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Purification of Antibiotics Produced by *Lentinus squarrosulus* and Preliminary Characterization of a Compound Active Against *Rigidoporus lignosus*

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Abstract. Purification and study of the antibiotic substances produced by *Lentinus squarrosulus* have been carried out. The substances, excreted in the culture medium, were extracted with *n*-butanol. The butanolic extract inhibited growth of *Rigidoporus lignosus*, the agent of white rot of *Hevea brasiliensis*, and also of *Mucor ramannianus*, *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*, and *Bacillus subtilis*. The antibiotic fractions were purified by silicic acid column chromatography, then by reversed-phase (C18) high performance liquid chromatography (HPLC) followed by preparative adsorption thin-layer chromatography on silica gel. Two purified compounds were obtained: Ls1, which was active against *B. subtilis*, and Ls2, which in addition was also active against *R. lignosus*, *M. ramannianus*, and yeasts. Only the Ls2 compound is analyzed in this work. It was characterized by chemical reactions, ultraviolet spectroscopy, infrared spectroscopy, and proton nuclear magnetic resonance spectroscopy, which indicated the hydrophilic character of the molecule and the presence of alcoholic functions as well as a glycosidic moiety. The properties differed from those of already known antibiotics produced by different species of *Lentinus*.

Lentinus squarrosulus Mont. [*Pleurotus squarrosulus* (Mont.) Singer] has already been studied as a potential antagonist against *Rigidoporus lignosus* [15], which attacks the roots of many tropical trees, causing serious damage especially in rubber plantations (*Hevea brasiliensis*) [4, 6]. Attempts have been made to control this disease with fungicides such as Tridemorphe [18], PCNB, sulfur, and an antibiotic cycloheximide [3], but repeated applications of the chemicals are necessary with the concomitant economic cost and environmental impact. Biological control of plant pathogens by antagonistic microorganisms offers an attractive alternative to existing pest management methods. Preliminary work has shown that *L. squarrosulus*, a non-pathogenic lignolytic Basidiomycete, produces antibiotic compounds active against *R. lignosus* [15].

The possible role of fungal antibiotic molecules in disease suppression has been shown to be of considerable importance, and the screening of mycelial cultures from Basidiomycetes has led to the discovery of a great number of metabolites [14]. Recently discovered molecules active against phytopathogenic fungi include Mycenon isolated from *Mycena sp.* with activity against *Nematospora coryli* [7] and Oudemansin X extracted from *Oudemansiella radicata*, which inhibits certain phytopathogenic fungi such as *Ascochyta pisi* and *Ustilago nuda* [2]. Strobilurin D was also recently isolated from cultures of *Cyphellopsis anomala* and is a potent inhibitor of *Curvularia lunata* and *Nematospora coryli* [19].

Several antibiotics have already been discovered from the genus *Lentinus*; *L. edodes* produces several antibiotics such as lenthionine and cortinellin, both having fairly strong inhibitory activities against a number of microorganisms including bacteria and fungi [8, 10, 11, 12]. In addition, several antitumor and antiviral substances were also produced by *L. edodes*, *L. lepideus*, and *L. trabeum* [5, 8, 9, 17].

The purpose of this paper is to report the

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extraction and purification of two antibiotics produced by *L. squarrosulus* and to characterize one of these antibiotics active against *R. lignosus*.

Materials and Methods

Fungal cultures. *Lentinus squarrosulus* Mont. strain 55A, isolated from the stumps of *Pycnanthus angolensis* in Cameroun, was obtained from the Technical Center of Tropical Forestry, Abidjan, Ivory Coast (Dr. Brunck). *Rigidoporus lignosus* (Kl.) Imazeki, strain F1, was obtained from the Rubber Research Institute, Paris (Dr. Tran Van Canh). This strain was isolated from *Hevea brasiliensis* roots in Ivory Coast. These strains were all kept at 30°C on 2% malt agar medium.

