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A BIOACTIVE TETRAPRENYLPHENOL FROM *LACTARIUS LIGNYOTUS*[1]

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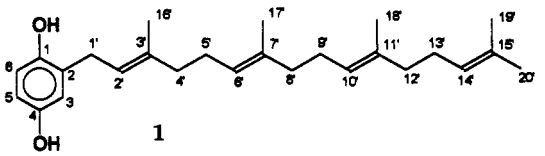
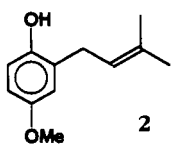
ABSTRACT.—2-Geranylgeranyl-1,4-dihydroxybenzene [**1**] and a mixture of fatty acid esters of this phenol were isolated from the fruiting bodies of *Lactarius lignyotus*. Compound **1** was highly active in the brine shrimp test and showed significant inhibitory activity on DNA, RNA, and protein synthesis in HeLa and HL-60 cell lines.

In the course of a screening program of European Basidiomycetes for bioactive compounds, and continuing our investigation on *Lactarius* species, we examined a CH_2Cl_2 extract of the inedible and uninvestigated species *Lactarius lignyotus* Fr. (Russulaceae) (2). The residue showed interesting activity ($LD_{50} \leq 10$ μg/ml) in the brine shrimp (*Artemia salina*) lethality assay (3), and very weak antimicrobial activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli,* and *Candida albicans*. Activity-directed chromatography led to the isolation of the bioactive compound **1** (LD_{50}=0.47 μg/ml), mp 35–36°, $C_{26}H_{38}O_2$ (hrms and ^{13}C nmr), identical (^1H nmr, ir, ms spectra) to all-*trans* 2-geranylgeranyl-1,4-dihydroxybenzene [**1**], which was previously isolated from the marine sponge *Ircinia muscarum* (4), and also as a potent contact allergen from the higher plants *Phacelia minor* and *P. parryi* (5). Besides the free hydroquinone **1**, free fatty acids, and triglycerides (see Experimental), a chromatographically inseparable mixture of new fatty acid diesters of compound **1** was isolated from the same extract. Methanolysis of this mixture gave the hydroquinone **1** and a mixture of the following methyl esters [gc and gc-ms analysis (6)]: tetradecanoic (0.6%), pentadecanoic (0.4%), (9Z)-hexadecenoic (4.1%), hexadecanoic (7.6%), (9Z,12Z)-octadecadienoic (15.1%), (9Z)-octadecenoic (50.3%), and octadecanoic (9.3%) acid methyl esters. This ester mixture of hydroquinone **1** showed a much lower activity (LD_{50}=119 μg/ml) than the free phenol **1** in the brine shrimp lethalithy assay (3). Moreover, free phenol **1** is slightly acrid, whereas the ester mixture is tasteless.

Polyprenyl hydroquinones are typi-





[1]Part 34 in the series, "Fungal metabolites." For part 33, see Garlaschelli *et al.* (1).

cal constituents of mushrooms of the genera *Croogomphus* (7) and *Suillus* (8–10). The latter hydroquinones possess, however, a third phenolic group on the aromatic ring. Among *Lactarius* species, prenylated hydroquinones have been isolated only from *L. flavidus* (11,12), *L. fuliginosus*, and *L. picinus* (13). They may be taxonomic markers, as the typical constituents of most *Lactarius* species are sesquiterpenoid compounds (14) that, in contrast, have not been isolated from *Lactarius* species affording phenolic compounds. It is noteworthy that *L. lignyotus, L. picinus,* and *L. fuliginosus* belong to the same section, Plinthogali (Bull.) Sing., of Bon's subdivision of the genus *Lactarius* (2).

By analogy with the biochemical transformations occurring in injured specimens of *L. picinus* and *L. fuliginosus* (13), intact fruiting bodies of *L. lignyotus* probably contain the 1,4-hydroquinone **1** protected as diacyl esters, which are hydrolyzed by lipases in disrupted tissues (13) during handling and extraction of the mushrooms. This may also occur when *L. lignyotus* is attacked by predators and parasites and, therefore, phenol **1** may function as a chemical defense protecting the mushroom (6,13). Some terpene hydroquinones have been reported to possess a demonstrable antitumor activity (7,15,16); however, information on the mechanism of action of these compounds is still lacking. It has been suggested that a relationship may exist between the antitumor and antioxidant activities of hydroquinones (7).

