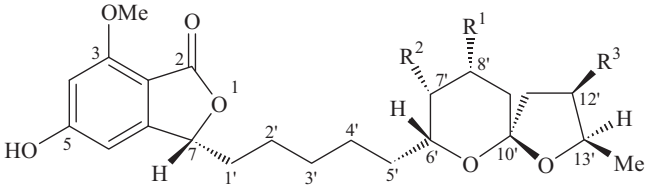


1a $R^1 = R^2 = R^3 = H$

1b $R^1 = OH;\ R^2 = R^3 = H$

1c $R^1 = H;\ R^2 = OH;\ R^3 = H$

1d $R^1 = R^2 = OH;\ R^3 = H$

1e $R^1 = R^2 = H;\ R^3 = OH$

Fig. 1. Hydroxy derivatives of spirolaxine **(1a)** by cultures of *Bacillus megaterium* and *Cunninghamella echinulata*.

analyses were performed using a LiChroCART column RP-18 250-4 (Merck) on an Agilent 1100 instrument; mobile phase: H_2O/MeCN (60:40); flow rate = 0.5 ml min^{-1}; isocratic. Flash CC was performed on Merck silica gel; TLC and PTLC with Merck HF_{254} silica gel. The purity of products was checked by TLC, NMR and MS and deemed sufficient for the purpose of structural determination.

2.2. Culture and screening procedures

Cultures were grown according to the standard two-stage fermentation protocol: stage I of fermentation was used for the growth of microorganisms, while the stage II was used for the biotransformation of **1a**.

Screening experiments of biotransformation were performed in conical Erlenmeyer flasks (300 ml) containing 100 ml (fungi) of sterile medium CSB (per litre): corn steep 10 g, glucose 30 g, $NaNO_3$ 2 g, K_2HPO_4 2 g, KH_2PO_4 1 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4{\cdot}7H_2O$ 0.02 g or 50 ml (bacteria) of sterile medium YMP2 (per litre): yeast extract 3 g, malt extract 2 g, peptone 10 g, K_2HPO_4 2 g, KH_2PO_4 1 g. Flasks were incubated at 28° on a rotary shaker at 180 rpm for growth of microorganisms, then to 250 rpm during the biotransformation. Stage I cultures in medium MPGGlB (per litre): malt extract 20 g, peptone 5 g, glucose 20 g, glycerol 8 ml, were inoculated with a water–spore suspension obtained from freshly grown agar slants. Flasks were incubated at 24 °C (fungi) or 28 °C (bacteria) on a rotary shaker at 180 rpm. After 48–72 h (ascomycetes) or 96–144 h (basidiomycetes), a 2–4% inoculum from stage I cultures was used to initiate stage II cultures, which were incubated for 48–96 h (180 rpm) before receiving 5 mg of substrates for flask, dissolved in 50 μl of DMSO and incubated at 28 °C (250 rpm). Culture controls consisted of fermentation blanks in which microorganisms were grown under identical conditions but without substrate. Substrate controls consisted of sterile medium containing the same amount of substrate and incubated under the same conditions. The fermentations were sampled periodically by TLC analysis, with pure samples as reference. Metabolite **2b** was a metabolite produced by fermentations of *A. cuneospora* ATCC 24693, while metabolite **3a** was produced by *T. hirsuta* sp. ICRM 197 (Istituto di Chimica del Riconoscimento Molecolare, Milano, Italy), using **1a** as a substrate.

2.3. Preparative biotransformation of *1a* to metabolite *2b* by A. cuneospora

48–72 h-old stage I cultures in medium MPGGlB, were transferred in thirty 300 ml Erlenmeyer flasks containing each 100 ml of CSB liquid medium. The flasks were incubated at 28 °C on an orbital shaker (180 rpm). After 48–72 h, a total of 300 mg of **1a**, dissolved in DMSO (3 ml), was evenly distributed among 30 flasks containing stage II cultures (final concentration 0.1 g/l for flask). After 8–10 days (250 rpm), the cultures were harvested, extracted two times with equal volumes of AcOEt/MeOH (100:1) and the organic phase was evaporated to dryness under reduced pressure. The crude residue (340 mg) was purified by column chromatography on silica gel with a stepwise elution with CH_2Cl_2/MeOH from 30:1 to 10:1. The fraction containing compound **2b** (160 mg) was further purified by preparative plates (PTLC) using CH_2Cl_2/i-Pr_2O/i-PrOH = 1:1:1 as eluent, to afford 96 mg (32% yield) of the metabolite.

