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Syringaldazine, an Effective Reagent for Detecting Laccase and Peroxidase in Fungi

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Mycologists and plant pathologists have long suspected a definite relationship between the production of phenol-oxidizing enzymes by wood rotting fungi and the ability of these fungi to attack and modify or destroy lignin. However, all details of the manner of action of these enzymes on lignin and the degree of their implication in the overall scheme of lignocellulose biodegradation have not been clearly established. One cause of this uncertainty has been the lack of good tests to detect and differentiate the various phenol oxidases produced by fungi, and other microorganisms and plants. In syringaldazine [*N, N'-bis*-(3,5-dimethoxy-4-hydroxybenzylidene) hydrazine] we have found an excellent substrate for easy and rapid detection of laccase, or in its absence, of peroxidase. The tests we have developed with this compound for these enzymes may help to clarify connections between phenol oxidase secretion and wood decay by microorganisms and may be of value in fungus taxonomy.

Phenolases, BAVENDAMM *reaction, and wood rotting*

Since the classical studies of BAVENDAMM[1,2], agar medium with 0.5% of either tannic acid or gallic acid has been used in a diagnostic test for detecting secretion of phenol oxidases by fungi. A positive test is indicated by the formation of a brown-colored zone around the fungal mycelium on a suitable medium in petri plates. Efforts have been made to correlate phenol oxidase production reflected in a positive BAVENDAMM reaction with the type of decay associated with numerous species of fungi. Most white-rot fungi, which destroy both the polysaccharide and the lignin components of wood[3], evoked a positive reaction[4–6], whereas most brown-rot fungi, which attack mainly the carbohydrate portion of wood and merely modify its lignin[3], gave a negative test[4–6]. However, discrepancies in these general results invariably occurred that indicated some significant shortcomings of this method as a taxonomic tool or a diagnostic test for rot type. The method was therefore critically examined by LYR[7], who proposed reducing the tannic or gallic acid to 0.08–0.1%, since he and earlier workers[4] had frequently encountered strong inhibition of growth at the 0.5% concentration. Even at lower levels, some organisms still failed to grow normally; this might be due to inhibition by even the lower level of acids or by trace amounts of quinones produced from their polyphenol structures by autoxidation or by the initial secretions of phenol oxidase. Furthermore, the reduction in concentration of gallic or tannic acid does not improve the ability of the test to predict rot type.

Some confusion exists concerning the particular enzymes that are responsible for producing a positive BAVENDAMM reaction and what their significance, if any, is in lignin biodegradation. LYR[7] suggested that an extracellular laccase (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2) was solely responsible for producing a positive BAVENDAMM test. Previous workers invoked laccase, tyrosinase (*o*-diphenol:oxygen oxidoreductase, EC 1.10.3.1), or peroxidase (donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7), enzymes for which both tannic and gallic acids are suitable substrates, as participants in the reaction[4–6,8–12]. The explanation for borderline cases in the reaction – with brown-rot fungi, at least – apparently lies in the release through autolysis of normally intracellular tyrosinase, which then produces a weak brown diffusion zone on the agar media[7,13].

* Maintained at Madison in cooperation with the University of Wisconsin.

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What is understood of the relationship between phenol oxidases and lignin biodegradation has recently been summarized[3]: First, these enzymes can in theory catalyze some oxidative degradations of lignin, but whether they are a really significant or a major factor in the overall scheme of degradation is not yet clear; and second, these enzymes apparently must be only a part of the entire microbiol enzyme complex that brings about complete destruction of lignin, because other oxidases, oxygenases, and enzymes of the tricarboxylic acid cycle apparently are involved. However, that phenol oxidases are of importance in the degradative conversion of lignin to humus seems very plausible.