The inhibitory activity of compound **1** on DNA, RNA, and protein syntheses was tested in two human tumor cell-line systems. In addition, the activity of **1** was compared with those displayed by a mixture of diacyl esters of **1** and by phenol **2**, respectively, previously isolated from *L. fuliginosus* (13), in order to ascertain whether it might be affected by protecting one or two phenolic groups and shortening the isoprenoid side-chain. Table 1 shows the results of [^{3}H]-thymidine, [^{3}H]-uridine, and [^{3}H]-leucine incorporation in HeLa cells (epitheloid carcinoma) and HL-60 cells (promyelocytic leukemia), respectively. The phenols **1** and **2** showed a significant inhibitory effect on macromolecular synthesis in both cell lines at relatively low concentrations (10–20 μg/ml), whereas upon acylation of the phenol groups of compound **1** with fatty acids, the macromolecular synthetic activity was strongly inhibited. The observed parallel inhibition of [^{3}H]-thimidine and [^{3}H]-uridine incorporation in cells suggests that the cytotoxicity of compounds

TABLE 1. Activity[a] of Compounds **1** and **2**, and a Mixture of Diacyl Esters of **1** on DNA, RNA, and Protein Synthesis in HeLa Cells and HL-60 Cells.

Test sample	Concentration (μg/ml)	HeLa			HL-60		
		[^{3}H]-Thy	[^{3}H]-Leu	[^{3}H]-Urd	[^{3}H]-Thy	[^{3}H]-Leu	[^{3}H]-Urd
1	10.4	100	100	96	55	85	95
	25	74	92	83	38	52	32
	50	83	67	91	20	52	36
	75	49	61	86	17	32	42
2	10.4	100	96	97	64	49	98
	25	59	43	86	32	38	50
	50	70	38	91	14	35	55
	75	31	37	90	1.5	32	38
Mixture of diacyl esters of **1**	25	83	80	100	—	—	—
	50	—	—	—	100	100	100
	75	83	100	97	85	63	65

[a]Measured as percent residual radioactivity of incorporated [^{3}H]-thymidine, [^{3}H]-leucine, and [^{3}H]-uridine into each cell line, to which each test sample was separately added at the given concentration.

1 and **2** may be due to an indirect inhibition of DNA synthesis through an inhibition of RNA synthesis (17,18).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—General procedures for ir, nmr, uv, and ms spectral determination, cc and tlc Si gel chromatography separations, and gc and gc-ms analysis have already been described (6). Methanolysis of triglycerides, as well as identification of fatty acid methyl esters thus obtained, was carried out as reported previously (6). Free fatty acids were methylated (CH_2N_2) and identified by the same procedure. [Methyl-^{3}H]-thymidine, 25 Ci/mmol, [5-^{3}H]-uridine, 29 Ci/mmol, and L-[4,5-^{3}H(N)]-leucine, 63 Ci/mmol, each 1 mCi/ml, were purchased from Amersham International plc, UK. Essential Medium Eagle (MEM), Roswell Park Memorial Institute Medium 1640 (RPMI 1640), and fetal calf serum were purchased from Flow Laboratories, UK; L-glutamine and gentamicin were obtained from Merck, Germany, and from Schering-Plough Corp., Kenilworth, NJ, respectively; Whatman GF/F glass microfiber filters, 2.5 cm, were used.

PLANT MATERIAL.—*Lactarius lignyotus* (0.6 kg) was collected in several pine forests of Tyrol, Austria, in September–October 1989, and was identified by Mr. Luigino Polita, of the Societé Mycologique de France. A voucher specimen has been deposited in the collection of the Mycological Group "F. Bresadola," Fara Novarese, Italy.

EXTRACTION AND ISOLATION.—The fruiting bodies were frozen at −20° and extracted three times with CH_2Cl_2 (2 liters each) at −20°. The combined extracts were dried rapidly ($MgSO_4$) and concentrated below 25° under reduced pressure to give a dark-brown residue (2.13 g). This residue (1.06 g) was adsorbed on a RP-18 (25–40 μm) column (110 g), eluted with the following solvent mixtures (100 ml each): MeOH-EtOAc-H_2O (5:5:2); MeOH-EtOAc-H_2O (5:5:1); MeOH-EtOAc (1:1); MeOH-EtOAc (1:2); EtOAc, 50 ml. Phenol **1** (32 mg), two mixtures (A, 40 mg; B, 76 mg) of free fatty acids, and a mixture of esters (300 mg), respectively, were isolated. The latter mixture was further chromatographed on a Si gel (40–63 μm) column (30 g) eluted with hexane-Et_2O (36:1) to give triglycerides (156 mg) and a mixture of diacyl esters of **1** (85 mg). Two samples of mixtures A and B were separately methylated (CH_2N_2) and analyzed by gc and gc-ms (6). Mixture A contained the following compounds: (9*Z*,12*Z*)-octadecadienoic (89.1%), octadecanoic (4%), pentadecanoic (1%), and tetradecanoic (1%) acids; traces of hexadecanoic, hexadecenoic, and (9*Z*)-octadecenoic acids; and unidentified acids (5%). Mixture B contained the following compounds: (9*Z*)-octadecenoic (70%), hexadecanoic (25.4%), and (9*Z*,12*Z*)-octadecadienoic (2%) acids; traces of pentadecanoic, hexadecenoic, and octadecanoic acids; and unidentified acids (2.6%). A sample of triglycerides was submitted to methanolysis and methyl esters of the following acids were identified by gc and gc-ms analysis (6): (9*Z*)-octadecenoic (71.6%), (9*Z*,12*Z*)-octadecadienoic (13.5%), hexadecanoic (10.2%), and (9*Z*)-hexadecenoic (1.5%) acids; traces of tetra- and pentadecanoic acids; and unidentified acids (3.2%).

