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Microbiological Systems in Organic Synthesis

1. The Stereospecific Oxidation of Glaucine by *Fusarium solani*

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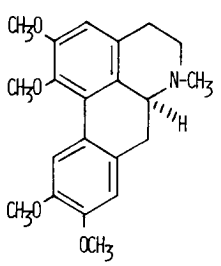
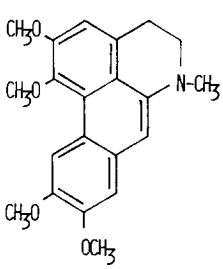
Fusarium solani stereospecifically and quantitatively oxidizes *S*-(+)-glaucine to dehydroglaucine. *R*-(−)-glaucine was not metabolized, and *R,S*-glaucine was oxidized to the extent of 50%. The reaction proceeds without production of complicating side products. The conversion represents a form of microbial destructive resolution of an alkaloidal racemate.

Microorganisms perform highly selective chemical modifications of a wide variety of organic compounds, and compendia are available to aid in the selection of specific microorganisms which might be capable of achieving selected chemical transformations (*1–6*). Such reactions are mediated by the enzyme systems of bacteria, fungi, and yeasts, and they are often characterized by high stereochemical and/or functional group selectivities (*7*). Indeed stereospecific reductions and oxidations have been used for the synthesis of chiral products from both chiral and achiral substrates (*7–12*). Further examples involve the enzymatic resolution of racemates (*13–15*). While the majority of such work has been conducted with aliphatic and alicyclic ketonic substrates, little has been done within the field of alkaloids and nitrogen heterocyclic compounds in general. This area of application is particularly appealing, since only one of several possible stereoisomers usually exists in nature. Consequently, little information about the biological properties of other possible stereoisomers is available.

We previously reported that the alkaloid glaucine (**I**) is stereospecifically oxidized to dehydroglaucine (**II**) by the fungus *Fusarium solani* (ATCC 12823)



I



II

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(*16*). While data from preparative scale conversions indicated a high-yielding reaction, the methodology utilized required a tedious isolation by preparative layer tlc, followed by quantitation by uv spectroscopy and optical purity determinations. It was difficult to draw firm conclusions from early experiments because of low and variable recoveries of (I) and (II) and also because the metabolism of the unnatural aporphine isomer was not examined. Difficulties experienced in the isolation of aporphines (*17*) have been bypassed by the application of an hplc method (*18*) which has allowed the accurate determination of the enantioselectivity achieved by *F. solani* on *R*-(−)-, *S*-(+)-, and *R,S*-glaucines. This report describes the stereospecific dehydrogenation of *S*-(+)-glaucine by *F. solani*.

EXPERIMENTAL

All solvents were analytical reagent grade or better in quality. Solvents for hplc were of hplc grade (Lichrosolv, MCB). Boldine (K&K Nutritional Biochemicals) and papaverine–HCl (Sigma) were homogeneous by tlc and gave melting points according to literature values. Diazald and a diazomethane-generating apparatus were purchased from Aldrich Chemical Company.

Nuclear magnetic resonance spectra were generated in $CDCl_3$ using a Varian Model HA-100 spectrometer with TMS as the internal standard. Mass spectra were taken on a Dupont Model 21491 mass spectrometer by direct probe insertion. Optical rotation data were measured on a Perkin–Elmer Model 141MC polarimeter using a 1-dm microcell (1.0-ml capacity). Infrared spectra were taken as KBr pellets using a Beckman Model IR-5A spectrometer. Melting points were taken on a Fisher Model 355 digital melting point apparatus, and are corrected.

All thin-layer chromatography (tlc) was performed on plastic-backed 0.25-mm silica gel GF_{254} plates (Polygam, Brinkman), eluted with benzene : methanol (4 : 1). Plates were visualized under 254- or 280-nm light, followed by spraying with either modified Dragendorff's reagent or cerric ammonium sulfate (1% in 50% phosphoric acid) as described earlier (*16*).

