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Microbial Transformation of Macrocyclic Trichothecenes

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A resting culture of *Rhizopus arrhizus* (ATCC 11145) transformed verrucarin A into 16-hydroxyverrucarin A, whereas *R. arrhizus* transformed verrucarin B into a mixture of 16-hydroxyverrucarin B and 3'-hydroxyverrucarin A. Relative to verrucarins A and B, the 16-hydroxy derivatives showed marked increases in activity, as tested in vivo against P388 mouse leukemia.

The trichothecene antibiotic complex has generated a great deal of interest in recent years due to the wide range of biological activity observed with this class of sesquiterpenes (3, 6, 13, 16). These mycotoxins, which are produced by a variety of common soil fungi (e.g., *Fusarium*, *Trichoderma*, and *Myrothecium* spp., etc.), are potent protein synthesis inhibitors and exhibit marked cytotoxicity and cytostaticity. It is these latter properties which make the trichothecenes attractive candidates as potential cancer chemotherapeutic agents.

Our interest has been centered on the macrocyclic trichothecenes (6, 15) (Fig. 1), since several members of this series have been shown to be exceptionally active in vivo against mouse P388 leukemia. In earlier work, we have shown that the potent antileukemic baccharinoids (baccharinol [11]) isolated from the Brazilian shrub *Baccharis megapotamica* are the result of a unique interaction between a soil microorganism and a higher plant (7). In its native habitat, *B. megapotamica* appears to acquire roridins (see legend to Fig. 1) produced by a nearby soil fungus, which are then taken up by the plant and transformed into baccharinoids. The hydroxylation at C-8 (see structure of baccharinol [see legend to Fig. 1]) transforms the P388 in vivo inactive roridins into baccharinoids which are quite active in this test system (7). In a related study, we have shown that, in the laboratory, fungus-produced macrocyclic trichothecenes which are inactive in vivo against P388 can be converted into highly active compounds by A-ring oxygenation (8).

Although this A-ring oxygenation can be carried out by chemical means, we sought other methods which might prove more selective and higher yielding. Though there are a few reports of biotransformations of simple trichothecenes (2, 4, 5, 12, 17), there have been no attempts made, to our knowledge, on microbial transformations of macrocyclic trichothecenes. We have randomly screened several fungi as well as bacteria obtained from the American Type Culture Collection (Rockville, Md.) and the Northern Utilization Research and Development Division (U.S. Dept. of Agriculture, Peoria, Ill.); in this paper, we report the transformations of verrucarins A and B by *Rhizopus arrhizus*.

MATERIALS AND METHODS

Verrucarins A and B were obtained from a culture of *Myrothecium verrucaria* as described elsewhere (9). *R. arrhizus* (ATCC 11145) was purchased from the American Type Culture Collection. The fungus was grown in medium composed of the following: glucose, 20 g; KH_2PO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 2 g; NH_4Cl, 3 g; $FeSO_4 \cdot 7H_2O$, 0.2 g; peptone, 2 g; yeast extract, 2 g; and malt extract, 2 g. The medium was made up to 1 liter with distilled water.

Melting points were determined on a Fisher-Johns hot stage melting point apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectra were determined in deuteriochloroform on a Varian XL-100 spectrometer with tetramethylsilane as an internal standard. Flash chromatography was carried out on silica gel (230 to 400 mesh; E. Merck AG, Darmstadt, Germany) as described by Still et al. (14). Thin-layer chromatography (TLC) was performed on precoated TLC sheets of silica gel F 254 (0.25 mm; Merck) supported on aluminum. Visualization usually involved viewing developed plates under short-wavelength UV light, later spraying the plates with vanillin-sulfuric acid (0.5 g of vanillin in 100 ml of H_2SO_4)-ethanol ([vol/vol] 4:1), and heating until the spots were colored. High-performance liquid chromatography was done with a Liquid Chromatograph 6000 (Waters Associates) coupled with model 660 Solvent Programmer and Spectraflow monitor SF 770. A Whatman Magnum 9 (10/50) semipreparative partisil column was used. Separations were monitored by UV absorbance at 260 nm.