Search for better tests for phenolases

The confusion about the nature and the release of these phenol oxidizing enzymes and about their roles in the BAVENDAMM reaction and possibly in lignin biodegradation has prompted several workers over the years to seek substances that are more tolerable to the fungi or more specific for each oxidase or preferably both. Suitable substrates would allow the organisms to grow normally and would not induce the organisms to secrete enzymes. In addition, they would be acted on by only one of the three oxidases or, if by more than one oxidase, in a different manner by each. Thus, for example, LYR developed a test with benzidine plus ascorbic acid in acetate buffer to detect peroxidase[14]. BOIDIN[5] used guaiacol to detect laccase and tyrosine to detect tyrosinase, and later employed a number of formulae including these, guaiacol plus glycine, and *p*-cresol[15]. TICHÝ and KLABANOVÁ[16] developed a guaiacol vapor diffusion technique to suppress inhibition of the fungal growth by this phenolic substrate. SCHANEL[17] also used a mixture of hydroquinone plus glycine to detect extracellular phenolases by formation of a red quinone-amino acid adduct. JØRGENSEN and VEJLBY[18] deployed discoloration of an anthocyanin dye in red cabbage extract as an indicator of oxidases. LYR[7] and CAPELLANO and DEMOULIN[19] used *p*-cresol plus glycine to indicate tyrosinase, whereas KÄÄRIK[20] used dilute solutions of *p*-cresol and other phenols and amines to distinguish laccase and tyrosinase. Recently, HOLUBOVÁ-JECHOVÁ[21] applied a number of previous methods to try to obtain clarity, and SUNDMAN and NÄSE[22] developed a direct method in which lignosulfonates are cleared from agar plates. HINTIKKA and LAINE[23] measured the respiration of actual samples of rotting wood with gallic acid as substrate, and applied benzidine solution to the decayed wood to differentiate between white and brown rot, but they repeated warnings of the carcinogenicity of benzidine.

All of these methods are still very arduous and time consuming; results can often be assessed only after several hours or days. Moreover, in work related to studies of the phenol oxidase involved in lignification, HIGUCHI[24] measured the oxygen uptake of a large variety of phenolic substrates on manometric respirometry, and found that most of the substrates used are not specific to any one phenolase type.

Gum guaiac test

NOBLES[8] tried to overcome these drawbacks by using tincture of guaiac as an indicator for rapid detection of phenol oxidase; she believed that this reagent, which gave an almost instantaneous blue coloration with most BAVENDAMM-positive cultures, was specific for extracellular laccase. However, MAEHLY[25] had already presented a valid objection to this reagent that the extract on standing formed peroxides that render it unsuitable for differentiating between laccase and peroxidase. Some furanoid or tetrahydrofuranoid structures found in the lignan constituents of the resin[26] could well give rise to these peroxides via air oxidation. Moreover, the active principle of the resin, furoguaiacin or 2,5-di-(4-hydroxy-3-methoxyphenyl)-3,4-dimethylfuran[27,28] is also oxidized to guaiacum blue by tyrosinase[28] and many nonenzymic oxidants and catalysts[25]. We have also found that tincture of guaiac prepared from fresh resin and stored for about 1 month gives a blue color with purified horseradish peroxidase *without addition of hydrogen peroxide;* sufficient endogenous peroxide has already formed to give a strong positive test.

Possibility of wood decay without phenolases

Some work by KIRK and KELMAN[29] indicated that a few wood-destroying fungi had some ability to degrade lignin without producing phenol oxidases. However, it was later found that changes these fungi evoked in

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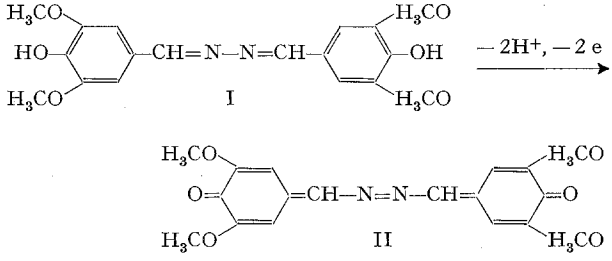
lignin models and in lignin itself could be ascribed to phenol oxidase reactions[30,31]. It therefore appears that these organisms do in fact produce the enzymes, but at a level too low for ready detection by any of the methods discussed thus far.

The foregoing, therefore, highlights the need for a more reliable, rapid method of detecting extracellular laccase. A suitable reagent should be specific, should not interfere with growth, and should not be complicated by contaminants.

