Demethylmedicarpin, a Product Formed from Medicarpin by *Colletotrichum coccodes*

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ABSTRACT

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The structure and antifungal activity of a product formed from medicarpin (3-hydroxy-9-methoxypterocarpan) by the tomato pathogen, *Colletotrichum coccodes* (=*C. phomoides*), was reinvestigated. The compound was identified as demethylmedicarpin (3,9-dihydroxypterocarpan) hereafter called CP-I) by ultraviolet, mass, and nuclear magnetic resonance spectroscopy; by chromatographic comparison with authentic material; by methylation, which yielded homopterocarpin (3,9-dimethoxypterocarpan); and by formation of the expected diacetoxy derivative upon acetylation. Contrary to an earlier report, CP-I was considerably less inhibitory than medicarpin in bioassays to test its effects on spore germination, germ tube elongation, and mycelial growth of *C. coccodes*. Tests with the vital stain fluorescein diacetate also confirmed that the effect of CP-I on the metabolic activity of growing germ tubes was less severe than that of medicarpin. Maackiain (3-hydroxy-8,9-methylenedioxypterocarpan) and homopterocarpin did not appear to be metabolized when incubated with *C. coccodes* under the conditions used for the conversion of medicarpin.

In earlier studies on the metabolism of pterocarpanoid phytoalexins by fungi, it was reported (8) that *Colletotrichum coccodes* (Wallr.) Hughes (=*Colletotrichum phomoides*) converted the alfalfa (*Medicago sativa*) phytoalexin medicarpin ((-)-6a*R*;11a*R*-3-hydroxy-9-methoxypterocarpan) (Fig. 1-I) to an initial product designated CP-I. More recently, studies on the conversion of medicarpin by other *Colletotrichum* species showed that an array of products was possible (9). One compound formed from (-)-medicarpin by *C. lindemuthianum* and identified as demethylmedicarpin (3,9-dihydroxypterocarpan) (Fig. 1-II) had spectral characteristics similar to those described for CP-I.

This report provides evidence that CP-I is demethylmedicarpin and reexamines its toxicity to *C. coccodes* by several types of bioassay. The ability of *C. coccodes* to be inhibited by and to modify two related pterocarpanoid compounds, maackiain (3-hydroxy-8,9-methylenedioxypterocarpan) (Fig. 1-III) and homopterocarpin (3,9-dimethoxypterocarpan) (Fig. 1-IV), was also tested.

MATERIALS AND METHODS

All pterocarpanoid compounds used were either crystalline or chromatographically pure. Medicarpin was isolated from jackbean cotyledons (6) or sweetclover leaflets (9) and maackiain was obtained from red clover roots (5). A sample of homopterocarpin was supplied by C. W. L. Bevan, Department of Chemistry, University of Ibadan, Nigeria. Isolates of *Colletotrichum coccodes*, *Helminthosporium carbonum* Ullstrup, and *Stemphylium botryosum* Wallr. (ATCC #26881) were those used in the previous study (7,8). Cultures of all three fungi were maintained as reported elsewhere (7).

Production, isolation, and quantification of CP-I. Pregerminated conidia of *C. coccodes* were grown in medicarpin-amended (20 μg/ml) half-strength Czapek Dox broth for 12 or 24 hr as previously described (5). Conversion products and medicarpin were isolated from the culture filtrates by extracting with ethyl acetate followed by chromatography of the ethyl acetate fraction on silica gel thin-layer plates by using chloroform/methanol (100:2, v/v) as the developing solvent. After elution of the appropriate areas of silica gel with ethanol, both CP-I (R_f 0.14) and medicarpin (R_f 0.44) were quantified by the absorbance of ethanolic solutions at 287 nm by using the extinction coefficient ($\log \epsilon = 3.89$) given by Harper et al (3) for medicarpin. An extinction coefficient for CP-I is not available but, because of its structural similarity to medicarpin, it is assumed that both compounds have comparable extinction coefficients.

Characterization of CP-I. *Methylation.* CP-I (about 100 μg) was dissolved in a mixture of methylene dichloride (2 ml) and methanol (1 ml) before being treated (1 min) with gaseous diazomethane prepared as described by Powell (14). After removal of solvent (in vacuo, 40 C), the residue was chromatographed (silica gel TLC) in cholorform/carbon tetrachloride (3:1, v/v), which yielded 3,9-dimethoxypterocarpan at R_f 0.78.