The hplc analyses were conducted using a Tracor Model 950 pump and 970A variable wavelength detector set at 280 nm. The unit was fitted with a Rheodyne Model 7125 injector with a 20-μl fixed volume loop (all injections were 20 μl). The column used was a μ-Bondapak Phenyl (Waters), 10 μm, 30 cm × 3.9 mm i.d. The mobile phase for isocratic elution was acetonitrile–methanol–0.05 *M* KH_2PO_4 (2 : 4 : 5). The mobile phase components were filtered separately (GF/F Whatman), then combined in the appropriate ratio, and degassed ultrasonically prior to use. The flow rate used was 2.0 ml/min (1450 psi). Under these conditions T_r glaucine = 3.4 min; T_r papaverine = 4.4 min; T_r dehydroglaucine = 9.1 min. A Hewlett–Packard Model 3380A electronic integrator, programmed for analysis by peak area, was used for chromatographic recording and data generation. Statistical analysis of the resultant data was performed on a Wang Model 600 computer.

Preparation of Substrates

S-(+)-Glaucine (**I**) was prepared by methylation of boldine using diazomethane according to the method of Neumeyer *et al.* (*19*). Dehydroglaucine (**II**) was prepared as described previously (*16*). *R,S*-Glaucine (*R,S*-**I**) was prepared by dissolving metal reduction of **II** according to the method of Cava *et al.* (*20*). *R,S*-Glaucine thus produced was resolved with (+)-tartaric acid as follows: the crude *R,S*-glaucine (1.8 g, 5 mmol) was dissolved in 12 ml abs ethanol and combined with 0.790 mg (5 mmol) (+)-tartaric acid (Aldrich) in 8 ml abs ethanol (both solutions boiling), and crystallization allowed to proceed at room temperature. Yield and optical rotation values on this and subsequent crystallizations were 1.170 g (−25.0°), 1.075 g (−28.22°), 0.900 g (−32.35°), and the final recrystallization yielded 825 mg (55% theoretical yield based on 2.1 g *S*-glaucine): $[\alpha]_D^{25}$ −33.35° (c = 1.003, H_2O) [rept. (21, 22), −33°]; mp 212–214°C [rept. (21, 22), 214–215°]. The tlc and hplc analyses indicated no dehydroglaucine (**II**).

Incubation Conditions

Stock cultures of *F. solani* (ATCC 12823, American Type Culture Collection, Rockville, Md.) were maintained on Sabouraud maltose-agar slants in sealed screw-cap tubes at 5°C with periodic (4-month) transfers to maintain viability. Incubations were conducted in 125-ml Bellco DeLong culture flasks as described previously using an NBS G-25 Environmental Shaker (New Brunswick Scientific, Edison, N.J.) at 250 rpm and 27°C. The medium (25 ml/flask) consisted of dextrose (20 g), NaCl (5 g), K_2HPO_4 (5 g), yeast extract (5 g), Acidicase peptone (BBL) (5 g), distilled deionized water (1 liter), and was adjusted to pH 7.0 prior to autoclaving (15 psi, 15 min). First-stage flasks were initiated by suspending spores from a slant into sterile medium and transferring the suspension to a 125-ml flask all under aseptic conditions. These cultures (Stage 1) were incubated as described above for 72 hr. A 2-ml portion was used to inoculate Stage 2 flasks which were allowed to incubate under the same conditions for 24 hr prior to the addition of substrate.