2.4. Preparative biotransformation of *1a* to metabolite *3a* by T. hirsuta

The strain of *T. hirsuta* was grown in 300 ml Erlenmeyer flasks containing each 100 ml of MPGGlB liquid medium. The flasks were incubated at 24 °C on an orbital shaker (180 rpm). After 96 h, 4% inoculum stage I culture was transferred in forty-five 300 ml flasks containing 100 ml of the CSB medium. Stage II cultures were incubated at 28 °C on an orbital shaker (180 rpm) for 96 h before receiving the substrate. **1a** (675 mg), dissolved in DMSO (6.75 ml), was evenly distributed among 45 flasks containing stage II cultures (final concentration 0.15 g/l). After ten days of fermentation (250 rpm) the cultures were harvested and filtered although the substrate was not completely consumed. The filtrate was subsequently extracted two times with equal volumes of AcOEt/MeOH (100:1) and evaporated to dryness under reduced pressure, to give 1.5 g of a brown oil. The residue was purified by column chromatography on silica gel with an stepwise elution with CH_2Cl_2/i-PrOH from 30:1 to 3:1 to afford 160 mg of **1a** (spirolaxine not consumed) and 360 mg of the metabolite **3a** (70%-yield calculated on the reacted substrate).

2.5. Isolation and purification of *1a*

The strain of *Sporotrichum laxum* CBS 578.63 [1] now *Phanerochaete pruinosum*, was inoculated in 40 Roux flasks containing RA (ground rice-agar, 90:20 g/l). After 30 days at 24 °C, the flasks were extracted twice with EtOAc containing 1% of MeOH. The crude extracts were washed with hexane to obtain 8 g of the residue; the mixture of metabolites, **1a**, sporotricale [1] and phanerosporic acid [1] were chromatographed on a column of flash silica gel filled with hexane–EtOAc (1:1) and using a mixture of hexane–EtOAc–MeOH as eluant; with hexane–EtOAc (1:2) was eluted sporotricale (250 mg) and with EtOAc–MeOH (2:1) a mixture of **1a** with phanerosporic acid. The fractions containing the last two metabolites were successively purified through a column filled with CH_2Cl_2–MeOH (9:1) to obtain pure **1a** (350 mg) that was crystallized from CH_2Cl_2 as a pale yellow solid.

2.6. Natural metabolite *2b*

Compound **2b** was isolated as an oil, $[\alpha]_D$ = +35.6 (*c* 0.05, MeOH); UV: 215, 252 and 293 nm (ε 48,610, 14,850 and 8570 l mol^{-1} cm^{-1}); IR (nujol) ν_{max} cm^{-1}: 3380, 1758, 1618 and 1456; EIMS *m*/*z* 567 $[MH]^+$; and was identified by NMR data and TLC as the compound obtained by synthesis (see point 2.8).

2.7. 5-β-Glucosylspirolaxine tetraacetate *2a*

1a (0.7 g; 1.7 mmol) was dissolved in dry CH_2Cl_2 (20 ml) and reacted with 2,3,4,6-tetra-O-acetyl-α-D-gluco-pyranosyl bromide (1.4 g), K_2CO_3 (1.2 g) benzyltributyl-ammonium chloride ($BzNBu_3Cl$) (0.1 g). The reaction was stirred at RT for about 15 h

Table 1
^{1}H and ^{13}C NMR data for compounds **2b** and **3a** in acetone-d_6.[a]