Acetylation. Acetic anhydride (3 ml) was added to a solution of CP-I (about 100 μg) in pyridine (1 ml). The mixture was then transferred to a watch glass and allowed to evaporate under a cool air stream (10 hr; 20 C). Silica gel TLC of the residue in chloroform gave 3,9-diacetoxypterocarpan at R_F 0.85.

Instrumentation. Mass spectra were determined as reported elsewhere (9,10). Optical rotation (taken in methanol at 589 nm) and NMR (measured at 360 MHz in acetone-d_6 with tetramethylsilane as the internal reference) data were obtained by using a Perkin-Elmer 141 polarimeter and a Brucker WH-360 FT spectrometer, respectively.

Bioassays. Germ tube growth bioassays were performed as described for the "non-continuous" method of Higgins (6) with

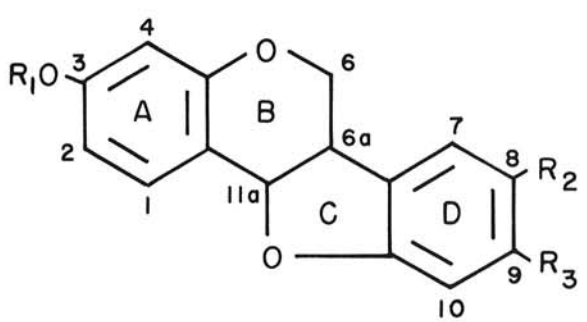

Fig. 1. Structural formulas of **I**, medicarpin ($R_1 = H$, $R_2 = H$, $R_3 = OCH_3$); **II**, demethylmedicarpin (CP-I) ($R_1 = H$, $R_2 = H$, $R_3 = OH$); **III**, maackain ($R_1 = H$, $R_2 = R_3 = -O-CH_2-O-CH_2-O-$); and **IV**, homopterocarpin ($R_1 = CH_3$, $R_2 = H$, $R_3 = OCH_3$).

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conidia incubated for 12 hr prior to addition of the test compounds and 6 hr after treatment. The longest germ tube of 25 or 50 conidia in each of four replicates was measured microscopically. For spore germination, a similar assay method was used, but the test compounds were added immediately after preparation of the spore suspension. Mycelial growth bioassays were performed according to the method described by Heath and Higgins (4) with CP-I or medicarpin concentrations of 20, 40, and 60 μg/ml. Radial mycelial growth was measured after 1, 2, and 3 days of incubation.

Vital staining with fluorescein diacetate (FDA) was used to compare the effect of test compounds on metabolic activity. Assays were prepared as for the germ tube growth bioassay, and at 1–3 hr after addition of the test compound, 10 μl of sterile medium containing 3% FDA (Sigma Chemical Co., St. Louis, MO 63178) and 1.5% acetone were added to the 50 μl drop of spore suspension. Germ tubes were immediately examined on a Zeiss Photomicroscope fitted with an Osram Mercury Super Pressure HBO 200 w/4 lamp and exciter filters BG12 and BG3 and barrier filters 65 and 47. Germ tubes were rated for degree of fluorescence on a scale of 0 to 4, in which 0 indicated no fluorescence and 4 equaled the average fluorescence of solvent controls. Controls lacking FDA were used to check for autofluorescence, which generally was not associated with young germ tubes.

Tests for metabolization of maackiain and homopterocarpin. Techniques similar to those used for the fungal conversion of medicarpin also were employed to test the ability of *C. coccodes* to metabolize the related pterocarpanoids, maackian and homopterocarpin. These compounds, or medicarpin, were added (to give 10 μg/ml) to spore suspensions (5 ml/50-ml flask) after 12 hr of preincubation. At 0, 4, 8, 12, and 24 hr thereafter, 0.2-ml samples were aseptically removed from each flask, evaporated to dryness, taken up in 10 μl of ethanol, and chromatographed by using the TLC system described earlier. Phenolic compounds were detected by spraying developed TLC plates with diazotized *p*-nitroaniline reagent, which gives brightly colored products with medicarpin and maackiain (both yellow) and CP-I (orange). Homopterocarpin does not react with the nitroaniline reagent, but a demethylated product should react. An alternte method used to measure the recovery of maackiain or homopterocarpin from 24-hr culture filtrates was to extract three times with carbon tetrachloride (CCl_4), reduce the CCl_4 to dryness in vacuo, dissolve the residue in ethanol, and determine the absorbance of this solution at the appropriate wavelengths. CP-I was not removed from aqueous solution in significant quantities by CCl_4.

