bination with the reference concentration of (*a*), gave a relative potency [as compared to the reference concentration of (*a*)] of greater than 110% or less than 90% has been termed the "interference threshold." These limits were selected because the 95% confidence range of an average microbiological assay is ±10%. Any assay within this range of theoretical is considered normal variation and any assay beyond this range is considered significant.

The lowest concentration of (*b*) alone which caused a measurable response in the assay procedure of (*a*) is called the "sensitivity threshold."

The interference thresholds and the sensitivity thresholds for the antibiotics and methods tested are given in Tables I and II, respectively.

## DISCUSSION

The information given in Table I makes it possible to predict whether the assay of an antibiotic will be complicated by interference from a second antibiotic included in the formulation, which then must be eliminated.

Table II describes the effects of single antibiotics on various assays. The information given in this table can be applied in numerous ways, such as: (*i*) a guide to determine if interference can be expected from different antibiotics in a given assay procedure; (*ii*) a guide to the specificity of the given assay procedure; (*iii*) a means of qualitative identification of unknowns; and (*iv*) selection of an alternative organism for various antibiotics and combinations of them. In dealing with the latter, caution must be exercised, because in many cases the zones are not as clear and well defined as those usually obtained with the original method.

It was noted in many cases that (*b*) alone produced inhibition in the assay for (*a*) at a much lower concentration than that which caused interference when combined with the reference concentration of (*a*). For example (see Table II), 200 mcg./ml. of viomycin will cause inhibition in the penicillin assay without penicillin, but (Table I) more than 500 mcg./ml. of viomycin is necessary to cause interference in the assay with penicillin present. This is due to the fact that assay conditions may be more favorable to (*a*) so that they mask the effect of (*b*). Factors such as pH and ionic strength of the solvent, composition of the nutrient medium, and incubation temperature are significant. Therefore, the data should not be construed to indicate any synergistic or antagonistic relationships.

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*Keyphrases*

Antibiotic combinations—microbial analysis

Interference threshold—antibiotics

Sensitivity threshold—antibiotics

Analysis specificity—interference elimination

# Conversion of Griseophenone A to (±)-Dehydrogriseofulvin in the Presence of Horseradish Peroxidase and Hydrogen Peroxide

By ALVIN SEGAL and ELMORE H. TAYLOR

**The horseradish peroxidase catalyzed conversion of griseophenone A (I) to (±)-dehydrogriseofulvin (V) has been demonstrated. The results support a one-electron oxidative coupling mechanism previously proposed.**

Barton and Cohen (1) first suggested that in the biosynthesis of the antibiotic (+)-griseofulvin (VII) (Scheme I), the chlorobenzophenone (I) could conceivably be converted to the spiran, (−)-dehydrogriseofulvin (V), *via* a 1-electron oxidative coupling mechanism proceeding through the formation of the intermediate diradical (III). Chemical synthesis of (±)-V using 1-electron oxidizing agents supports this hypothesis (2–4). The final step was the stereospecific enzymatic reduction of (−)-V to form (+)-VII (5–7).

(+)-Griseofulvin (VII) was first isolated from the mycelium of *Penicillium griseofulvum* (8). It was subsequently shown to be a metabolic product of many species of *Penicillia* (9).

The enzyme peroxidase has been demonstrated to be present in species of *Penicillia* (10), and has been implicated in the biosynthesis of fungal metabolites such as the ergot alkaloids (11). The peroxidase catalyzed O—C oxidative coupling of phenols has been reported (12). In addition, 1-electron transfer mechanisms have been proposed for coupled oxidations involving peroxidase (13). On the basis of this information the authors decided to investigate the possibility of converting I to V *via* peroxidase catalysis.

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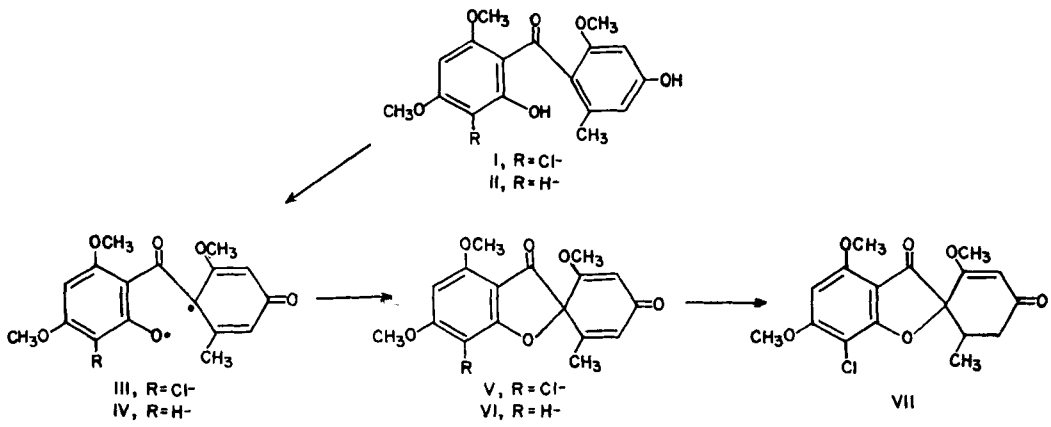