Proton[b]	**2b** (δ_H, J in Hz)	**3a** (δ_H, J in Hz)	Carbon[c]	**2b** (δ_C)	**3a** (δ_C)
H-4	6.75 *d* (1.8)	6.68 *d* (1.8)	C-2	167.7	167.7
H-6	6.79 *dd* (1.8, 0.9)	6.80 *dd* (1.8, 0.9)	C-2a	106.6	106.6
H-7	5.35 *dd* (7.7, 3.9)	5.33 *dd* (7.8, 4.0)	C-3	160.4	160.4
OCH3	3.90 *s*	3.92 *s*	C-4	101.2	101.4
			C-5	165.3	165.3
H-1′a	2.05–1.99 *m*	2.04–1.98 *m*	C6	102.1	101.8
H-1′b	1.69–1.62 *m*	1.73–1.66 *m*	C-6a	155.7	155.9
H-2′,3′,4′,5′	1.44–1.27 *m*	1.47–1.29 *m*	C-7	80.2	80.2
H-6′	3.68–3.64 *m*	3.72–3.66 *m*	OCH_3	56.3	56.3
H-7′a	1.52 *m*	1.52, *m*			
H-7′b	1.11 *m*	1.11, *m*	C-1′[d]	35.2	35.5
H-8′a	1.80 *m*	1.80 *m*	C-2′[d]	29.9	31.1
H-8′b	1.60 *m*	1.60 *m*	C-3′[d]	26.0	26.6
H-9′a + H-9′b	ca. 1.60 *m*	ca. 1.60 *m*	C-4′[d]	25.2	25.5
H-11′a	1.79 *ddd* (12.5, 9.1, 4.9)	1.80 *ddd* (12.6, 8.8, 5.1)	C-5′[d]	36.6	36.9
H-11′b	1.69 *ddd* (12.5, 10.2, 7.3)	1.60 *ddd* (12.6, 10.4, 7.2)	C-6′	70.6	70.5
H-12′a	2.05 *ddt* (11.9, 8.9, 7.2)	2.08 *ddt* (11.9, 8.9, 7.1)	C-7′	31.5	31.9
H-12′b	1.34 *dddd* (11.9, 10.5, 6.9, 5.1)	1.36 *dddd* (11.9, 10.5, 7.0, 5.1)	C-8′	20.9	21.2
H-13′	4.09 *sext.* (6.3)	4.11 *sext.* (6.3)	C-9′[d]	34.1	34.5
CH3-13′	1.13 *d* (6.2)	1.15 *d* (6.2)	C-10′	108.4	108.6
			C-11′	38.4	38.7
H-1″	5.09, *d* (7.5)	5.14, *d* (6.7)	C-12′	31.9	32.2
H-2″	3.50, *dd* (9.0, 7.5)	3.54–3.49 *m*	C-13′	74.4	74.3
H-3″	3.54, *t* (8.8)	3.54–3.49 *m*	CH_3-13′	21.4	21.6
H-4″	3.40, *dd* (9.8, 8.8)	3.60–3.96, *m*			
H-5″a	3.58, *ddd* (9.8, 6.3, 2.3)	3.48, *dd* (11.4, 9.6)	C-1″	101.6	102.1
H-5″b	-	3.95, *dd* (11.4, 4.9)	C-2″	74.1	74.2
H-6″a	3.64, *dd* (11.9, 6.3)	–	C-3″	77.4	77.4
H-6″b	3.89, *dd* (11.9, 2.3)	–	C-4″	70.9	70.6
			C-5″	77.8	66.6
OH[e]	4.63, *s br*	4.65, *s br*	C-6″	70.6	–
OH[e]	4.40, *s br*	4.37, *s br*			
OH[e]	4.34, *s br*	4.33, *s br*			
OH[e]	3.76, *s br*				

[a] ^{1}H NMR at 500 MHz and ^{13}C NMR at 125 MHz.
[b] Assignments are based on homonuclear COSY and NOESY experiments.
[c] Assignments are based on heteronuclear HSQC and HMBC experiments.
[d] Assignment may be interchanged.
[e] Not assigned.