RESULTS

Identification of CP-I. The medicarpin metabolite had UV maxima in ethanol and ethanol plus aqueous sodium hydroxide (0.1 N; two drops per milliliter of CP-I solution) identical with those previously reported for 3,9-dihydroxypterocarpan (9). Similarly, CP-I and the authentic dihydroxypterocarpan could not be separated by silica gel TLC in five different (acidic, basic, and neutral) solvent systems. Mass spectroscopy (MS) gave the molecular ion (M^+) at 256 (corresponding to $C_{15}H_{12}O_4$), while diazomethane methylation yielded a nonphenolic dimethyl ether (M^+284) indistinguishable (by UV, MS, and TLC) from homopterocarpin (Fig. 1-IV). The dihydroxy nature of CP-I was also supported by formation of a diacetoxy derivative upon reaction with acetic anhydride (M^+340, prominent fragments at *m/e* 299, 298, 257, 256 (base peak), 255, 147, and 134; UV maxima at 210, 226 sh, 278 sh, and 284 nm). Confirmation of the suspected 3,9-substitution pattern of CP-I was provided by its nuclear magnetic resonance (NMR) spectrum which revealed signals at δ 6.28 (1H, doublet, J = 2.2 Hz, H-10), 6.35 (1H, doublet, J = 2.4 Hz, H-4), 6.37 (1H, quartet, J = 8.1, 2.2 Hz, H-8), 6.55 (1H, quartet, J = 8.3, 2.4 Hz, H-2), 7.15 (1H, doublet, J = 8.1 Hz, H-7), and 7.32 (1H, doublet, J = 8.3 Hz, H-1) almost exactly coincident with values reported for the corresponding aromatic (A/D) ring protons of 3,6a,9-trihydroxypterocarpan (12). Heterocyclic (B/C) ring signals at δ 3.55 (2H, multiplet, H-6a, H-6ax), 4.26 (1H, multiplet, H-6eq), and 5.47 (1H, doublet, J = 5.9 Hz, H-11a) were identical with those determined (in acetone-d_6) for the model pterocarpan phaseollidin (3,9-dihydroxy-10-isopentenylpterocarpan) and served to eliminate any possibility of substitution or other modification in this non-aromatic portion of the CP-I molecule. Finally CP-I was found to be strongly levorotatory ($[\alpha]_{589nm}$ - 204°; ~0.15 mg in 1 ml of methanol). Because the absolute sterochemistry of pterocarpans can be deduced from their optical rotation (13), it is possible (in conjunction with UV, TLC, MS, and MNR data) to fully and unequivocally define the structure of CP-I as (-)-6a*R*; 11a*R*-3,9-dihydroxypterocarpan (demethylmedicarpin) (Fig. 1-II).

Yields of CP-I. Although production and isolation of CP-I was as efficient as could be expected based on the kinetics of the system as previously described (8), yields varied considerably between experiments. Typical yields for two incubation schedules are given in Table 1; the values were not corrected for losses incurred during isolation. Because small quantities of several other conversion products were detected on TLC plates, it is assumed that additional losses reflect the further metabolism of CP-I possibly by introduction of a nonaromatic hydroxyl group (to give 3,6a, 9-trihydroxypterocarpan) or by C-ring cleavage (to give 7,2′,4′-trihydroxyisoflavan) (9,18).

Antifungal activity of demethylmedicarpin. Initial bioassays (10–40 μg/ml) against germ tube growth of *C. coccodes* gave ED_{50} values of ~10 μg/ml and >40 μg/ml for medicarpin and demethylmedicarpin, respectively. A bioassay designed to separate effects on germination from effects on germ tube growth was also used to compare CP-I, medicarpin, maackiain, and homopterocarpin at 20 μg/ml. This study (Table 2) confirmed the reduced antifungal activity of CP-I in relation to medicarpin with respect to both spore germination and germ tube growth. CP-I and homopterocarpin had no effect on spore germination after 11 hr of incubation, whereas in the presence of medicarpin and maackiain germination was markedly reduced or delayed. Although CP-I and homopterocarpin were found to reduce germ tube of growth of *C. coccodes*, inhibition caused by medicarpin and maackiain was considerably greater. In these bioassays, appressorium formation was frequent (>50%) in control and CP-I treatments, but it was very rarely observed (<1%) in other treatments. Results of two experiments comparing the effects of medicarpin and CP-I (at 20 μg/ml) on germ tube growth of *H. carbonum* showed that medicarpin reduced growth to 40 and 35% of the control, whereas corresponding values for CP-I were 58 and 63%. In this comparison of relative biological activity of the two compounds we assumed similarity of their extinction coefficients; however, the differences in activity against *C. coccodes* are sufficiently large that a small disparity in the extinction coefficients would not change the conclusion that CP-I is less active.