Metabolism of R-, S-, and R,S-glaucines by F. solani (ATCC 12823)

F. solani was grown according to the procedure described above, and 12.5 mg of each of the glaucines (expressed as free alkaloid bases) were added to 24-hr-old Stage 2 culture flasks as follows: *S*-(+)-glaucine (free base), 12.5 mg in 50 μl DMF plus 1 ml sterile distilled water; *R*-(−)-glaucine-(+)-tartrate, 17.8 mg in 1 ml sterile water plus 50 μl DMF; and *R,S*-glaucine, 6.25 mg *S*-(+)—glaucine in 50 μl DMF plus 8.90 mg *R*-(−)-glaucine-(+)-tartrate in 1 ml sterile water. A variety of controls were used to confirm the metabolizing capability of cultures containing *R*- and *R,S*-glaucines, and to evaluate the extent of spontaneous air oxidation of glaucine to dehydroglaucine (*16*). Triplicate controls of autoclaved 24-hr Stage 2 cultures containing 12.5 mg *S*-(+)-glaucine were incubated along with live cultures for 7 days, and pH 6 and 9 (0.05 *M* phosphate) buffers containing the same level of substrate were incubated for 72 hr.

Flasks were harvested immediately following substrate addition (T_0) and at 24-hr intervals over a 7-day period, and were stored frozen until required for analysis. Triplicate flasks were harvested for each substrate at each time interval (24 independent analyses for each substrate) and were analyzed independently for remaining glaucine substrate and for dehydroglaucine formed.

Culture Analysis

In experiments where pH and/or glucose (Tes-Tape) were measured, cultures were analyzed at room temperature after thawing prior to the addition of internal standard.

Flasks were thawed and 2.5 ml of the papaverine–HCl internal standard solution (1.40 g in 100 ml 0.2 *N* HCl) added. The addition of the internal standard at the first step negates any problems of culture-volume variation. Flasks were incubated at 250 rpm for 15 min and then fully homogenized (Polytron, Brinkman). All glassware used beyond this point was fully silylated with 10% trimethylchlorosilane (Pierce) in toluene prior to use. A 5-ml aliquot of the culture homogenate was alkalinized with 1 ml 10% potassium hydroxide, extracted with 2 ml ethyl acetate by gentle rocking (18 rpm) for 15 min, and centrifuged. A total of 50 μl of the organic layer was taken to dryness and reconstituted in 1 ml acetonitrile : methanol (1 : 1) prior to hplc analysis.

RESULTS AND DISCUSSION

Microorganisms and enzymes derived from them have been applied as "reagents" in organic synthesis (*1–6*), largely due to the high degree of stereoselectivity often observed vs conventional reagents (*7*). One of the most useful applications of microbial/enzymatic systems is in the resolution of stereoisomeric mixtures of compounds. Such reactions become important in preparing and evaluating unnatural stereoisomers as potential drug substances (*23, 24*). The use of whole cells in such reactions overrides the tedious process of isolating purified enzymes, and makes them simple to use and ideal reagents.

The mode of glaucine transformation by microorganisms was elucidated in a previous study (*16*). Dehydroglaucine (**II**) was by far the major product formed when glaucine was used as substrate with *F. solani,* and low levels of the oxoaporphine (oxoglaucine) were produced as an artifact by the well-known air oxidation of dehydroglaucine (*16, 17*). Since the dehydrogenation reaction involved the sole chiral center of glaucine, we suggested and demonstrated chiral specificity for the *S*-enantiomer (**I**). However, neither the extent of metabolism nor the degree of stereoselectivity could be accurately ascertained. The development of a new analytical high-performance liquid chromatographic method (*18*) allowed for the determination of residual glaucine, and the product dehydroglaucine, in a quantitative manner.