until **1a** disappeared; the formation of the derivative **2a** was monitored on TLC with CH_2Cl_2–MeOH (50:1) as an eluent. The reaction mixture was neutralized with 10% HCl and the organic layer was separated and washed with water satd. with $NaHCO_3$, dried over Na_2SO_4 and evaporated. Oil (1.7 g, 85%), EIMS *m*/*z* 735 $[MH]^+$. ^{1}H NMR ($CDCl_3$), δ: 6.52 (2H, *s*, H-4 and -6), 5.4–5.1 (5H, *m*, H-7, -1″, -2″, 3″ and -4″), 4.28 and 4.20 (2H, *m*, H_2-7″), 4.15 (1H, *m*, H-13′), 3.96 (3H, *s*, 3-OMe), 3.95 (1H, *m*, H-5″), 3.68 (1H, *m*, H-6′), 2.10, 2.08, 2.07 and 2.06 (12H, *s*, 4×OAc), 2.1–1.0 (20H, *m*, H_2-1′, -2′, -3′, -4′, -5′, -7′, -8′, -9′, -11′ and -13′), 1.23 (3H, *d*, *J* = 6.2 Hz, H_3-14′).

2.8. 5-β-Glucosylspirolaxine **2b**

Compound **2a** (300 mg) was dissolved in MeOH (10 ml) and MeONa (400 mg) was added at RT with stirring; after 10 h, the mixture was concentrated and 100 ml of H_2O were added. **2b** was extracted with EtOAc and the residue was chromatographed on flash conditions on a SiO_2 column filled with EtOAc; with EtOAc–MeOH (70:30), 250 mg (yield, 85% from **2a**) of compound **2b** were eluted, oil, EIMS *m*/*z* 567 $[MH]^+$ (5%), 405 (100), 163 (15) and 145 (30). HPLC: MeCN-H_2O (60:40); flow rate = 0.5 ml min^{-1}; retention time = 5.67. ^{1}H and ^{13}C NMR data are reported in Table 1.