Cultures of *L. squarrosulus* and detection of antibiotics. One liter of 1.5% malt extract liquid medium was poured into a 3-L Erlenmeyer flask. The surface of the medium was inoculated with 10 discs of *L. squarrosulus* solid medium (7-mm diameter) punched out from the edge of a 7-day-old colony grown on malt agar. The fungus was grown at 30°C for different periods of time without agitation. Dry weight of the mycelium and pH values of the liquid culture were measured during the period of the experiment. Antibiotic production was tested by a growth-inhibitory test (antibiographic test) developed against *R. lignosus* in liquid culture. 240 µl of *L. squarrosulus* culture filtrate was added to 2.5-ml vials, each containing 1 ml of 1.5% malt extract liquid medium. Blank tests were carried out with culture filtrates that had not been used for growing the fungus. The media were inoculated with a 1-mm disc of agar bearing a 7-day-old colony of *R. lignosus* which floated on the surface of the medium. The colonies were grown at 30°C for 3 days. At the end of the incubation period the mycelium was collected, dried to a constant weight in a ventilated oven at 100°C, and weighed. The results are expressed as percentage of inhibition with respect to the control.

Extraction of antibiotics. After 21 days of culture at 30°C, the mycelia were removed from the culture media and discarded. The liquid media were filtered through a paper filter and extracted twice with one volume of *n*-butanol for one volume of culture filtrate. The butanol extract was dried under vacuum, with a 40°C water bath and a rotary evaporator, then redissolved in methanol. Aliquot extracts were transferred to 2.5-ml vials and dried by sterilized air to remove methanol. The residues were weighed, and the vials were filled with 1 ml of 1.5% malt liquid medium and inoculated with a 1-mm disc of *R. lignosus* agar culture which was placed on the surface of the liquid medium. Antifungal activities were determined by weighing the colonies as described above.

Thin-layer chromatography and bioautography. Thin-layer chromatography (TLC) was carried out for analytical purposes with silica gel plates (Merck 60 F 254, 0.1-mm thick, 20 × 5 cm). Butanolic extracts were deposited as spots of 5 µl, and after drying the chromatograms were developed in a butanol–acetic acid–water mixture (3:1:1, vol:vol:vol). The spots were detected by bioautography on silica gel plates seeded with *Mucor ramannianus*, *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*, or *Bacillus subtilis* and by being viewed under ultraviolet radiations at 254 nm or 366 nm. Because *R. lignosus* does not differentiate spores, bioautography directly performed on silica gels cannot be used with this fungus, and the growth-inhibitory test carried out in liquid culture was used to estimate inhibitory activities of the extracts. Silica gel was removed, dissolved in methanol, and after its elimination the residues were redissolved in malt liquid medium which was inoculated with *R. lignosus* as indicated above. For preparative purposes, butanolic extracts were separated by using larger and thicker silica gel plates: Merck, Kieselgel G Nr. 7731; 2 mm thick, 20 × 20 cm, which were used according to the same protocol.

Purification and characterization of the antibiotics. For a large-scale purification, butanolic extracts were purified by silicic acid column chromatography. The extracts were applied to a column (2 × 37 cm) filled with silica gel silicic acid (Merck 60, Nr. 9385) mixed with kieselguhr (Merck, Nr. 10601) (1:1, wt:wt) and eluted with steps of increasing concentrations of methanol in dichloromethane: 0, 5, 10, 20, 30, 100%. The active fractions detected by bioautography were further purified by two successive reversed-phase C18 HPLCs (column Deltapak Millipore-Waters, 30 cm × 7.8 mm). Elution was performed with water containing 70% methanol at a flow rate of 2 ml min^{-1}. Absorbance of the effluent was monitored at 220 nm, and active peaks were retained. This purification step was followed by two successive preparative adsorption thin-layer chromatographies on silica gel plates as described above. Two active bands were removed, extracted by methanol, dried under vacuum as described above, then redissolved in methanol. The fractions that displayed anti-*R. lignosus* activity were further characterized by chemical reactions, UV absorbance (Shimadzu UV-260 spectrophotometer) and infrared spectroscopy (Shimadzu IR-470 spectrophotometer). For this last technique, 1 mg of extract was examined in KBr, and the percentage of transmittance versus wave number (4000 – 400 cm^{-1}) was plotted. The preparation was further characterized by proton nuclear magnetic resonance (NMR) spectrometry (Bruker AM-400 spectrometer) with DMSO as solvent. The spectrum of the compound was recorded at 400 MHz. Mass spectra were measured on an LKB 2091 spectrometer by the DCI-NH_3 method.

