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ANTIFUNGAL TERPENOIDS PRODUCED BY CYPRESS AFTER INFECTION BY *DIPLODIA PINEA* f. sp. *CUPRESSI*

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Key Word Index—*Cupressus sempervirens*; Cupressaceae; *Diplodia pinea* f. sp. *cupressi*; *Seiridium cardinale*; terpenoids; phytoalexin; antifungal agents; cupressotropolones.

Abstract—Two antifungal terpenoids, 6-isopropyltropolone β-glucoside and 5-(3-hydroxy-3-methyl-*trans*-1-butenyl)-6-isopropyl-tropolone β-glucoside, named by us cupressotropolone A and B, respectively, were isolated from the bark of Italian cypress, in response to infection by the fungus *Diplodia pinea* f. sp. *cupressi*. These tropolone glucosides inhibited *in vitro* germination of spores of *Diplodia pinea* f. sp. *cupressi*, *Seiridium cardinale*, *Alternaria alternata* and *Verticillium dahliae*.

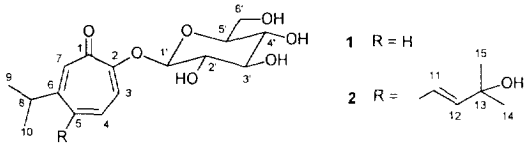
INTRODUCTION

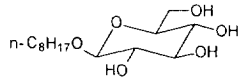
The production of the antimicrobial compounds phytoalexins, by a plant in response to microbial infection or other physiological stimuli (UV, heat, wounding), is an important disease resistance mechanism [1–3]. Recently we have become interested in the defence mechanisms of Italian cypress (*Cupressus sempervirens* L.) against the fungal pathogen *Diplodia pinea* f. sp. *cupressi*. Little is known about phytoalexins in conifers, particularly in the Cupressaceae [1]. There have been several reports on the fungitoxicity of conifer monoterpenes [4, 5]. Correlations between monoterpenes in the stem xylem and branch cortex of loblolly pine and resistance to fusiform rust were reported by Rockwood [6]. The monoterpenoid β-thujaplicin, isolated from the heartwood of *Thuja plicata* D. Don, has been shown to possess antifungal and antibacterial activity [7]. Preliminary experiments indicated that production of two antifungal compounds was induced in Italian cypress bark in response to infection by *D. pinea* f. sp. *cupressi*. We describe the isolation, identification and antifungal activity of these two substances, which we have named cupressotropolone A (**1**) and B (**2**). The former is the glucoside of β-thujaplicin, while the latter is a glucosylated sesquiterpenic tropolone.

RESULTS AND DISCUSSION

Indentification of antifungal compounds

Two antifungal compounds, **1** and **2**, were isolated from the bark of Italian cypress infected by *Diplodia pinea* f. sp. *cupressi*. The elucidation of their chemical structures





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was based mainly on a full analysis of their NMR data (^1H and ^{13}C), which was aided by several 2D techniques (COSY, one-bond and long-range ^1H–^{13}C heteroCOSY), and was confirmed by the observation of quasimolecular ions in the DCI-MS.

The most conspicuous features in the NMR spectra of both compounds are the presence of a β-glucoside unit, which could be identified by its vicinal HH coupling constants and ^{13}C chemical shifts (Table 1). As a model, we have included in the table the corresponding data for *n*-octyl β-glucoside (**3**) in the same solvent; the main differences are significant deshieldings of H-1′, H-2′ and C-1′.