2.9. Compounds **3a** and **3b**

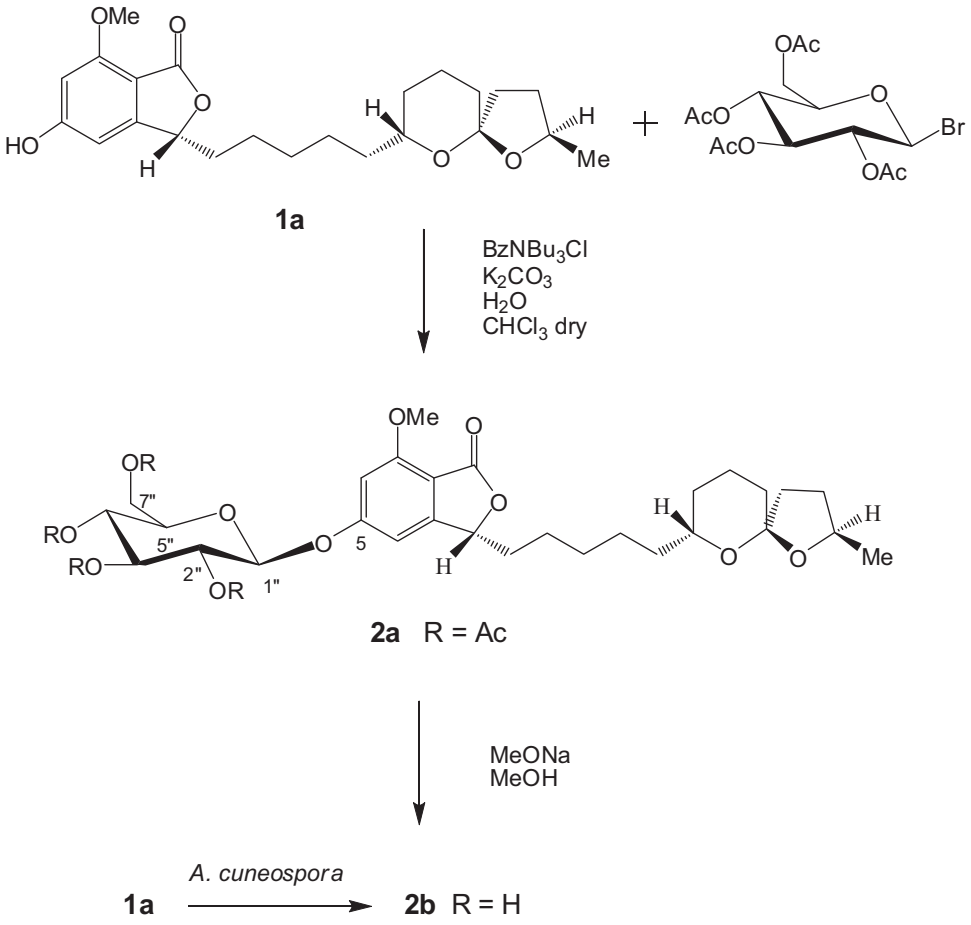
Compound **3a** was isolated as an oil, $[\alpha]_D$ = +10.4 (*c* 0.01, MeOH); UV: 215, 250 and 294 nm (ε 47,800, 14,240 and 8550 l mol^{-1} cm^{-1}); IR (nujol) ν_{max} cm^{-1}: 3393, 2942, 1762, 1618 and 1456; ESIMS, *m*/*z* 559 $[M+Na]^+$, 537 $[MH]^+$; ^{1}H and ^{13}C NMR spectra are in Table 1; the metabolite was successively acetylated (py/AC_2O) to give the triacetylderivative **3b**; oil, ESIMS, *m*/*z* 663 $[MH]^+$ and 685 $[M+Na]^+$; ^{1}H NMR ($CDCl_3$), δ: 6.53 (1H, *dd*, *J* = 1.8, 0.8 Hz), 6.52 (1H, *d*, *J* = 1.8 Hz), 5.33 (1H, *d*, *J* = 5.2 Hz), 5.28 (1H, *dd*, *J* = 8.0, 3.8 Hz), 5.24 (1H, *t*, *J* = 7.2 Hz), 5.18 (1H, *dd*, *J* = 7.3, 5.3 Hz), 5.01 (1H, *td*, *J* = 6.6, 4.4 Hz), 4.25 (1H, *dd*, *J* = 12.3, 4.3 Hz), 4.14 (1H, *sext.*, *J* = 6.2 Hz), 3.95 (3H, *s*), 3.69 (1H, *m*), 3.64 (1H, *dd*, *J* = 12.3, 6.4 Hz), 2.13 (3H, *s*), 2.109 (3H, *s*), 2.105 (3H, *s*), 2.101 (3H, *s*), 1.99–1.25 (19H, *m*), 1.22 (3H, *d*, *J* = 6.2 Hz), 1.14 (1H, *m*).

2.10. Hydrolysis of compound **3a**

3a (100 mg) was dissolved in MeCN (8 ml) and trifluoroacetic acid (TFA, 1 ml) was added; after 1 h at RT, the solvent was removed and PTLC of the residue in CH_2Cl_2:MeOH (95:1) gave **1a** and compound **4**; **4** was acetylated (py/AcO_2) to obtain **5** which is composed of a mixture ca. 4:6 of α-D-xylose and β-D-xylose tetraacetates. ^{1}H NMR (α-D-xylose tetraacetate, $CDCl_3$), δ: 6.26 (1H, *d*, *J* = 3.6 Hz), 5.46 (1H, *t*, *J* = 9.8 Hz), 5.05–4.96 (2H, *m*), 3.93 (1H, *dd*, *J* = 11.2 and 5.9 Hz), 3.71 (1H, *t*, *J* = 11.0 Hz), 2.17 (3H, *s*), 2.05 (3H, *s*), 2.04 (3H, *s*), 2.02 (3H, *s*). ^{1}H NMR (β-D-xylose tetraacetate, $CDCl_3$), δ: 5.72 (1H, *d*, *J* = 6.8 Hz), 5.21 (1H, *t*, *J* = 8.1 Hz), 5.05–4.96 (2H, *m*), 4.15 (1H, *dd*, *J* = 12.0 and 5.0 Hz), 3.53 (1H, *dd*, *J* = 12.0 and 8.3 Hz), 2.10 (3H, *s*), 2.06 (3H, *s*), 2.05 (6H, *s*).