Results

Cultures of *Lentinus squarrosulus* and production of antibiotics. Growth of the fungus, pH of the medium, and antifungal activity of the culture filtrates against *Rigidoporus lignosus* were determined for 35 days of culture. As shown in Fig. 1, during the growth period

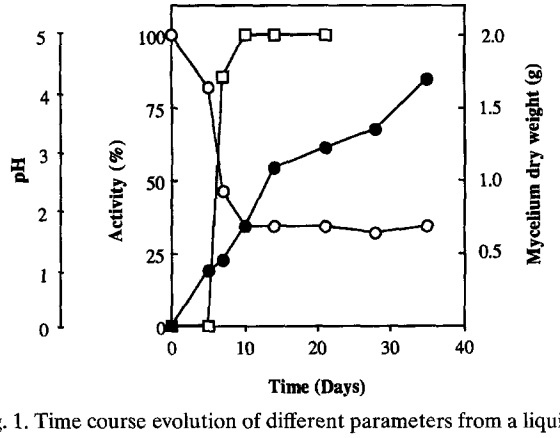

Fig. 1. Time course evolution of different parameters from a liquid culture of *Lentinus squarrosulus*. Dry weight of the mycelium (●); pH (○); liquid culture activity against *Rigidoporus lignosus* (□). Activity was determined by addition of 240 µl of culture filtrate to 1 ml of 1.5% malt liquid extract which was inoculated with *R. lignosus*, the latter being weighed after 3 days of incubation.

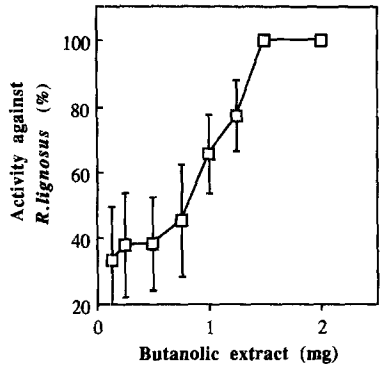

Fig. 2. Activity against *R. lignosus* of different quantities of butanolic extract. Vials containing 1 ml of 1.5% malt extract were inoculated with *R. lignosus*, which was weighed after 3 days of incubation. Values are means of five independent replicates ± S.E.M. (vertical bars).

the pH value decreased from 5.0 to 1.7. The antifungal activity was detectable after 5 days and reached a maximum after 10 days of culture under our standard conditions. For the extraction of the antibiotics, culture media were subsequently used after 21 days of incubation.

Extraction of antibiotics. The *n*-butanolic extracts inhibited growth of *R. lignosus*, causing a total inhibition when 1.5 mg of the dried extract was redissolved in 1 ml of 1.5% malt liquid medium (Fig. 2). Such a quantity of dried extract corresponded to about 500 µl of culture filtrate. The butanolic extracts also inhibited *Mucor ramannianus*, *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*, and *Bacillus subtilis* (not shown).

Detection of the antibiotics on thin-layer chromatography. On silica gel thin-layer chromatograms, butanolic extracts were separated into three bioautographic spots with different R_fs (0.75, 0.50, 0.17) which were all shown to be active against *Bacillus subtilis*. Only the upper spot with an R_f value of 0.75 was found to be significantly active against *R. lignosus* (Fig. 3). In addition, this spot was also inhibitory against *M. ramannianus* and the yeasts. Occasionally, a minor spot at R_f: 0.30 was detected and was active against *B. subtilis*. and *R. mucilaginosa* (not shown).