Fermentation and isolation. (i) Transformation of verrucarin A. *R. arrhizus* was grown in the above medium (5 liters; 1 liter of medium per 4-liter flask). After 2 days, the culture was preincubated with verrucarin A (4 μg/ml). One day later, the mycelia were separated from the medium, washed with water, and resuspended in water (5 liters; 1 liter per flask). Verrucarin A (100 mg per flask) was added, and the transformation was monitored by TLC. At the end of

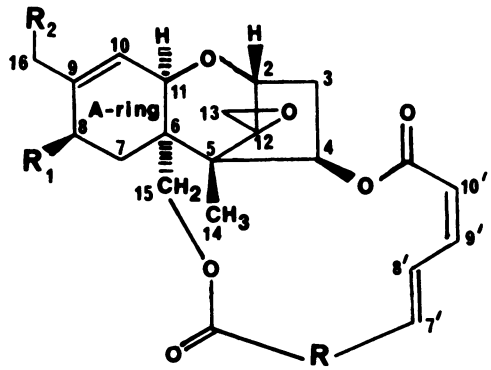

FIG. 1. General structure of macrocyclic trichothecenes. Definitions of R groups for several trichothecenes are as follows.

Verrucarin A, $R_1 = R_2 = H$; R = $-\overset{2'}{C}H(OH)-\overset{3'}{C}H(CH_3)-\overset{4'}{C}H_2-\overset{5'}{C}H_2-\overset{6'}{C}(=O)O-$ (12')

16-Hydroxyverrucarin A, $R_1 = H$; $R_2 = OH$; R = $-CHOHCHCH_3CH_2CH_2OC(=O)-$

Verrucarin B, $R_1 = R_2 = H$; R = $-CH(O)-CCH_3CH_2CH_2OC(=O)-$

16-Hydroxyverrucarin B, $R_1 = H$; $R_2 = OH$; R = $-CH(O)-CCH_3CH_2CH_2OC(=O)-$

3'-Hydroxyverrucarin A, $R_1 = R_2 = H$; R = $-CHOHCOH(CH_3)CH_2CH_2OC(=O)-$

Baccharinol, $R_1 = OH$; $R_2 = H$; R = $-CH(O)-CCH_3CHOHCH_2OCHCHOHCH_3$

Roridin A, $R_1 = R_2 = H$; R = $-CHOHCHCH_3CH_2CH_2OCHCHOHCH_3$

10 days, the mycelia were separated from the fermentation beer, and the latter was extracted with ethyl acetate. The mycelia were extracted with methanol, concentrated, diluted with water, and extracted with ethyl acetate. Both mycelia and beer extracts were combined and evaporated. The crude extract was subjected to flash column chromatography. Elution of the column with 1% methanol in methylene chloride yielded unchanged verrucarin A (0.40 g [80%]). Elution with 3% methanol in methylene chloride furnished a more polar compound (0.05 g [10%; 50% based on recovered verrucarin A]), which was further purified by high-performance liquid chromatography and crystallization from acetone-hexane to yield a white crystalline solid. This compound was shown to be identical to 16-hydroxyverrucarin A (8) by comparison with an authentic sample by TLC and NMR spectroscopy.

(ii) Transformation of verrucarin B. *R. arrhizus* was grown in the above medium (1 liter per 4-liter flask). After 2 days, the culture was preincubated with verrucarin B (4 μg/ml), and 1 day later, verrucarin B was added in a higher concentration (100 μg/ml [0.10 g]). The transformation was monitored by TLC, and 7 days after the addition of verrucarin B, the culture was processed as described above. The extract was subjected to flash chromatography. Elution with ethyl acetate yielded two new trichothecenes, namely, 3'-hydroxyverrucarin A (0.022 g [22%]) and 16-hydroxyverrucarin B (0.007 g [7%]). The latter decomposed at temperatures >230°C. Methanolysis of 16-hydroxyverrucarin B yielded 16-hydroxyverrucarol which was shown to be identical to an authentic sample by TLC comparison.

For 16-hydroxyverrucarin B, mass spectrum (chemical ionization, methane gas reagent), *m/e* 517.2068 ($M^+ + H$ calculated 517.2072); ^1H NMR ($CDCl_3$) δ 0.90 (3H, s, 14-H), 1.58 (3H, s, 12'-H), 3.00 (2H, AB, J = 4 Hz, 13-H), 3.41 (1H, s, 2'-H), 3.60 (1H, d, J = 5 Hz, 2-H), 3.89 (1H, d, J = 5 Hz, 11-H), 5.74 (1H, d, J = 5 Hz, 10-H), 6.08 (1H, d, J = 16 Hz, 8'-H), 6.18 (1H, d, J = 11 Hz, 7'-H), 6.66 (1H, d, d, J = 11 and 11 Hz, 10'-H), 7.94 (1H, d, d, J = 11 and 16 Hz, 9'-H).