Phenol oxidation of syringaldazine

We have now discovered such a reagent in syringaldazine, a pale yellow crystalline compound that can be synthesized easily in high purity from syringaldehyde and hydrazine hydrochloride[32]. (Syringaldazine is available from Aldrich Chemical Co., 940 West St. Paul Ave., Milwaukee, Wis. 53233 USA.)

Dilute (approx. 0.1%) solutions of syringaldazine (I) in ethanol, a mixture of alcohols, or dimethyl sulfoxide turn from yellow to deep purple on treatment with laccase and air or peroxidase plus hydrogen peroxide. The color change results from twofold phenol dehydrogenation of I and from intramolecular pairing of the free radicals produced to yield the highly colored ($\varepsilon \simeq 65{,}000$ at 525 nm in 50% aqueous methanol), highly conjugated tetramethoxy-azo-bis-methylene-quinone (II) shown in the following:



The color formation is rapid at all pH levels, but the color tends to fade rapidly outside the pH 3 to 7 range, presumably by addition of acid or alkali across the two quinone methide structures. The azine reacts instantly with purified laccase of *Polyporus versicolor* [= *Coriolus versicolor* (L.) Quél.][33] or with horseradish peroxidase plus H_2O_2, and produces a deep-purple solution, or, if enough reagent is used, a heavy purple precipitate. No reaction occurs with tyrosinase, or with peroxidase or with hydrogen peroxide alone; since the azine cannot give rise to peroxides, it can be used with H_2O_2 in the absence of laccase to test for peroxidase.

A sample of the purple azo-*bis*-quinone methide II was prepared by oxidizing a large volume of a saturated solution of syringaldazine in a 1:1 ethanol/water mixture with peroxidase (Type 1, from Sigma Biochemical Corp., 3500 De Kalb, St. Louis, Mo. 63118 USA) and 0.3% aqueous hydrogen peroxide; a purified laccase[33] was inactivated by this high concentration of ethanol. The purple pigment was filtered off through a fine sintered glass funnel, and purified by washing with hot ethanol. The IR-spectrum of the product showed it to be practically free from entrapped, insolubilized azine (Elemental analysis: calc. 60.3% C, 5.0% H, 7.8% N, found 59.93, 59.82% C, 5.36, 5.30% H, 6.25, 5.60% N). The electronic spectrum in methanol/water (1:1 v/v) of our preparation showed a rather sharp maximum at 525 nm ($\varepsilon \simeq 65{,}000$) and an inflexion at 457 nm ($\varepsilon \simeq 30{,}000$ (cf.[32]). The yellow azine has a broader absorption maximum at 420 nm. The IR-spectrum of II in KBr showed major bands at 1627 (vs), 1611 (s), 1592 (m), 1554 (s), 1293 (vs), 1233 (m), 1205 (m), 1176 (m), 1115 (m), and 1108 (s) cm^{-1} plus weak bands at 1464, 1424, 1338, 1020, 978, 920, 860, 815, 795. 760, 655, and 626 and 502 cm^{-1}. The azine I showed prominent absorption bands at 1625 (m), 1603 (s), 1515 (vs), 1345 (s), 1308 (m), 1278 (m), 1247 (m), 1215 (s), and 753 (m), and weak bands at 3010, 2940, 2845, 1042, 990, 955, 915, 849, 828, 645 and 618 cm^{-1}. The spectrum of the time-averaged proton magnetic resonance of II in $CDCl_3$ (Varian T-60 instrument) showed singlets for the methoxyl groups at 3.95 δ, for the benzyl protons at 8.83 δ, and for the aromatic protons at 7.15 δ. The parent azine resonated with singlets at 4.00 δ (methoxyl), 7.15 (aromatic H), 8.84 (benzyl protons), and 5.87 (hydroxyl proton, signal disappears with D_2O).

Unlike guaiac resin and its ethanolic extracts, syringaldazine is not readily oxidized by many non-enzymic reagents. Although strongly oxidized by lead dioxide and halogens, it is only weakly oxidized by activated manganese dioxide and silver oxide. Moreover, no color is formed with most heavy metals, hydrogen cyanide, urea, ammonia, methylamine, or copper sulfate or ferrous sulfate in the presence of H_2O_2, although all of these substances oxidize α-guaiaconic acid (furoguaiacin) to guaiacum blue[25].