While a mycelial growth bioassay (performed only once owing to limited amounts of CP-I) confirmed the reduced fungitoxicity of the metabolite (Table 3), inhibition even with medicarpin was not marked. However, the degree of inhibition associated with medicarpin remained relatively constant over the 3-day incubation period, whereas inhibition caused by CP-I gradually increased with

TABLE 1. Yield of demethylmedicarpin (CP-I) from medicarpin after incubation with germinated conidia of *Colletotrichum coccodes*

Germination period (hr)[a]	Period in medicarpin (hr)[b]	Medicarpin added (μg)	Medicarpin recovered (μg)[c]	CP-I isolated (μg)[c]
15 (shake)	24	1,120	200 (18%)[d]	480 (43%)
12 (still)	12	1,235	150 (12%)	750 (61%)

[a] Conidia (~5×10^5 spores per milliliter) in half-strength Czapek Dox broth in 0.05 M citrate-phosphate buffer at pH 6.0 (5 ml/50 ml flask) were germinated in the dark at 25 C with or without shaking.

[b] Medicarpin in ethanol was added to give ~20 μg medicarpin per milliliter and 1% ethanol.

[c] Medicarpin and CP-I, a product formed from medicarpin by *C. coccodes*, were isolated from culture filtrates by partitioning with ethyl acetate followed by TLC on silica gel.

[d] Values in parentheses are yields as a percentage of the total medicarpin added.

TABLE 2. Relative inhibitory effects of medicarpin, demethylmedicarpin (CP-I), maackiain and homopterocarpin on germination and germ tube growth of *Colletotrichum coccodes*

Incubation schedule[a]	Parameter	Germination or germ tube length as % of control[b]			
		Medicarpin	CP-I	Maackiain	Homopterocarpin
A	Germination	33	100	5	100
	Germ tube length	17	79	9	54
B	Germ tube length	27	60	28	45

[a] For schedule A, the test compounds were added (to give 20 μg/ml) as soon as the spore suspensions were prepared. The spores were incubated for 11 hr at 25 C. for schedule B, the test compounds were added (to give 20 μg/ml) to germinated spores that had been incubated for 11 hr and these spores were incubated for an additional 6 hr.

[b] Twenty-five (schedule B) or 50 (schedule A) spores in each of four replicates were examined and, if germinated, the longest germ tube was measured. Values given are calculated as the percentage of the germination rate (98%) or germ tube length (51.5 ± 35.9 μm - A, 316.3 ± 89.0 μm - B) of the solvent control. The average germ tube lengths in all treatments were significantly different (Student's *t*-test, $P < 0.05$) from each other except for the medicarpin and maackiain treatments.

TABLE 3. Comparison of the effect of medicarpin and the conversion product demethylmedicarpin (CP-I) on mycelial growth of *Colletotrichum coccodes*

Compound	Rate (μg/ml)	Mycelial growth as % of the control[a]		
		1 day	2 days	3 days
Medicarpin	60	64.7	69.2	70.2
	40	80.1	78.2	81.3
	20	87.6	84.6	87.6
CP-I	60	91.5	80.7	79.7
	40	95.2	87.2	82.9
	20	95.2	97.0	95.5

[a] Mean diameters for colonies in the control treatments at 1, 2, and 3 days of incubation were 4.4, 13.0, and 21.1 mm, respectively. All values are calculated from means of three or four replicates per treatment.

time.

In bioassays with FDA, the fluorescence of medicarpin-treated *C. coccodes* germ tubes was consistently lower than that of germ tubes exposed to CP-I. Thus, in a typical test made between 1 and 2 hr after treatment, average fluorescence ratings of 25 germ tubes per treatment were: control = 3.4; 20 μg/ml medicarpin = 1.3; and 20 μg/ml CP-I = 2.2. Similar tests on germ tubes of *Stemphylium botryosum* and *H. carbonum* also demonstrated that medicarpin consistently reduced FDA fluorescence to a greater extent than did CP-I.

Metabolization of maackiain and homopterocarpin. No phenolic conversion products of maackiain or homopterocarpin were detected at any harvest period up to the longest, which was 24 hr. In contrast, CP-I was first detected in medicarpin treatments at 8 hr, and by 24 hr over half (55%) of the added medicarpin was isolated as the major conversion product. Maackiain and homopterocarpin were recovered from 24-hr culture filtrates at levels not significantly different from those of the sterile medium controls. For example, in one experiment recovery of maackiain and homopterocarpin was 91 and 100%, respectively.