The aglycone of **1** contains an isopropyl group and seven sp^2 carbons (of which one is a probable carbonyl), suggesting a monoterpenoid. The ^1H spectrum shows a sequence of one isolated and three adjacent CHs in the 7.1–7.4 ppm region. This is not, however, a *meta*-substituted benzene, in view of the large vicinal coupling constants. Instead, the data are fully consistent with a substituted tropolone. The position of the alkyl residue is

Table 1. ^1H and ^{13}C NMR data*

H/C	1†	2†	3†§	1‡	2‡	3‡¶
1	—			181.4	181.3	—
2	—	—	—	163.4	162.0	—
3	7.37 *dd*, 10, 1	7.37 *s*‖	—	120.1	120.4	—
4	7.28 *dd*, 11, 10	7.37 *s*‖	—	134.7	133.3	—
5	7.15 *ddd*, 11, 1.5, 1	—	—	133.2	144.8	—
6	—	—	—	161.8	160.5	—
7	7.25 *d*, 1.5	7.34 *s*	—	135.7	135.3	—
8	2.89 *septet*, 7	3.27 *septet d*, 6.5, 1	—	39.7	34.3	—
9	1.26 *d*, 7	1.25 *d*, 6.5	—	23.4	23.4	—
10	1.26 *d*, 7	1.24 *d*, 6.5	—	23.4	23.4	—
11	—	6.89 *dd*, 15.5, 1	—	—	127.8	—
12	—	6.15 *d*, 15.5	—	—	145.1	—
13	—	—	—	—	71.5	—
14, 15	—	1.38 *s*	—	—	29.9	—
1′	5.04 *d*, 7.5	5.02 *d*, 7.5	4.24 *d*, 8	101.8	101.9	104.3
2′	3.63 *dd*, 9.5, 7.5	3.60 *dd*, 9, 7.5	3.16 *dd*, 9, 8	74.3	74.4	75.1
3′	3.55 *dd*, 9.5, 8.5	3.51 *dd*, 9, 8.5	3.35**	77.1	77.2	78.1
4′	3.43 *dd*, 9.5, 8.5	3.39 *dd*, 9.5, 8.5	3.29**	71.2	71.3	71.6
5′	3.56 *ddd*, 9.5, 5.5, 2	3.54 *ddd*, 9.5, 6, 2	3.25**	78.5	78.7	77.8
6′	3.93 *dd*, 12, 2 3.71 *dd*, 12, 5.5	3.93 *dd*, 12, 2 3.69 *dd*, 12, 6	3.86 *dd*, 12, 2 3.66 *dd*, 12, 5.5	62.4	62.5	62.8

* In CD_3OD.
† ^1H. ‡ ^{13}C.
§ Side-chain: 3.90 (*dt*, 9.5, 7, H-1), 3.53 (*dt*, 9.5, 6.5, H-1), 1.62 (*m*, H-2), 1.37 (*m*, H-3), 1.30 (*m*, H-4 to 7), 0.90 (*t*, 6.5, H-8).
¶ Side-chain: 70.9 (C-1), 30.8 (C-2), 27.1 (C-3), 30.3 and 30.5 (C-4 and 5), 33.0 (C-6), 23.7 (C-7), 14.4 (C-8).
‖ In a 1:1 CD_3OD: d_6-acetone solution, this two-proton singlet splits into an AB *q* with $J = 11$ Hz and $\Delta\delta 0.04$ ppm.
** δs obtained from a COSY spectrum, but multiplets not analysed.

established by the detection—in double resonance experiments—of an allylic coupling (*ca* 0.5 Hz) between the isopropyl CH and the isolated olefinic hydrogen. Confirmation of the isopropyl substitution on position 6 rather than 4 is provided by an analysis of the ^{13}C data. Bagli and St.-Jacques [8] have shown that the most shielded sp^2 carbon in tropolone methyl ether is C-3. This must correspond to the resonance at 120.1 ppm in **1**, which is correlated to the hydrogen at δ7.37 by a 2D experiment. The latter signal is enhanced (NOE = 5%) by irradiation of the anomeric H-1′. Relevant long-range ^{13}C–^1H correlations were observed between C-6 and the methyl hydrogens, and between C-8 and H-7.

