Plant Growth Regulatory Effects and Stereochemistry of Cladosporin

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Cladosporin has been isolated from a new source, *Aspergillus repens* De Bary. The metabolite and its diacetate inhibit growth of etiolated wheat coleoptiles at 10^{-3}, 10^{-4}, and 10^{-5} M. Cladosporin does not affect the growth of greenhouse-grown tobacco or corn. However, the diacetate induces chlorosis and stunting in corn at 10^{-2} and 10^{-3} M but produces no visible response in tobacco. The complete stereochemistry of cladosporin is presented for the first time.

In our search for fungal metabolites which show plant growth regulating activity, we isolated from *Aspergillus repens* De Bary a compound which inhibits the growth of etiolated wheat coleoptiles. Physical and chemical characterization of the purified active component showed that it was a previously described compound, cladosporin (I) (Figure 1) (identical with asperentin). Cladosporin (I) has been isolated before from various fungal sources including *Cladosporium cladosporioides* (Scott et al., 1971), *Aspergillus flavus* (Grove, 1972), an unidentified *Aspergillus* (Ellestad et al., 1973), and several *Eurotium* species (Anke et al., 1978). Cladosporin (I) inhibits the germination of spores of several *Penicillum* and *Aspergillus* species at ≤40 μg/mL. Also, it completely inhibits the growth of several dermatophytes on agar medium at 75 μg/mL (Scott et al., 1971). In *Bacillus brevis*, cladosporin (I) completely inhibits the uptake of leucine and uracil into the trichloroacetic acid insoluble fraction of cells without inhibiting the incorporation of thymidine (Anke et al., 1978; Anke, 1979). Later results showed that the antibiotic also inhibits the incorporation of uridine, but not leucine, into cells of the ascitic form of Ehrlich carcinoma in mice (Anke, 1979). We will describe both the plant growth regulatory properties of cladosporin (I) and its diacetate (II) (Figure 1) and its absolute stereostructure.

MATERIALS AND METHODS

Production and Purification of Cladosporin. *A. repens* De Bary (ATCC accession no. 38646) was transferred from contaminated home-processed tomato paste to potato dextrose agar slants and grown at 26 °C for 1 week. Cultures were then maintained at 5 °C until they were transferred to shredded wheat medium in Fernbach flasks (2.8 L). Each flask contained 100 g of shredded wheat, 200 mL of Difco mycological broth (pH 4.8), 2% yeast extract, and 20% sucrose (Kirksey and Cole, 1974). After inoculation flasks were incubated for 11 days at ~26 °C; 300 mL of acetone was then added to each flask and the contents were macerated in a Super Dispax homogenizer. The pulp was squeezed in cheesecloth bags, and the filtrate was put through filter paper on a Buchner funnel. The clear filtrate was reduced in volume under vacuum at 50 °C to an aqueous phase which was extracted twice with equal volumes of ethyl acetate. The extracts were combined, dried over sodium sulfate, and reduced in volume under vacuum at 50 °C to yield a crude solid. This crude solid contained a high yield of crystalline material which was inactive in the coleoptile assay. The remainder of the crude fraction was placed on a 9.0 × 10 cm silica gel column slurry packed in benzene. The column was then eluted with 1.5 L of benzene and 1.3 L each of diethyl ether, ethyl acetate, acetone, and methanol. Individual solvent eluates were reduced in volume under vacuum and tested for plant growth inhibitory activity with wheat coleoptiles. Activity was found in the ether fraction. Therefore, this concentrated eluate was placed on a 4.0 × 50 cm silica gel column again slurry packed in benzene; ~1.0 L of benzene was put through the column, followed by a linear gradient of benzene to acetone (1.0 L of benzene and 1.0 L of acetone). Twenty-milliliter fractions were collected and 25-μL aliquots of these were bioassayed.

Physical and Chemical Analyses. The ultraviolet (UV) spectrum of cladosporin (I) was determined in 95% ethanol with a Beckman Model 35 spectrophotometer. Infrared (IR) spectra were obtained from thin film samples on KBr windows by using a Beckman IR 4210 equipped with a 4X beam condenser. Uncorrected melting points were determined with a Hoover capillary melting point apparatus. ^1H and ^{13}C NMR spectra were obtained on a Varian XL-100-12 spectrometer. Samples were prepared in $CDCl_3$ with Me_4Si as an internal standard. ^1H spectra were obtained in CW mode while ^{13}C spectra were obtained in FT mode. Typical operating parameters for ^{13}C spectra were as follows: spectral width, 5000 Hz; pulse angle, 15°; repetition rate, 1 s; data points, 8K; 100-Hz square-wave modulated proton decoupling. Low-resolution (LRP) mass spectra were measured with a Hewlett-Packard 5985 spectrometer at 70 eV by direct probe. The circular dichroism (CD) measurements were made in methanol with a Jasco J-41A spectropolarimeter. X-ray diffraction measurements were made with Cu Kα radiation (λ = 1.5418 Å) by using a Syntex $P2_1$ automatic diffractometer.