DISCUSSION

The tomato pathogen *C. coccodes* demethylated medicarpin to demethylmedicarpin (3,9-dihydroxypterocarpan; CP-I) a product previously isolated following inoculation of *Melilotus alba* leaflets with *Botrytis cinerea* or *Colletotrichum lindemuthianum* (9). In both situations, demethylmedicarpin was assumed to have arisen via fungal modification of medicarpin produced by the host tissue, although critical in vitro experiments were not performed to firmly exclude the possibility that it was of plant origin. Apart from the studies involving *Colletotrichum* and *Botrytis*, it has recently been reported that *Fusarium proliferatum* also has the ability to demethylate pterocarpans at C-9 (18). This *Fusarium* likewise demethylates at the 3-position, a feature characteristic of *Ascochyta pisi* as well as various other *Fusarium* species (2,11,17).

In previous work on the interaction between *C. coccodes* and the nonhost alfalfa, both medicarpin and its conversion product (CP-I) were reported to have comparable fungitoxic activity (7). However, the earlier bioassays were undertaken with ethyl acetate extracts of culture filtrates rather than with chromatographically pure CP-I as in the present investigation. Differences in purity may perhaps explain why the activity of CP-I against *C. coccodes* now appears to be less than that of medicarpin in bioassays measuring mycelial growth, spore germination and germ tube elongation. This reduced activity was also demonstrated by fluorescein diacetate staining, a common test for cell viability (15). It has been found that germ tubes that cease growth even temporarily due to the effects of pterocarpanoid phytoalexins have a decreased ability to fluoresce in the presence of FDA, an effect that is both time- and concentration-dependent (V. J. Higgins, *unpublished*). The observation that *C. coccodes* germ tubes still formed appressoria after exposure to CP-I whereas medicarpin-treated germ tubes did not, is further evidence for a difference in activity. Maackiain and medicarpin were similarly reported (1) to prevent appressoria formation by germ tubes of *H. carbonum*. This activity could be useful for assaying the sensitivity of those fungi that consistently form conspicuous appressoria on glass slides. The low fungitoxicity of demethylmedicarpin against mycelial growth of *H. carbonum* and *B. cinerea* was noted by Ingham (9). The use, in the present study, of FDA staining and germ tube growth bioassays supports these earlier observations on *Helminthosporium* and *Botrytis*.

The *C. coccodes*-alfalfa interaction has been used as an example of a situation in which a nonpathogen degrades a host phytoalexin to form an equally toxic product (8). As the metabolite CP-I has now been proven to be less inhibitory than medicarpin, further research is clearly needed to investigate the activities of the other conversion products formed in this system. In fact, very small quantities of several additional phenolic compounds were detected on TLC plates but no attempt was made to determine their chemical constitution. Such products would have been present in the ethyl acetate extracts bioassayed in the previous study (8) and may conceivably have been responsible for the observed level of toxicity. The gradually increasing degree of inhibition caused by CP-I in mycelial growth bioassays supports the possibility that this substance is metabolized by *C. coccodes* to form more toxic products.

In an attempt to clarify some structure-activity relationships of pterocarpanoid compounds, maackiain and homopterocarpin were also used in bioassays and conversion experiments with *C. coccodes*. The failure to detect metabolites of homopterocarpin produced by *C. coccodes* under conditions similar to those used for metabolism of medicarpin suggests the involvement of a highly specific fungal demethylase, since homopterocarpin differs from medicarpin only by having an $-OCH_3$ instead of an -OH group at carbon-3 (Fig. I-IV). The absence of homopterocarpin conversion products is also surprising because recent in vitro studies have shown that this pterocarpan is readily metabolized by *F. proliferatum* to isomedicarpin (3-methoxy-9-hydroxypterocarpan) as well as to demethylmedicarpin and its isoflavan derivative (7,2′, 4′-trihydroxyisoflavan) (18). The possibility that *C. coccodes* might metabolize homopterocarpin under other cultural conditions and incubation periods was not examined. The reduced toxicity of

demethylmedicarpin and homopterocarpin is likewise not easily explained. Van Etten and Pueppke (16) suggested that failure to find a clear relationship between activity and structure is due to different modes of action by different compounds. Hopefully, the continued accumulation of structure-activity data will allow some clarification of this problem.

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