Glucoside **2** is very similar to 1 (Table 1), but one of the tropolone ring hydrogens has been replaced by a side-chain, leaving one isolated and two adjacent CHs. Arguments similar to those in the previous paragraph show that the substituents are in positions 5 and 6. The latter carries the isopropyl group (a 5% enhancement of H-7 on irradiation of H-8), and the former a C_5 fragment which can be identified as a 3-hydroxy-3-methyl-*trans*-1-butenyl residue. The aglycone of **2** is therefore a sesquiterpene.

For a final confirmation of the structures, one would like to be able to determine their molecular weights by mass spectrometry; the major challenge is thus to find an ionization mode which would provide quasimolecular ions with a minimum of decomposition.

For these highly polar and thermally labile compounds, desorption chemical ionization (DCI) was found to be the most suitable technique. In contrast to conventional solid probe electron impact or chemical ionization, the spectra obtained by direct sample insertion into the reagent gas plasma contain (and often are dominated by) ions indicative of the molecular weight, as well as structurally significant fragment ions (Fig. 1).

Using this technique, **1** gives an intense protonated ion (327, 52%) followed by a very stabilized hydrogenated species (329, 100%). Such a hydrogen addition under CI-MS conditions is known for quinones [9]; **1** contains a tropolone ring and might be expected to behave similarly, yielding $[M+H]^+$ and $[M+3H]^+$ ions; thus, the molecular weight of **1** is 326. This determination is supported by the detection of the MNH_4^+ complex (344, 7%) which appears in the DCI/NH_3 spectrum. Other peaks arise from the loss of the glucose moiety ($C_6H_{10}O_5$) from the $[MH]^+$ (165, 68%) and a retro-Diels–Alder decomposition yielding the ion $[C_2H_4Glu]^+$ (207, 75%). To verify the assumption of hydrogenation under CI conditions only, we checked the EI spectrum of **1**. While no molecular ion appears in this mode, the tropolone, the sole part which might undergo hydrogenation, gives the

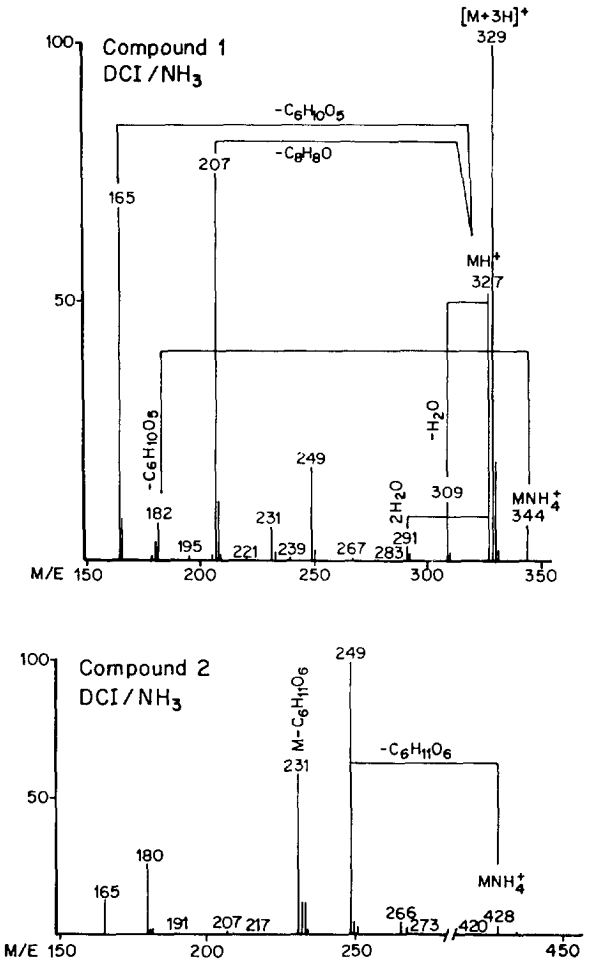

Fig. 1. Mass spectra of **1** and **2**.

ions $[C_{10}H_{12}O_2]^+$ (164, 54%) and $[C_{10}H_{13}O_2]^+$ (165, 65%); fragments like $[C_{10}H_{14}O_2]^+$ or $[C_{10}H_{15}O_2]^+$ ($m/z = 166$ and 167, respectively), expected for the hydrogenated species, do not appear.