2.11. Acetylation of D-(+)-xylose

A sample (10 mg) of D-(+)-xylose was acetylated in the same conditions as above, and the tetraacetylxylose isolated,



Scheme 1.

corresponds to the acetyl derivative of the product deriving from hydrolysis of **3a** (β-D-xylose tetraacetate; ^{1}H NMR spectra, see above).

3. Results and discussion

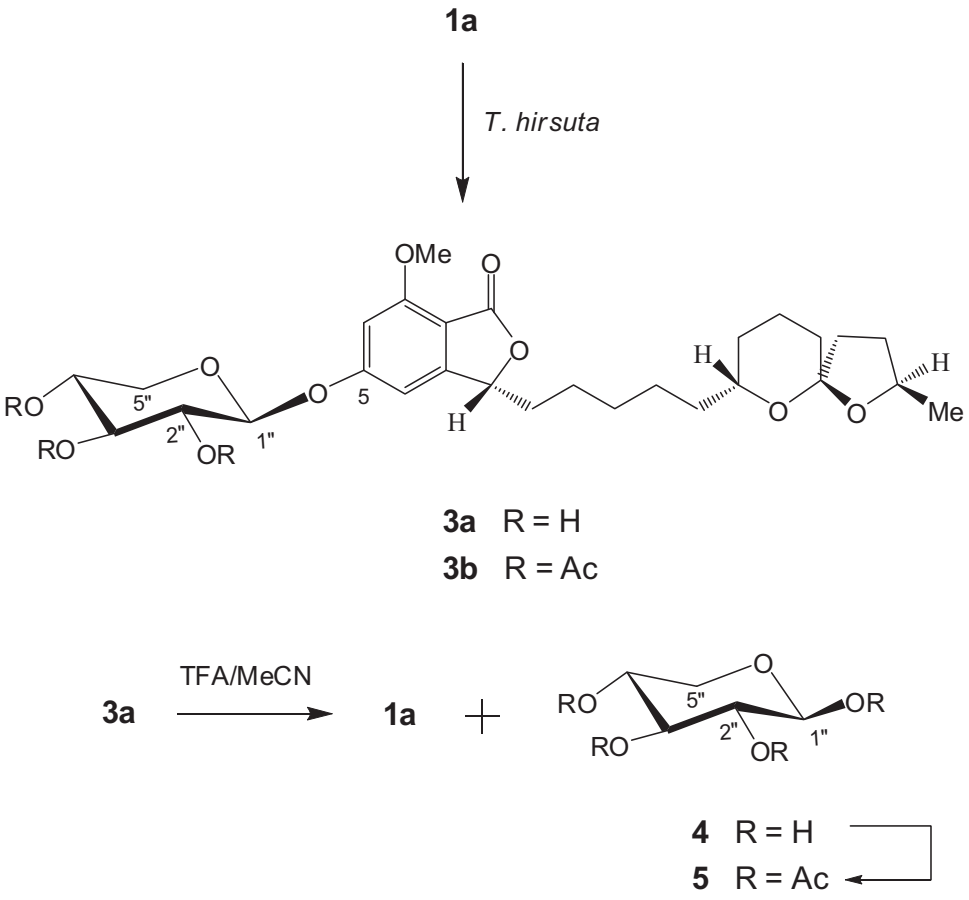
Incubation of **1a** with *A. cuneospora* yielded a new metabolite **2b** as an oil; it revealed an $[MH]^+$ ion peak at *m*/*z* 567, suggesting that the compound contains an additional glycosidic moiety in five position, confirmed by synthesis. The ^{1}H NMR spectrum of **2b**, besides the signals of a **1a** moiety, showed a group of resonances characteristic of a glucopyranosyl residue. Diagnostic are the values of the ring vicinal coupling constants which fall in the range 7.5–9.8 Hz (Table 1), typical for the *trans* diaxial arrangement of the protons of a glucopyranoside ring. The structure was subsequently confirmed by synthesis from **1a** and 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide using a solid–liquid phase transfer and $BzNBu_3Cl$ as catalyst [10] (see Scheme 1) to obtain compound **2a** that was successively deacetylated to **2b**.