Purification of the antibiotics. The butanolic extract was further purified by silicic acid column chromatography. Three peaks of antibiotic activity named Ls2, Ls1, and Ls0 were detected (Fig. 4). Ls1, first characterized by thin-layer chromatography, was eluted after Ls2 in the same solvent system (dichloromethane containing 5% methanol). The Ls2 peak was active against *R. lignosus* and was further characterized by bioautography with *B. subtilis*, *M. ramannianus*, and yeasts on thin-layer chromatography and showed a single spot at R_f 0.75 (Fig. 5A). The Ls1 peak did not inhibit *R. lignosus*. Bioautography on thin-layer chromatography revealed a single spot at R_f: 0.50, which was antagonistic only to *B. subtilis*

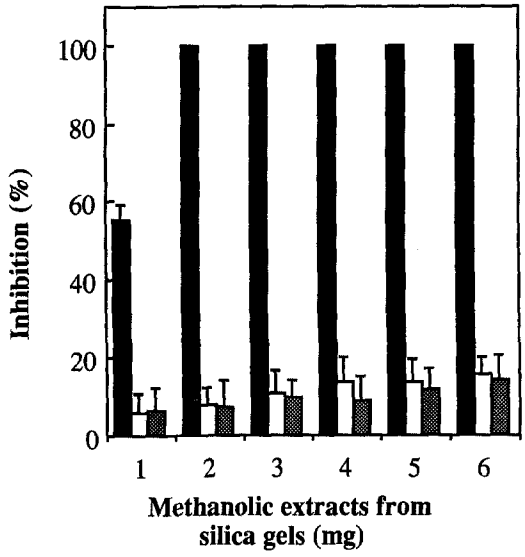

Fig. 3. Activity against *R. lignosus* of the active substance with an R_f value of 0.75, separated with preparative adsorption thin-layer chromatography (■). Other less active substances with R_f 0.50 (□) and 0.17 (▨). Silica gel parts corresponding to the different R_fs were removed and eluted in methanol. After being dried, the residues were weighed and dissolved in 1 ml of 1.5% malt liquid extract. Inhibition tests with *R. lignosus* were performed as indicated in Figs. 1 and 2. Values are means of six independent replicates ± S.E.M. (vertical bars).

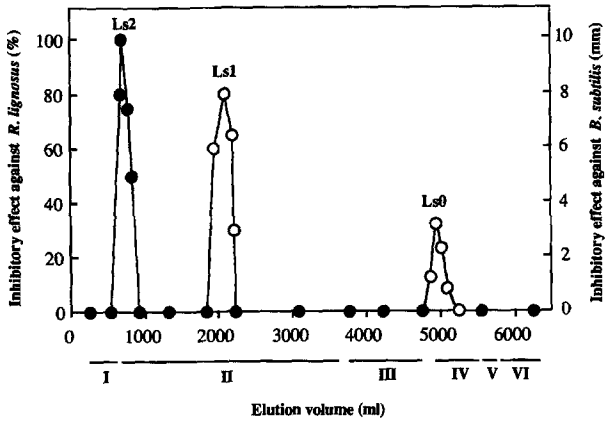

Fig. 4. Elution profile of the butanolic extract on silicic acid column chromatography. Fractions were eluted with gradient elution of methanol in dichloromethane: I. 0%, II. 5%, III. 10%, IV. 20%, V. 30%, VI. 100%. The inhibitory effect of the fractions active against *R. lignosus* (Ls2 compound) (●) were detected by the antibiographic test on liquid culture. The inhibitory effect of the fractions active against *B. subtilis* (Ls1 and Ls0 compounds) (○) were detected by measuring the diameter of the inhibition zone on silica gel plates.