Compound **2** is very labile even in the DCI mode. While the quasimolecular ion $[MH]^+ = 410$ is missing, we can detect the $[MNH_4]^+$ complex (428, 0.5%). The main fragments arise from the loss of the glucose moiety ($C_6H_{11}O_6$) from the $[MNH_4]^+$ (249, 100%) and from the whole molecule (231, 59%). Thus, the mass spectral data confirm the structures proposed by NMR and are in good agreement with them.

The two natural products give very similar UV spectra, with maxima at 240 and 330 nm for **1**, and at 240 and 340 nm for **2**.

Antifungal activity

There was a negative correlation between the concentration of both compounds and germination of spores of *Diplodia pinea* f. sp. *cupressi* (Fig. 2 A, B). The ED_{50} values for the inhibition of spore germination by cupressotropolones A (**1**) and B (**2**) were 3.3 $\mu g\,disc^{-1}$ and 3.7 $\mu g\,disc^{-1}$, respectively. The two compounds were inhibitory to various phytopathogenic fungi (Fig. 3 A, B). Spore germination of *Diplodia pinea* f. sp. *cupressi*, *S. cardinale* and *Alternaria alternata* was completely inhib-

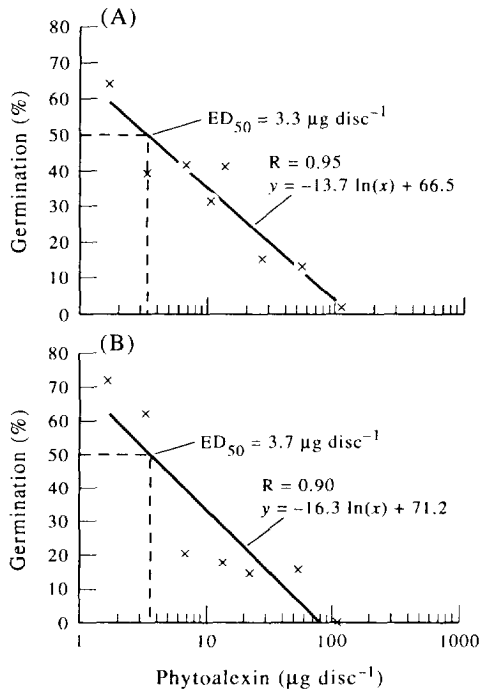

Fig. 2. Germination of spores of *D. pinea* f. sp. *cupressi* at different doses of cupressotropolone A and B.

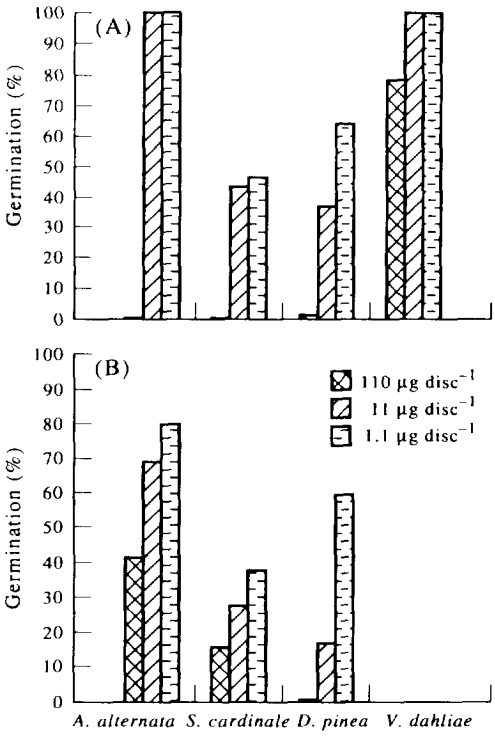

Fig. 3. Germination of spores of various phytopathogenic fungi at different doses of cupresssotropolone A and B.