Tests for phenol oxidases in microbial cultures with syringaldazine

To test fungal cultures, whether on slants, agar plates, or in liquid culture, minute amounts of reagent solution are added, for example, dropwise from a capillary pipette. Some organisms produce a deep-purple color almost immediately where the solution is

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applied; others form the color more slowly and less intense or not at all. The intensity of the color and the rapidity of its formation give a rough indication of the amount of laccase present. If no pink or red color develops, laccase is absent and dilute hydrogen peroxide can be added to test for the presence of peroxidase, or *fresh* tincture of guaiac – preferably of furoguaiacin[27,28] – can be applied to test for tyrosinase.

The great advantage of syringaldazine is that no inhibition of growth has been observed when it is used. With typically strong producers of extracellular laccase, such as *Coriolus versicolor* (L.) Quél., a single small drop of azine solution on the culture surface immediately evokes an extremely intense purple color. Here, and with some other fungi, the color fades within a few minutes, and the culture continues to grow. With other organisms, the color fades very slowly, but growth continues. The decoloration probably occurs via acid-catalyzed addition of water onto the two methylenequinonoid portions of the pigment to give α,α'-azo(syringyl alcohol); this diphenol may be oxidized further by the laccase and thereby decomposed. Syringaldehyde has been detected among other products on decomposition of a sample of purple pigment made with peroxidase and hydrogen peroxide under mildly acidic conditions comparable with the pH of many fungal cultures.

Thus, fungal growth is obviously not inhibited by the decolorized pigment or by any portion of the decomposed azine. The amount of reagent needed is so small that growth interference by the ethanol solvent is negligible. The same culture can be tested time and again, and will continue to grow. The reagent could therefore be useful for measuring the stages of maturity at which laccase secretion commences in some organisms.

A few organisms tested thus far for phenol oxidase secretion with the reagent are listed in Table I. An extensive testing program with fruiting bodies of mushroom samples collected in the field and with the cultures of the USDA collection of wood-rotting fungi is in progress, and results will be published.

Cultures 1–11 in Table I were grown on plates for 14 days on Difco malt agar medium, cultures 12–41 for 24 days on Fleischmann's diamalt syrup agar medium, and cultures 42–48 for 10 weeks and culture 49 for 4 weeks on malt agar slants. Several replicates of each culture were tested. Unfortunately cultures 1–11 were not tested with furoguaiacin[27,28], which would have revealed tyrosinase activity in the absence of laccase.

Most organisms that gave a positive BAVENDAMM reaction also gave a strongly positive laccase test with syringaldazine, whereas most of those that gave a negative BAVENDAMM test also gave negative tests for laccase and peroxidase (Table I). However, some exceptions were noted. No. 9, *Ganoderma applanatum* (Pers. ex Wallr.) Pat., gave only a weak laccase reaction, but a strong peroxidase and BAVENDAMM reaction. No. 42, *Fomes cajanderi* Karst., and No. 45, *Phaeolus schweinitzii* (Fr.) Pat., gave strong peroxidase reactions, but negative laccase and BAVENDAMM tests. No. 46, *Phellinus pini* (Thore ex Fr.) Pil., and one isolate of *Phlebia radiata* Fr., No. 48, gave positive BAVENDAMM tests, but negative tests for laccase. The *Phlebia* also gave a negative test for peroxidase, whereas different cultures of the same isolate of the *Phellinus* gave either positive or negative indications of peroxidase. Lastly, No. 14, *Tyromyces subcartilagineus* (Overh.) Dom., gave no BAVENDAMM reaction, but with both furoguaiacin and syringaldazine gave a strongly positive test for laccase.

Growth substrate influence on phenolase level

One further finding is of interest. The fungus *Cryptoderma yamanoi* Imaz. causes white rot of wood, but gave a negative test in culture for extracellular laccase and peroxidase with syringaldazine and for tyrosinase with furoguaiacin (Table I, Nos. 6 and 49). This organism has the ability to deplete the lignin component of wood faster than the polysaccharides[34,35]. It might therefore seem able to decompose the lignin in wood without producing extracellular phenolases (cf.[29]). However, tests with the azine and the furoguaiacin of the same isolate allowed to grow for 4 weeks on sterile blocks of Douglas-fir and Tupelo-gum wood under the conditions of the soil-block test[36] revealed a readily detectable production of laccase on this substrate. The laccase reaction was equally strong on blocks pretreated with catalase solution; this eliminates the possibility of peroxidase plus endogenous H_2O_2 as the source of the blue and purple stains obtained with furoguaiacin and syringaldazine, respectively.