Preliminary diffraction experiments indicated a crystal symmetry of $P2_12_12_1$ with a = 8.689 (1) Å, b = 11.722 (1) Å, and c = 14.272 (2) Å with a calculated density of 1.34 g/cm^3 for Z = 4. Of the 1151 reflections measured, 1053 (91%) were observed ($I \geq 3\sigma I$). The structure was solved by using standard direct methods techniques (Main et al., 1978) and refined by using full matrix least squares (Stewart et al., 1972) by minimizing $\sum[\omega(|F_o| - |F_c|)^2]$ with $\omega = (1/\sigma F_o)^2$. The final unweighted R factor using anisotropic temperature parameters for the nonhydrogen atoms and fixed isotropic temperature parameters for the hydrogens was 0.058.

The diacetate (II) was made by dissolving I in pyridine and acetic anhydride (1:2) overnight under nitrogen at

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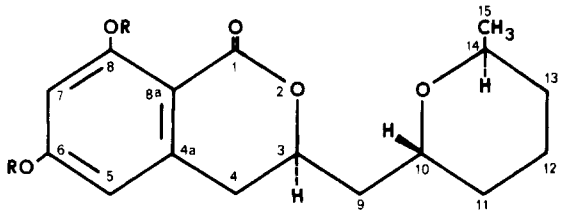

I. R=H
II. R=CH_3CO

Figure 1. Structure and numbering scheme of cladosporin (I) and its diacetate (II).

room temperature. The reaction was quenched with water and ice, and the product was partitioned into ether which was evaporated to give solid II.

Bioassays. Wheat coleoptile sections were cut 4 mm long from etiolated, 4-day-old *Triticum aestivum* L. cv. Wakeland seedlings grown in the dark at 22 ± 1 °C. They were incubated in pH 5.6 buffered solutions of phosphate–citrate containing 2% sucrose (Nitsch and Nitsch, 1956). Cladosporin (I) or its diacetate (II) was dissolved in acetone (Cutler, 1968) and added to the assay solution to produce concentrations of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M. Ten coleoptile sections were introduced to each test solution (2 mL) and were incubated for 24 h at 22 °C in a roller-tube apparatus that rotated at 0.25 rpm. Sections were then measured (X3 images produced by a photographic enlarger) and data were statistically analyzed (Kurtz et al., 1965).

Solutions of I and II were tested on intact greenhouse-grown corn and tobacco plants at concentrations of 10^{-2}, 10^{-3}, and 10^{-4} M. Formulations consisted of 10% acetone plus 0.1% Tween-20 in water. Six-week-old tobacco seedlings (*Nicotiana tabacum* L. cv. Hicks) were individually treated with a 1-mL aerosol per plant. Ten-day-old corn seedlings (*Zea mays* L. cv. Norfolk Market White) were treated with 100 μL of each test solution pipetted into each leaf whorl. Four corn seedlings were used per treatment and all treatments were triplicated.

RESULTS AND DISCUSSION

Physical and Chemical Analysis. *A. repens* produced ~1 g of cladosporin (I) from 72 flasks of shredded wheat media in 11 days. The R_f values for I and II were 0.64 and 0.68, respectively, on silica gel TLC plates developed with toluene–ethyl acetate–formic acid (5:4:1). Both I and II appeared as dark blue spots at 254 nm, while II was visible as a bright yellow fluorescent spot at 366 nm. The melting points for I and II are 180–184 and 72–73 °C, respectively. The UV spectra of I showed maxima at 217 nm (log ε = 4.36), 224 nm (shoulder), 270 nm (log ε = 4.16), and 303 nm (log ε = 3.78). The IR spectra of I gave peaks at 3220, 2945, 2920, 2865, 1615, 1590, 1575, 1160, 1110, 1035, 835, and 795 cm^{-1} while II showed peaks at 2930, 2860, 1770, 1715, 1605, 1365, 1250, 1183, 1130, 1082, 1045, and 900 cm^{-1}. The LRP analysis of I gave a molecular ion peak (M^+) at *m/e* 292.3 consistent with a formula of $C_{16}H_{20}O_5$ with fragment ions at *m/e* 274 ($M^+ - H_2O$), 256 ($M^+ - 2H_2O$), and 207 ($M^+ - C_5H_9O$). The M^+ for II was observed at *m/e* 376.3 with prominent ion fragments at *m/e* 334 ($M^+ - CH_2CO$) and 292 ($M^+ - 2CH_2CO$).