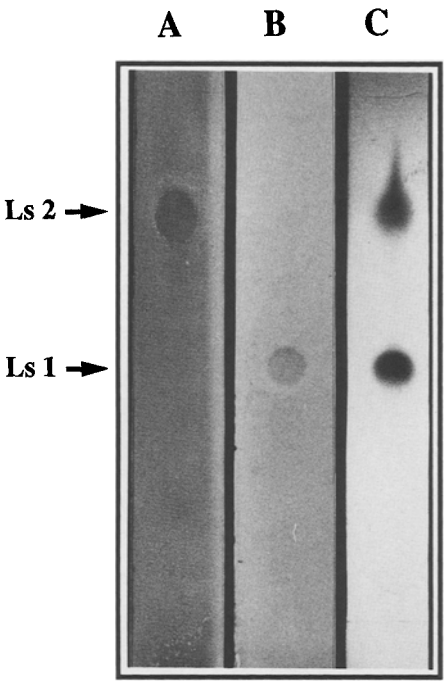

Fig. 5. Thin-layer chromatography of antibiotics Ls1 and Ls2 purified by silicic acid column chromatography. (A) Bioautography of the Ls2 compound with *B. subtilis*; (B) bioautography of the Ls1 compound with *B. subtilis*; (C) chemical revelation of a mixture of Ls1 and Ls2 with the vanillin–sulfuric acid mixture after heating at 105°C for 10 min. The antibiotics are revealed as black and violet spots respectively. Silica gels were developed with butanol–acetic acid–water, as indicated in the Materials and Methods section.

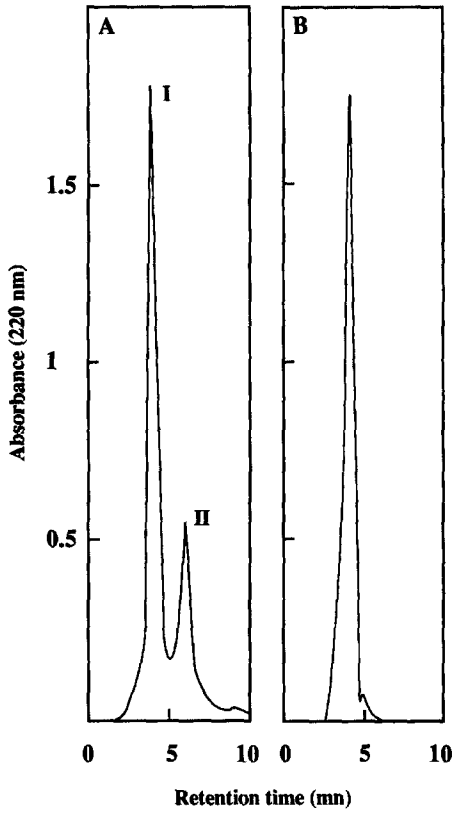

Fig. 6. HPLC elution profiles of the Ls2 compound on a reversed-phase C18 column. (A) Profile obtained with the Ls2 peak eluted from the silicic acid column. (B) Profile obtained with the peak I eluted from the C18 column and rechromatographed on the same column.

(Fig. 5B). The Ls0 peak was eluted with 20% methanol in dichloromethane (Fig. 4). On thin-layer chromatography it was revealed with *B. subtilis* as a single spot at R_f: 0.17 (not shown). This peak, less active than Ls1, was not retained for a further purification.

The spot detected in the butanolic extract at R_f: 0.30 was not found after purification on the silicic acid column.