Possible interference from fungal hydrogen peroxide

One reason for carrying out the pretreatment with catalase mentioned previously was that a recent report[37] indicated that many wood-destroying fungi produce low levels of extracellular hydrogen peroxide when grown on a malt extract medium with either heated sheep's blood or bovine hemoglobin powder. Even in low concentrations, these secretions of peroxide could perhaps interfere with or confound

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Table I. Fungal cultures tested on agar plates and slants for laccase and peroxidase by adding a drop of 0.1% syringaldazine in 95% ethanol with or without 0.3% hydrogen peroxide

Culture condition and organism	Type of rot (B = brown; W = white)	BAVENDAMM reaction (N = negative; P = positive)	Test with furoguaiacin[a]	Indication with syringaldazine of	
				Laccase (N = negative; P = positive)	Peroxidase
14 Days, Difco malt agar, plates					
1. *Gloeophyllum trabeum* (Pers. ex Fr.) Murr.	B	N		N	N
2. *Lentinus lepideus* Fr.	B	N		N	N
3. *Poria monticola* Murr.	B	N		N	N
4. *Bondarzewia berkeleyi* (Fr.) Bond. et Sing.	W	P		P	—[b]
5. *Coriolus versicolor* (L.) Quél.	W	P		P	—[b]
6. *Cryptoderma yamanoii* Imaz.	W	P		N	N
7. *Dichomitus squalens* (Karst.) Reid	W	P		P	P
8. *Fomitopsis ulmaria* (Sow. ex Fr.) Bond. et Sing.	W	N/P[c]		P	—[b]
9. *Ganoderma applanatum* (Pers. ex Wallr.) Pat.	W	P		weak[d]	P
10. *Gloeoporus dichrous* (Fr.) Bres.	W	N		P	—[b]
11. *Polyporus giganteus* Pers. ex Fr.	W	P		P	—[b]
24 Days, FLEISCHMANN's diamalt syrup agar, plates					
12. *Poria subvermispora* Pil.	B	P	P	P	—[b]
13. *Serpula pinastri* (Fr.) Bond.[e]	B	N	N	N	N
14. *Tyromyces subcartilagineus* (Overh.) Dom.	B	N	P	P	—[b]
15. *Aporpium caryae* (Schw.) Teix.[e]	W	P	P	P	—[b]
16. *Coriolus biformis* (Klotch) Pat.[e]	W	P	P	N	N
17. *C. biformis* (Klotch) Pat.	W	P	P	P	—[b]
18. *Fomitopsis ohiensis* (Berk.) Bond. et Sing.	W	P	P	P	—[b]
19. *F. ulmaria* (Sow. ex Fr.) Bond. et Song.	W	N/P[c]	P	P	—[b]
20. *Gloeocystidiellum lactescens* (Berk.) Boid.	W	P	P	P	—[b]
21. *Hericium coralloides* Pers. ex S.F. Gray[e]	W	P	P	P	—[b]
22. *Hyphoderma puberum* (Fr.) Wallr.[e]	W	P	P	P	—[b]
23. *Laeticorticium roseum* (Pers. ex Fr.) Donk	W	N	N	N	N
24. *Phlebia radiata* Fr.[e]	W	P	P	P	—[b]
25. *Polyporus arcularius* Batch. ex Fr.[e]	W	P	P	P	—[b]
26. *Poria alachuana* Murr.[e]	W	P	P	P	—[b]
27. *P. cinerascens* (Bres.) Sacc. et Syd.[e]	W	P	P	P	—[b]
28. *P. conferta* Overh.[e]	W	P	P	P	—[b]
29. *P. pannocincta* (Rom.) Lowe[e]	W	N	N	N	N
30. *P. sanguinolenta* (Alb. et Schw. ex Fr.) Cke.[e]	W	P	P	P	—[b]
31. *P. spissa* (Schw.) Cke.[e]	W	N	N	N	N
32. *P. stellae* Pilát	W	P	P	P	—[b]
33. *P. subincarnata* (Pk.) Murr.	W	P	P	P	—[b]
34. *P. tarda* (Berk.) Cke.[e]	W	N	N	N	N
35. *P. tenuis var. pulchella* (Schw.) Lowe[e]	W	P	P	P	—[b]
36. *Radulum casearium* (Morg.) Lloyd[e]	W	P	P	P	—[b]
37. *Spongipellus fissilis* (Berk. et Curt.) Murr.[e]	W	P	P	P	—[b]
38. *Steccherinum ciliolatum* Berk. et Curt.) Gilbertson	W	P	P	P	—[b]
39. *Trametes trogii* Berk.[e]	W	P	P	P	—[b]
40. *Trechispora candidissima* (Schw.) Bond. et Sing.[e]	W	P	P	P	—[b]
41. *Tyromyces fumidiceps* Atk.[e]	W	P	P	P	—[b]
10 Weeks, malt agar, slants					
42. *Fomes cajanderi* Karst.	B	N	P	N	P
43. *Laetiporus sulphureus* (Bull. ex Fr.) Murr.	B	N	P	N	N
44. *Odontia* sp. FP 133125-Sp	B	P	P	N	N
45. *Phaeolus schweinitzii* (Fr.) Pat.	B	N	N/P[c]	N	P
46. *Phellinus pini* (Thore ex Fr.) Pil.	W	P	P	N	N/P[c]
47. *P. pini* (Thore ex Fr.) Pil. var. *abietis* (Karst.) Pil.	W	P	P	N	N
48. *Phlebia radiata* Fr.	W	P	P	N	N
4 weeks, malt agar, slants					
49. *Cryptoderma yamanoii* Imaz.	W	P	N	N	N