Table I gives the ^1H and ^{13}C NMR parameters for cladosporin (I) and its diacetate (II). Chemical shifts were assigned on the basis of the single frequency, off-resonance proton decoupled spectra, chemical shifts in similar compounds (Stothers, 1972; Sankawa et al., 1978), and the differences expected between the hydroxy and acetate functional groups (Wehrli and Wirthlin, 1976). The CD

Table I. ^{13}C Chemical Shifts[a] for Cladosporin (I) and Its Diacetate[b] (II)

carbon	I	II
1	169.5	161.2
3	76.0 (4.76)	75.3 (4.70)
4	38.7 (2.85)	39.1 (2.90)
4a	141.2	142.4
5	106.8 (6.27)	118.1 (6.95)
6	163.9[c]	154.2[c]
7	101.4 (6.20)	116.3 (6.90)
8	164.4[c]	152.7[c]
8a	100.4	115.3
9	33.5 (2.05)	34.4 (2.03)
10	66.2[d] (4.00)	66.3[d] (3.97)
11	30.5[e] (1.33, 1.60)	30.8[e] (1.33, 1.65)
12	18.2 (1.60)	18.2 (1.65)
13	31.1[e] (1.33, 1.60)	30.9[e] (1.33, 1.65)
14	66.9[d] (4.00)	67.3[d] (3.97)
15	19.2 (1.18, J = 6.6)	19.1 (1.15)
CH_3CO		20.9, 21.1 (2.3)
CH_3CO		167.2, 169.0

[a] In ppm downfield from Me_4Si. [b] Proton data are in parentheses. [c–e] Assignments may be reversed.

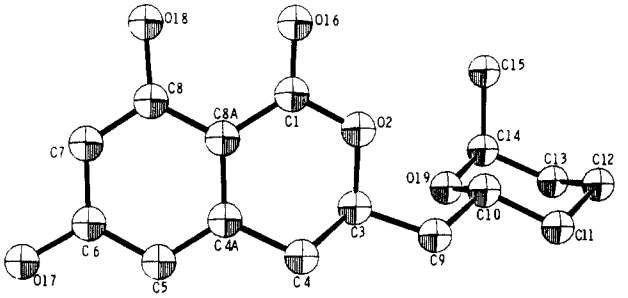

Figure 2. Computer-generated perspective drawing of cladosporin (I) from X-ray coordinates with hydrogens omitted for clarity.

spectra measured at 157 μM gave maxima and minima with the following characteristics [λ (nm), Δε (M^{-1} cm^{-1})]: 303, –0.72; 268, 2.35; 248, –0.67; 234, 2.27.

The above data are totally consistent with the structure reported for cladosporin (I) (Scott et al., 1971; Grove, 1972). The crystal structure determination of I established the stereochemistry of the tetrahydropyran ring at positions C10 and C14. The absolute stereochemistry at position C3 was previously determined to be *R* by comparison of the CD spectra with a known compound (Grove, 1972). Consequently, the present X-ray structure completes the absolute stereochemical assignment of cladosporin (I) as being C10 (*R*) and C14 (*S*). The results are shown in Figure 2 (Johnson, 1970) and in Tables II, III, and IV which can be found in the supplementary material (see paragraph at end of paper regarding supplementary material).

Biological Results. Both cladosporin (I) and cladosporin diacetate (II) significantly ($P < 0.01$) inhibited the growth of etiolated wheat coleoptiles at 10^{-3}, 10^{-4}, and 10^{-5} M. The results for both sets of data are virtually identical (Figure 3). Generally, alteration of a compound is readily manifested in the sensitive coleoptile assay (Cutler et al., 1978, 1979).

Neither I nor II produced any visible effects when sprayed onto 6-week-old tobacco seedlings. When 10-day-old corn seedlings were treated with I, no changes in plant growth were noted, but when treated with II the response was dramatic. Within 24 h, the 10^{-2} and 10^{-3} M treated plants were very chlorotic compared to the controls. After 48 h, the 10^{-2} M treated plants were both chlorotic and necrotic with symptoms of stem collapse. Plants treated at 10^{-3} M were chlorotic; however, some patches of green tissue (<10%) were visible on the leaves. After

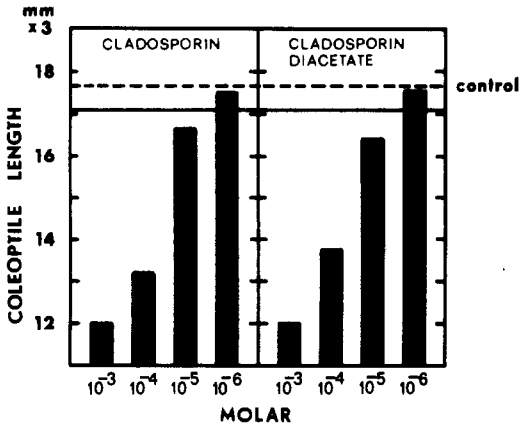

Figure 3. Growth inhibitory activity of cladosporin (I) and cladosporin diacetate (II) in etiolated wheat coleoptile bioassays (*T. aestivum* L. cv. Wakeland). Control: dashed line. Significant inhibition: below solid line ($P < 0.01$).

12 days, the growth of the 10^{-2} and 10^{-3} M treated plants was inhibited relative to that of controls.

Two questions and speculations concerning the biological properties of cladosporin (I) arise as a result of this study. First, can I, a demonstrated antifungal agent, be chemically manipulated into an economical higher plant growth regulator? Second, can the wheat coleoptile assay be used more generally to detect fungistatic and fungitoxic agents? We intend to explore these possibilities further.

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Supplementary Material Available: Tables II, III, and IV containing the fractional coordinates and temperature parameters, bond distances, and bond angles of I from the X-ray experiments (4 pages). Ordering information is given on any current masthead page.

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