For 3'-hydroxyverrucarin A, mass spectrum (chemical ionization, methane gas reagent), *m/e* 519.2225 ($M^+ + H$ calculated 519.2228); ^1H NMR ($CDCl_3$) δ 0.82 (3H, s, 14-H), 1.25 (3H, s, 6'-H), 1.74 (3H, s, 16-H), 2.97 (2H, AB, J = 4Hz, 13-H), 3.60 (1H, d, J = 5Hz, 2-H), 3.84 (1H, d, J = 5Hz, 11-H), 4.47 (2H, d, J = 10 Hz, 15-H), 5.43 (1H, d, J = 5 Hz, 10-H), 6.02 (1H, d, J = 16 Hz, 8'-H), 6.12 (1H, d, J = 11 Hz, 11'-H), 6.72 (1H d, d, J = 11 and 11 Hz, 10'-H), 8.06 (1H, d, d, J = 11 and 16 Hz, 9'-H).

RESULTS AND DISCUSSION

The following cultures were screened for the transformation of verrucarin A: *Wojnovia graminins* (NRRL 2472), *Hypholoma* sp. (NRRL 2471), *Bacillus megaterium* (NRRL 3938), *Hendersonia acicola* (NRRL 2595), *Curvularia lunata* (NRRL 2380), *Cephalosporium acremonium* (NRRL 3092), *Rhizopus arrhizus* (ATCC 11145), and *Streptomyces roseochromogenus* (ATCC 13400). Only *R. arrhizus* and *C. acremonium* transformed verrucarin A into more polar products. *C. lunata* consumed verrucarin A rapidly,

but no transformation products were observed on TLC. *R. arrhizus* transformed verrucarin A to 16-hydroxyverrucarin A. The identity of this compound was confirmed by co-TLC and by comparison of its NMR spectral data with that of an authentic sample of 16-hydroxyverrucarin A that was synthesized from verrucarin A in our laboratories (8). It is interesting that allylic hydroxylation of verrucarin A with selenium dioxide yielded 8β-hydroxyverrucarin A as the major product (48%) and 16-hydroxyverrucarin A as the minor product (8%). However, microbial hydroxylation of verrucarin A gave 16-hydroxyverrucarin A as the sole observed product, though the yield was only 50%, based on recovered verrucarin A. 16-Hydroxyverrucarin A exhibited substantial activity against P388 mouse leukemia with a T/C value of 252 at a 4-mg/kg dose level, whereas the parent compound (verrucarin A) shows a T/C value of only 127 at a dose level of 2 mg/kg. (T/C = [days test animals live/days control animals live] × 100. A T/C value of ≥125 is considered significant.) Hence, this transformation is very useful to convert toxic verrucarin A to a more active antileukemic compound.

R. arrhizus was also screened for the possible transformation of verrucarin B. Verrucarin B was modified by *R. arrhizus* to form two more polar compounds. The less polar of the two transformation products, I, was formed in 22% yield. Comparison of the ^{1}H-NMR spectrum of product I with that of verrucarin B revealed one easily noticeable difference: the singlet at δ 3.40 which is due to 2′-H of the epoxide on verrucarin B was absent. Also, the 12′-CH_3 attached to C-3′ of the epoxide shifted from δ 1.58 in verrucarin B to δ 1.25 in product I. These spectral features indicate that the 2′,3′-epoxide was probably hydrated to form 3′-hydroxyverrucarin A. ^{13}C-NMR spectral data for I supports the above structure assignment. The signals due to C-2′ and C-3′ of verrucarin B at 58.0 ppm and 61.1 ppm, respectively, have moved downfield to 72.0 ppm, indicating the presence of hydroxyl groups at C-2′ and C-3′. Hence, based on NMR spectral data and mass spectral data, product I was shown to be 3′-hydroxyverrucarin A.

The more prevalent transformation product II exhibited proton NMR signals very similar to those of verrucarin B except for the absence of the 16-CH_3 signal and for the downfield shift of H-10 from δ 5.45 to δ 5.74. These two differences in NMR signals between the parent and the transformation product indicate that the latter is probably 16-hydroxyverrucarin B. Methanolysis of the above compound yielded 16-hydroxyverrucarol (Fig. 2), and hence transformation product II is confirmed to be 16-hydroxyverrucarin B.

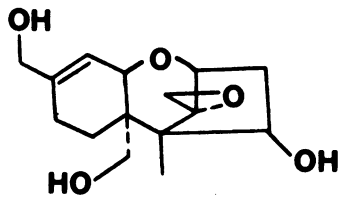

FIG. 2. Structure of 16-hydroxyverrucarol.

The microbial transformation discussed above is very useful for obtaining 16-hydroxy derivatives of verrucarins which are more active against P388 leukemia than the parent compounds. Also, this particular transformation is unusual for *R. arrhizus*. To our knowledge, *R. arrhizus* usually oxidizes methylene groups to secondary alcohols (1, 10), whereas, in verrucarin A, the vinyl methyl group is oxidized to a primary alcohol.

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