[a] Furoguaiacin is the active principle of gum guaiac[28]; a positive reaction (intense royal blue formed within less than 1 min) in the absence of a positive test for laccase with syringaldazine indicates the presence of an extracellular tyrosinase. [b] Test for peroxidase is invalid in presence of laccase; most of the organisms exhibit strong catalase activity indicated by effervescence on addition of the hydrogen peroxide solution. An intensified positive reaction on addition of hydrogen peroxide could thus be ascribed to peroxidase plus a stronger laccase reaction caused by the high local oxygen concentration released from the peroxide. [c] Separate cultures of same isolate behaved differently. [d] Faint purple developed after 15 min; strong purple color formed rapidly on addition of hydrogen peroxide. [e] Two separate isolates behaved identically.

tests for laccase with syringaldazine. The H_2O_2 release is thought to occur regardless of the culture medium used. However, we did not obtain any evidence of the presence of peroxide on cultures that gave us positive tests for laccase on our media; in fact, all seemed to have extremely strong catalase activity, evidenced by immediate effervescence when dilute aqueous hydrogen peroxide was added to the surface of the cultures. Moreover, addition of a solution of catalase to any culture a few minutes before applying the azine solution did not change the outcome of the test. Consequently, the phenomenon of hydrogen peroxide production by some fungi, presumed to occur via sugar oxidases or an oxalic acid decarboxylase[37], would apparently affect long-duration tests for phenol oxidases such as the BAVENDAMM test rather than the syringaldazine test. For example, protracted release of even minute amounts of peroxide on tannic acid or gallic acid medium could perhaps evoke browning even without enzymatic intervention; thus the aim of the BAVENDAMM test would be frustrated.

However, perhaps the most conclusive argument against the possibility that fungus-secreted hydrogen peroxide could upset the test under our conditions is provided by the observation that some species gave positive tests for peroxidase when hydrogen peroxide was added after they had given negative tests for laccase before the peroxide was added (Table I, Nos. 9, 42, 45, 46). Had endogenous peroxide been present, the peroxidase would have reacted with it to give a spurious positive test for laccase.