When **1a** was biotransformed with *Tramentes hirsuta* as a bioagent, **3a** was obtained in high yield (see Scheme 2); positive-ion ESIMS data showed a $[M+Na]^+$ ion peak at *m*/*z* 559, suggesting that **3a** is larger than **1a** by a pentose unit. The presence of such unit was confirmed by the NMR spectra (Table 1). Unfortunately the proton spectrum showed extensive signal overlapping, thus precluding the structural assignment. Instead, comparison of the carbon spectrum with the literature data suggested that the glycosyl residue is a β-xylopyranoside ring. The glycosidic bond between the sugar moiety and the spirolaxine residue of **3a** occurs at carbon C-5 of the aromatic ring of spirolaxine as indicated by the correlation observed between the anomeric proton H-1″ (5.14 ppm) and C-5 (165.3 ppm) in the HMBC spectrum. The same spectrum allowed to uniquely identify C-5 and in particular to distinguish it from C-3 because C-5 shows correlations with protons H-4 and H-6 in addition to that with H-1″ while C-3 shows correlations with H-4 and the OCH_3 group.

The structural assignment of the sugar moiety was confirmed performing an acidic hydrolysis with TFA of the metabolite, which afforded spirolaxine **1a** and D-(+)-xylose **4**. Compound **4** was converted to the tetraacetyl derivative **5**, that appeared identical to the tetraacetyl derivative obtained from a sample of authentic D-(+)-xylose.

From a biological point of view, compound **2b** was inactive in cytotoxicity test towards bovine microvascular endothelial cells (BMEC) [IC_{50} (μM) > 200; IC_0 (μM) 25] with respect to the parent compound **1a** [IC_{50} (μM) 51; IC_0 (μM) 0.4]. In a CAM assay, widely used as a model to examine antiproliferative activity, compound **2b** inhibited like **1a**, angiogenesis induced by growth factors such as bFGF (basic fibroplast growth factor) [9,11].

Biotransformation continues to be an important tool in the structural modification of bioactive compounds [12]; there has been increasing interest in aromatic O-glycosylation reactions in recent years. However, glycosylation of phenols is still hard for traditional synthesis to achieve due to the electron-withdrawing properties of aromatic rings [13]. Although microbial O-glycosylation of phenols is well documented [14], in the literature we find a biotransformation of paenol into both glucopyranoside and xylopyranoside derivatives by cell cultures of *Panax ginseng* [15], it has been demonstrated that the fungi of the genus *Absidia* and *Trametes* are capable to perform efficient reactions of



Scheme 2.

hydroxylation, oxidation and reduction on specific sites and rearrangement reactions of taxoids [16,17] but to our knowledge, this is the first example of the formation of sugar derivatives of a complex phenol by the two bioagents *A. cuneospora* and *T. hirsuta*.

4. Conclusions

In summary, biotransformation of **1a** by *A. cuneospora* and *T. hirsuta* was studied; both microorganisms were shown to transform **1a** into its 5-O-β-D-glucopyranoside **2b** and 5-O-β-D-xylopyranoside **3a**, respectively; compound **2b** and **3a** are new glycosides. Compound **2b** demonstrated a good antiangiogenic activity.

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