ited by 110 μg disc^{-1} of **1**. At this dose, *Verticillium dahliae* was only slightly inhibited (Fig. 3 A). At the highest dose, **2** was less inhibitory to the test fungi with the exception of *D. pinea* f. sp. *cupressi*, which was completely inhibited (Fig. 3 B). The spores of *D. pinea* f. sp. *cupressi*, the germination of which was completely inhibited by the highest dose (110 μg disc^{-1}) of either compound, were transferred to cellulose discs saturated with H_2O. The conidia germinated normally, indicating that the antifungal action was fungistatic. The fungitoxic compounds were absent from the bark of intact or mechanically wounded plants. Production of these antifungal terpenoid compounds was found to be induced also by *S. cardinale*, a pathogen causing cankers on Italian cypress.

In this study we demonstrated for the first time the presence of the glucosides of β-thujaplicin and a sesquiterpenoid in cypress bark in response to infection by *D. pinea* f. sp. *cupressi*. Synthesis of the antifungal terpenoid compounds was induced in response to fungal infection, and therefore these compounds can be defined as phytoalexins.

EXPERIMENTAL

NMR spectra. Taken in a Bruker AM-300 instrument (300.1 and 75.5 MHz for ^1H and ^{13}C, respectively), in CD_3OD solns, with TMS as int. ref. Chemical shifts are reported in ppm and coupling constants in Hz.

Mass spectra. The DCI-MS were recorded in a Finnigan MAT 4021 quadrupole instrument. A rhenium wire of 0.1 mm diameter is mounted on top of an inlet rod, which is introduced into the ion source via a standard vacuum lock. A droplet of the dissolved substance is brought to the wire loop with a syringe; after evapn of the solvent, the wire is introduced into the plasma of the CI source. During a rapid heating program of the wire, DCI-mass spectra are obtained. Operating parameters: ion source, 230°; DCI wire programmed with 7.5 mA sec^{-1} up to 2.5 A; electron energy, 70 eV; emission current, 0.28 mA; reagent gas, ammonia; 0.25 Torr pressure inside the CI chamber.

Plants and fungal inoculation. Four-year-old cypress saplings were inoculated with *D. pinea* f. sp. *cupressi* as described previously [10].

Purification of the phytoalexins. Infected bark tissue with 2–3 mm margins were removed from plants, cut into small pieces and extracted with MeOH (1:10, w/v) for several days at 4°. The crude extract was filtered through filter paper (Whatman no. 1) and evapd at 40° to dryness. The residue was redissolved in a small vol. of 30% MeOH. The extract was chromatographed on a polyvinylpyrrolidone column (20 × 1.8 cm) and eluted with 30% MeOH. The compounds were loaded on TLC plates (silica gel 60 F254 with concentrating zone, 0.25 mm, Merck) and developed (× 3) with EtOAc–EtOH–H_2O (85:6:9). The 2 bands (UV detection), at R_f 0.22 and R_f 0.18, were scraped off and eluted with MeOH.

Antifungal bioassay. The purified compounds were dissolved in MeOH and bioassayed as follows: a 13-mm cellulose disc (AA) was placed in a Petri dish and the test soln (110 μl) was dripped on to it. The solvent was evapd, and the disc was wetted to over-satn with 110 μl of distilled H_2O. Then a 3-mm moistened cellophane disc was placed above the cellulose disc, and conidia suspension (15 μl) of the test fungus was dripped on to it. The dish was covered with a moistened filter paper attached to the inner side of the lid. Conidia germination percentage (100 spores per disc, 3 discs per test) was determined after 18 hr of incubation at 25°.

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