Tests with syringaldazine for peroxide production by fungi

Tests were, nonetheless, conducted to examine whether production of peroxide by some fungi could indeed cause specious indications of laccase in the syringaldazine test. Syringaldazine itself was used as the reagent for testing for peroxide. The fungi selected for examination were mostly isolates found by KOENIGS[37] to produce high levels of hydrogen peroxide. Two of them, *Coniophora puteana* (Schum. ex Fr.) Karst. and *L. lepideus* Fr. had given no evidence of laccase or peroxidase in previous tests (see Table I).

Three white rots and 3 brown rots were grown for 1–4 weeks on Fleischmann's diamalt syrup agar in Petri dishes. Tests were made at weekly intervals. To ensure that ample peroxidase was definitely present, a drop of horseradish peroxidase solution was added to a spot on each culture that was to be examined. A drop of syringaldazine solution was then added at the same spot. Purple color formation would have indicated the presence of endogenous peroxide. No color was ever formed with any of the brown rots examined (see Table II). The white rots, too, initially gave no color, but later gave purple stains (Table II). However, these can probably the ascribed to laccase activity, since no difference in the rate or intensity of color formation could be observed whether horseradish peroxidase was added or not or whether catalase was added or not. Blue colors were also formed analogously in these cases with furoguaiacin, but not at all with the brown rots, so extracellular tyrosinase was also absent.

Table II. Test[a] for production by fungi of extracellular hydrogen peroxide that might interfere with syringaldazine tests for fungal laccase

Fungus and isolate number	Micromoles of H_2O_2/min/mg O.D. Mycelium[b]	Test results after (N = negative)			
		7 days	14 days	21 days	28 days
White rots					
1. *Armillaria mellea* (Vahl.) Quél. OKM 3812-s	(0.04)[c]	N	'N'[d] SG[e]	N	N
2. *A. mellea* (Vahl.) Quél. MAD 504		N	'N' SG	'N' SG	SG
3. *Clitocybe tabescens* (Scop. ex Fr.) Bres. FP 103160-sp.	4.54	N	N	N	'N' SG
4. *C. tabescens* FP 104175-sp.	—	N	'N' SG	'N' SG	'N' SG
5. *Flammulina velutipes* (W. Curt. ex Fr.) Singer MJL 1544-sp.	(0.35–2.9)[c]	N	N	'N' SG	'N' SG
Brown rots					
6. *Coniophora puteana* (Schum. ex Fr.) Karst. MAD 515	0.08	N	N	N	N
7. *C. puteana* (Schum. ex Fr.) Karst. FP 59130-R	(11.64)[c]	N	N	N	N
8. *Gleophylum trabeium* (Pers. ex Fr.) Murr. MAD 617	11.28	N	N	N	N
9. *Lentinus lepideus* Fr. MAD 534	15.04	N	N	N	N

[a] Two drops of a solution of horseradish peroxidase (Sigma Chemical Co.) followed by 1 drop of either syringaldazine or furoguaiacin test solution on each plate; coloration would indicate the presence of hydrogen peroxide. [b] After J. W. KOENIGS, Phytopathology, *62*, 100 (1971). [c] Same species as that used by KOENIGS, but a different isolate. [d] 'N' indicates the test was judged negative as no increase or lessening in the rate or intensity of coloration was observed in cases where laccase was present when the culture was pretreated with peroxidase or catalase. [e] SG indicates that laccase was detected with both syringaldazine and furoguaiacin.

Thus, it appears certain that fungal cultures do not have an amount of endogenous hydrogen peroxide high enough at any moment to cause a color with syringaldazine through peroxidase action that would simulate a positive test for laccase[38, 39].