Purification of the Ls2 compound was further performed by two successive reverse-phase (C18) HPLCs. The HPLC profile of the first chromatography showed two peaks of absorption at 220 nm, which were eluted with 70% methanol in water (Fig. 6A). Only the first peak (I) with a retention time of approximately 4.8 min was active against *R. lignosus* and also showed a fairly good activity against *B. subtilis* (not shown). On thin-layer chromatography carried out with *B. subtilis*, this peak gave rise to one spot detected at R_f: 0.75 corresponding to the Ls2 compound already shown in Fig. 5A. Peak number II did not show any antifungal activity against *R. lignosus* and was discarded for further analyses. When the pooled fractions of peak I were rechromatographed with the same solvent, a single peak was detected at a retention time of 4.8 min (Fig. 6B), which showed an anti-*R. lignosus* activity. However, chromogenic reactions performed with vanillin–sulfuric acid and anisaldehyde–sulfuric acid revealed a few impurities along the chromatogram (not shown). The Ls2 spot was consequently removed from the silica gel, redissolved in methanol, and submitted to a second thin-layer chromatography. When this spot was rechromatographed on TLC, it appeared as having been purified to homogeneity. When this Ls2 compound was mixed with the Ls1 compound (also purified with the same protocol), two spots were revealed with vanillin–sulfuric acid, which corresponded to the spots detected by bioautography (Fig. 5C).

After the last step of purification, the purified Ls2 compound was tested against *R. lignosus* by the growth-inhibitory test in liquid culture and against *R. mucilaginosa* and *B. subtilis* by bioautography. The antibiotic was found to be highly active against all three organisms. In addition, the amount of antibiotic provided by about 500 µl of culture medium used to

grow *L. squarrosulus,* caused a total inhibition of *R. lignosus* in our standard test conditions. However, the lack of a substantial amount of purified compound did not allow quantifying susceptibilities of these organisms to the pure antibiotic, and minimal inhibitory concentrations (MICs) will have to be determined in the future.

Physico-chemical properties of the antifungal compound Ls2. After the last step of purification, the Ls2 compound was found to be very soluble in DMSO, water, and methanol, weakly soluble in chloroform and dichloromethane, and insoluble in benzene and hexane.

The antibiotic gave a positive chromogenic reaction after being heated with vanillin–sulfuric acid (violet), vanillin–perchloric acid (violet), ethanolic anisaldehyde–sulfuric acid (violet), and Nelson reagent (blue). In addition, a positive chromogenic reaction was obtained after heating with anisaldehyde-sulfuric acid, suggesting the presence of glycosidic residues. No color response was found with the following reagents: ninhydrin, silver nitrate, diazoted benzidin, and ferric choride.

The UV spectrum in methanol showed a maximum absorption at 200–210 nm, and no absorption occurred above 300 nm, which demonstrates the absence of a quinone moiety.

The infrared spectrum exhibited absorption bands at 3400 (strong), 2900 (very weak), 1580 (strong), 1420 (medium), 1030 and 1010 (medium), and 660 (medium) cm^{-1}.

Proton NMR spectroscopy (Fig. 7) revealed several signals with the following chemical shifts (δ in ppm): 0.85 (weak), 1.2, 1.7, and 3.5 (strong).

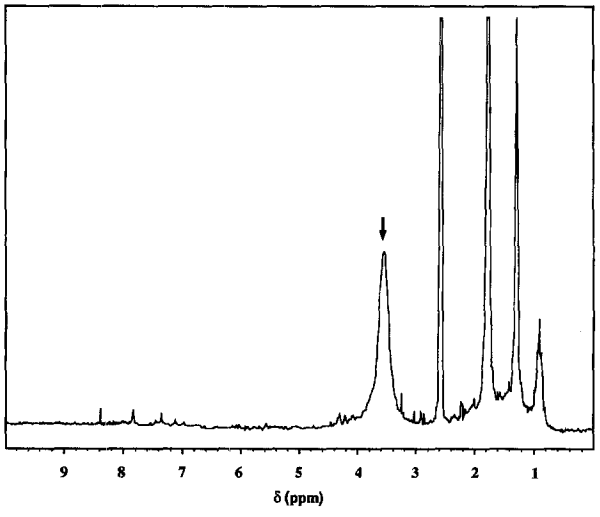

Fig. 7. NMR spectrum of the purified Ls2 compound. The molecule was dissolved in d_6-DMSO. Tetramethylsilane was used as a reference. The OH-groups are indicated by the arrow at 3.5 ppm.