Recoveries of glaucine and dehydroglaucine were nearly quantitative in all

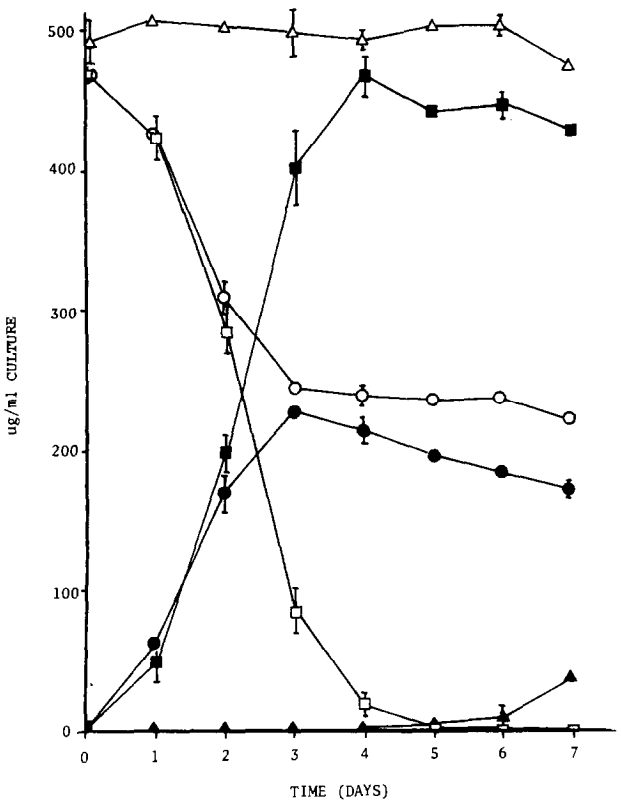

FIG. 1. Time course of glaucine metabolism by *Fusarium solani*. □, *S*-(+)-Glaucine (I); ■, dehydroglaucine (II) generated from *S*-(+)-glaucine (I); △, *R*-(−)-glaucine (R-I); ▲, dehydroglaucine (II) generated from *R*-(−)-glaucine (R-I); ○, *R,S*-glaucine (RS-I); ●, dehydroglaucine (II) generated from *R,S*-glaucine (RS-I).

cases examined, and the results obtained with *S*-(+)-, *R*-(−)-, and *R,S*-glaucines are presented in Fig. 1. *F. solani* completely converted *S*-(+)-glaucine (I) to dehydroglaucine (II) over a 5-day period, and during the incubation period the pH steadily rose from 6.55 to 8.81. *R*-(−)-Glaucine is not used by *F. solani* for the first five days of incubation, but low levels of II are observed at later times. A total of 6% of glaucine substrate is used, and control experiments suggest that the observation is not artifactual. One-half of the racemic glaucine (Fig. 1) is metabolized to dehydroglaucine. We had shown earlier that unused and recovered glaucine substrate gradually became enriched in the unnatural *R*-(−)-glaucine enantiomer (*16*). It is interesting to note that the rate of racemic glaucine oxidation (78 mg/ml/day) is considerably slower than the metabolic rate for pure *S*-glaucine (120 mg/ml/day). While an acceptable rate of metabolism of the *S*-enantiomer occurs in the racemic mixture, it is apparent that the presence of *R*-glaucine inhibits the rate of dehydrogenation.

A slight, but essential, modification of the original incubation medium (*16*) was important to the completion of this study. Substitution of Acidicase peptone for

the original particulate soybean meal medium component provided a completely soluble medium, desirable for analytical work. Instead of the usual white pelleted growth obtained in the other medium, a greenish-black and homogeneous growth occurred with the peptone present. Thin-layer chromatographic analysis indicated that no oxoglaucine was formed during the course of incubations with the new medium, which may be due to the presence of a "natural antioxidant."

These analytical studies show conclusively that the oxidation of glaucine by *F. solani* is both stereospecific and quantitative. This work strongly suggests the potential synthetic utility of this microbial reaction for application in the "destructive" resolution of racemic aporphines. This is important because most naturally occurring aporphines are of the *S*-(+)-configuration, and their biological activities are normally determined on a single isomer (*25*). Many aporphines can now be racemized by dehydrogenation (*26*) followed by nonstereoselective reduction (*16, 20*) or catalytic racemization (*27*). However, one of the major yield limitations in these processes is associated with the loss of compound during classical resolution workup procedures. The dehydrogenation of *S*-(+)-glaucine by *F. solani* is a facile, uncomplicated procedure, and the culture or purified enzymes from it may provide a convenient, general biochemical method for resolving racemic aporphine alkaloids. The scope and specificity of this reaction are under investigation with aporphines of synthetic and pharmacological interest.

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