(2′*E*,6′*E*,10′*E*)-2-GERANYLGERANYL-1,4-DIHYDROXYBENZENE.—Phenol **1**, mp 35–36°, was identified by comparison of ms, ir, and ^{1}H-nmr ($CDCl_3$, 300 MHz) data with those reported in the literature (4,5). ^{13}C nmr ($CDCl_3$, 75.5 MHz) δ 149.2 (s, C-4), 148.1 (s, C-1), 138.6 (s, C-3′), 135.5 (s, C-7′), 134.9 (s, C-11′), 131.2 (s, C-15′), 128.1 (s, C-2), 124.3 (d, C-14′), 124.1 (d, C-10′), 123.6 (d, C-6′), 121.1 (d, C-2′), 116.4 (d, C-3), 116.4 (d, C-6), 113.6 (d, C-5), 39.6 (t, C-8′), 39.6 (t, C-12′), 39.5 (t, C-4′), 29.6 (t, C-1′), 26.6 (t, C-5′), 26.5 (t, C-9′), 26.3 (t, C-13′), 25.6 (q, C-20′), 17.6 (q, C-19′), 16.1 (q, C-16′), 16.0 (q, C-17′), 15.9 (q, C-18′). The following sets of assignments are interchangeable: C-7′ and C-11′; C-6′, C-10′, and C-14′; C-16′, C-17′, and C-18′; C-4′, C-8′, and C-12′.

DIACYL ESTER MIXTURE.—Ir (dry film) ν max 2928, 2857, 1763, 1664, 1491, 1464, 1376, 1133, 963, 915, 831, 758, 721 cm^{-1}; uv (CH_2Cl_2) λ max 245 and 265 nm; ^{1}H-nmr ($CDCl_3$, 300 MHz) and ^{13}C-nmr ($CDCl_3$, 75.5 MHz) data are available from the authors. A sample (30 mg) of this diester mixture was stirred in 5 ml 15% methanolic MeONa at room temperature for 20 h. The mixture was carefully acidified with 10% HCl, diluted with Et_2O (50 ml), and extracted with H_2O, then with brine. The organic phase was dried ($MgSO_4$), concentrated, and separated on a Si gel column with a hexane/EtOAc mixture. Phenol **1** (7 mg) was identified from its ir and ^{1}H-nmr spectra. Fatty acid methyl esters (16 mg) were obtained as an inseparable mixture and were identified by gc and gc-ms according to a previously described procedure (6).

CANCER CELL-LINE BIOASSAYS.—HeLa S3 cells were grown in suspension cultures at 37° in MEM, supplemented with 20% heat-inactivated fetal calf serum, 300 mg/liter L-glutamine, and 40 μg/ml gentamicin. HL-60 cells were grown in a 5% CO_2 incubator at 37° in RPMI 1640 supplemented as described above. Exponentially growing cells were used in all experiments. The incorporation of [^{3}H]-thymidine, [^{3}H]-uridine and [^{3}H]-leucine into acid-insoluble material was used as a measure of DNA, RNA, and protein synthesis; 5 μl of each [^{3}H]-labeled precursor were added to 2.5×10^5 cells in 250 μl of medium. The cells were

then incubated, in the presence or absence of each test sample, at 37° for 45 min, and 130-µl aliquots were spotted onto GF/F filters, which were washed with 5% trichloroacetic acid (19). The remaining radioactivity was measured in a liquid scintillation counter (Kontron Betamax V). The inhibitory activity of each sample on macromolecular synthesis was determined (Table 1) as percent residual radioactivity compared to the control.

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