Zusammenfassung

Das Syringaaldehyd-azin ist eine hellgelbe, kristalline und leicht zu synthetisierende Verbindung, die in verdünnter alkoholischer Lösung von Laccase + Luftsauerstoff bzw. von Peroxydase + H_2O_2 unter Bildung eines intensiv purpurnen Pigments rasch oxydiert wird. Diese hochempfindliche Reaktion ist als Tüpfelprobe leicht anzuwenden und ergibt einen sehr spezifischen Nachweis dieser Phenoloxydasen in Pilzkulturen: Trägt man einige Tropfen verdünnter Azinlösung auf eine Pilzkultur auf, bildet sich an der Luft bei Anwesenheit von Laccase ein purpurner Fleck. Ist nur wenig Laccase vorhanden, wird die Probe nur rosa bis rot; bleibt sie farblos, ist keine Laccase vorhanden. Im letzteren Fall kann man verdünnte H_2O_2-Lösung an derselben Stelle auftropfen, um Peroxydase nachzuweisen. Färbt sich der Fleck dann lila rot, ist eine Peroxydase vorhanden. Der Nachweis von Peroxydase ist bei Anwesenheit von Laccase nicht möglich. Ist keine Laccase vorhanden, kann man eine Furoguajacin-Lösung (bzw. frisch hergestellte Guajaktinktur) an einer anderen Stelle der Kultur auftragen, um Tyrosinase nachzuweisen. Die Tyrosinase oxydiert das Azin überhaupt nicht. Mit Hilfe dieser eindeutigen und schnellen Nachweismethoden wird man den Zusammenhang zwischen der Bildung der verschiedenen Phenoloxydasen durch holz-zerstörende Pilze und deren Abbau von Holz und von Lignin besser untersuchen können.

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[39] Mention of trade names and products is solely for identification and does not imply endorsement by the USDA Forest Service.

SPECIALIA

Les auteurs sont seuls responsables des opinions exprimées dans ces brèves communications. – Für die Kurzmitteilungen ist ausschliesslich der Autor verantwortlich. – Per le brevi comunicazioni è responsabile solo l'autore. – The editors do not hold themselves responsible for the opinions expressed in the authors' brief reports. – Ответственность за короткие сообщения несёт исключительно автор. – El responsable de los informes reducidos, està el autor.

Configuration absolue de l'acide bongkrékique*

L'acide bongkrékique, antibiotique toxique produit par *Pseudomonas cocovenenans*, a la structure **1**[1]. Toutefois les configurations absolues des deux centres asymétriques présents dans la molécule n'avaient pu être établies de manière certaine.

Pour déterminer la chiralité des centres C-6 et C-17 de la molécule, nous l'avons scindée par ozonisation réductive[2], isolé les fragments C-5 à C-8 et C-15 à C-18 contenant ces deux centres asymétriques et synthétisé ces fragments à partir de précurseurs optiquement actifs de configuration connue.

L'action de l'ozone sur une solution méthanolique d'acide bongkrékique à 0° pendant 3 h conduit, après traitement par $NaBH_4$, à un mélange d'hydroxy-acides et de diols. La fraction neutre est composée du méthyl-2 butanediol-1,4 **2a**, du dihydroxy-1,4 méthoxy-3 pentane **3a**, les deux fragments recherchés, ainsi que du butanediol-1,4 et de l'éthylèneglycol.

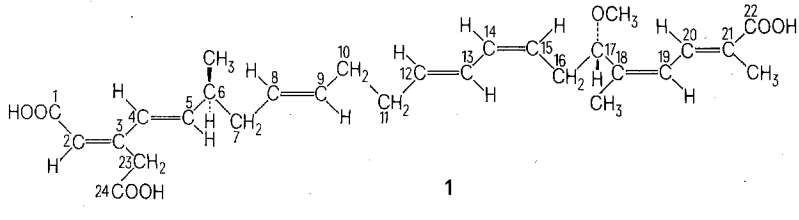
Le mélange est tritylé puis chromatographié sur colonne de gel de silice ce qui a permis d'isoler le composé monotritylé **3b** et le composé ditritylé **2b** optiquement actifs.

Composé **2b**. Le composé **2b**, obtenu de la manière indiquée, n'est pas pur mais accompagné du dérivé ditritylé du butanediol **4b** (F. 182–184°). La faible quantité de produits récupérés n'a pas permis une sépa-

* Dédié au Professeur E. LEDERER en l'honneur de son 65e anniversaire.

[1] J. DE BRUIJN, D. J. FROST, D. H. NUGTEREN, A. GAUDEMER, G. W. M. LIJMBACH, H. C. COX et W. BERENDS, Tetrahedron, sous presse (1973).

[2] D. G. M. DIAPER et D. L. MITCHELL, Can. J. Chem. *38*, 1976 (1960).



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