Mass spectrometry yielded fragment ions of m/z: 484 (467 + 17), 467, 424 (407 + 17), and 407 by the DCI–NH_3 method.

Assignments to the presence of alcoholic functions could be made for the peaks at 3400 and 1030–1010 cm^{-1} in I.R. spectrometry and at 3.5 ppm in ^{1}H-NMR spectroscopy. The compound did not contain numerous methyl groups (weak peaks at 2900 cm^{-1} in I.R. and 0.85 ppm in ^{1}H-NMR). In addition, it did not seem to contain nonpolar carbonyl or quinone groups.

Discussion

Lentinus squarrosulus cultivated in malt extract acidified the culture medium and produced several antibiotic substances that were excreted in the culture medium. Preliminary results have shown that oxalic acid was the main organic acid detected in the culture medium (unpublished results). Acidification due to the production of oxalic acid was probably responsible for half of the inhibitory effect of the culture medium, because 240 µl of the medium led to a total inhibition of *R. lignosus* (Fig. 1), whereas 500 µl of culture medium, once extracted with butanol, was necessary to give a similar result (Fig. 2). Purification by silicic acid adsorption, reversed-phase high performance and thin layer chromatographies led to the separation of two main antibiotics named Ls1 and Ls2. The former had antibacterial but not antifungal activity, whereas the latter possessed both antibacterial and antifungal activities, especially against *R. lignosus*.

The spectroscopic characteristics of the Ls2 compound are different from those of known antibiotics biosynthetized by several *Lentinus* species. Indeed, there was no absorption at 2200 cm^{-1} in the infrared spectrum characteristic of the acetylene group (C≡C) such as in polyacetylenic antibiotics produced by *Lentinus lepideus* [1]. The strong UV absorption above 300 nm by quinone metabolites synthesized by *Lentinus degener* [13] was not detected in the UV spectrum of Ls2. The latter compound is different from the sulfur-containing metabolites produced by *Lentinus edodes*, such as lentinic acid and lenthionine (thiepane metabolite). Lentinic acid showed an absorption band in infrared spectroscopy at 1310 cm^{-1} characteristic of a sulfonyl group [16, 20]. Ls2 is also different from lenthionine, which gave a single peak at 4.3 ppm in the ^{1}H-NMR spectra (–S–CH_2–S–) [11].

Some properties of the Ls2 compound, such as

the hydrophilic character, reactions with chromogenic reactants of sugars, and spectroscopic evidence of the presence of alcoholic functions, suggest that the molecule contains a glycosidic part. Further work is in progress to verify this hypothesis and to establish the complete structure of the compound.

Preliminary attempts have been carried out over the last years in Ivory Coast to test the effectiveness of *L. squarrosulus* for controlling white rot disease in *Hevea brasiliensis* plantations (Gener P, personal communication). Hevea wood blocks, previously colonized by *L. squarrosulus*, were deposited on one side and in contact with lateral roots of 10-year-old Hevea trees. *R. lignosus* inocula were also brought as colonized wood blocks which were placed on the other side of the lateral roots. Control experiments were prepared in the same way but without *L. squarrosulus*. After 4 months of incubation, the soil was removed, and it was found that in the presence of *L. squarrosulus*, roots were not invaded by the pathogenic fungus, whereas they were colonized to a large extent in the control tests. In addition, it was noted that *L. squarrosulus* was not pathogenic to *Hevea brasiliensis* because the fungus did not spread over the lateral roots. Although biocontrol capabilities of *L. squarrosulus* seem to be obvious, it will be of value to know whether this organism is capable of being maintained in field conditions. Direct applications of the purified antibiotic can also be envisaged assuming that the molecule is not toxic for the trees and is resistant to degradation under field conditions.

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