*Scheme I*

## EXPERIMENTAL

Melting points were determined on a Leitz Wetzlar apparatus. Infrared spectra were determined in KBr disks using a Perkin-Elmer model 137 Infracord spectrophotometer. Ultraviolet spectra were determined in 95% ethyl alcohol using a Perkin-Elmer model 202 ultraviolet-visible spectrophotometer. NMR spectra were determined in deuteriochloroform solutions with tetramethylsilane as internal standard using a Varian A-60A spectrometer. Optical rotations were measured in acetone solutions using a Carl Zeiss polarimeter. Analytical and preparative thin-layer chromatography (TLC) were performed on plates covered with Silica Gel $HF_{254}$ (Merck). The staining system used was 5% potassium carbonate–0.2% potassium permanganate. Solvent systems used were (*a*) butyl acetate (technical)–acetone, 4:1, and (*b*) chloroform–methanol, 98:2. Column chromatography was performed using Florisil (100–200 mesh) (Fisher Scientific Co., St. Louis, Mo.). Micro-analysis was performed by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

**(−)-Dehydrogriseofulvin (V)**—This substance was prepared essentially following the method of Taub (4). A suspension of 20 Gm. of (+)-VII (m.p. 219–220.5°; pure by TLC analysis using solvent systems *a* and *b*) and 40 Gm. of selenium dioxide in 1.2 L. of *tert*-butanol was refluxed for 65 hr. The hot reaction mixture was filtered through diatomaceous earth[1] and the solvent evaporated at 45° *in vacuo* to yield a red oil. TLC analysis of the reaction mixture with solvent system *a* revealed a series of spots from the origin to the solvent front, indicating a complex mixture, and containing VII ($R_f$ 0.49). The reaction products were suspended in 200 ml. of benzene and chromatographed on a column of Florisil (453 Gm., 77 × 3.7 cm.). The column was eluted with benzene, mixtures of benzene and chloroform, chloroform, and mixtures containing V and VII were then eluted from the column with chloroform–methanol (1–3%). A series of yellow solids (11 Gm.) which remained after evaporation of solvent melted between 260–265°. TLC analysis of the solids with solvent system *a* revealed the presence of V ($R_f$ 0.47) and VII ($R_f$ 0.49), with solvent system *b* the presence of V ($R_f$ 0.65) and VII ($R_f$ 0.68). The presence of VII could be detected (before permanganate oxidation of the plates) by its brilliant blue fluorescence under ultraviolet irradiation. An attempt was made to purify V using preparative TLC (plates 0.5 mm. thick). The product obtained was a white powder which was recrystallized from chloroform–ether and then benzene to yield white crystals, m.p. 276–279° with phase change at 262–263°; $[\alpha]_D^{25}$ −30° (c 0.5); $\lambda_{max.}$ infl. 230 mμ (ε, 25,000); 292 mμ (ε, 35,000); infl. 320 mμ (ε, 7000). [Lit. (4) m.p. 276–279° with phase change at 260°; $[\alpha]_D$ −31.6° (acetone); $\lambda_{max.}$ infl. 230 mμ (ε, 25,000); 292 mμ (ε, 33,000); infl. 320 mμ (ε, 6800).] The infrared spectrum was essentially identical to that reported for (±)-V (14).

*Anal.*—Calcd. for $C_{17}H_{15}ClO_6$: C, 58.21; H, 4.31. Found: C, 58.31; H, 4.52.

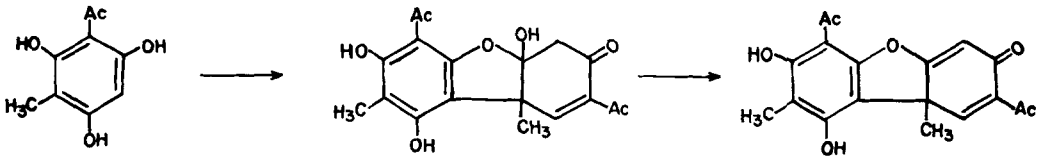
TLC analysis of the purified V (m.p. 277–279°) with solvent system *a* revealed the presence of a single spot ($R_f$ 0.47, V) after permanganate oxidation; ultraviolet irradiation revealed a faint blue fluorescent spot of $R_f$ 0.49 indicating a trace amount of VII. Analysis with solvent system *b* revealed the presence of a single spot ($R_f$ 0.65, V) and ultraviolet irradiation revealed a faint blue fluorescent spot of $R_f$ 0.68, again indicating the presence of a trace amount of VII.

**Griseophenone A (I)**—The method of Taub (4) was used to convert 800 mg. of (−)-V (m.p. 260–265°) to 280 mg. of I, using the zinc dust–acetic acid procedure. The yellow crystalline product melted at 212–214°; $\lambda_{max.}$ 297 mμ (ε, 18,800); 332 mμ (ε, 6300). [Lit. (4) m.p. 210–212°; $\lambda_{max.}$ 297 mμ (ε, 18,500); 332 mμ (ε, 6200).]

TLC analysis of I with solvent system *a* revealed a single spot of $R_f$ 0.34.

**(±)-Dehydrogriseofulvin (V)-From I by Enzymatic Conversion**—Griseophenone A (I) (100 mg., 285 μmoles) was stirred in 2 L. of phosphate buffer (pH 7.6) containing 15 ml. of $H_2O_2$ (0.3%) and 100 mg. of horseradish peroxidase (200 units per mg., Nutritional Biochemical Corp., Cleveland, Ohio). The mixture was left stationary in the dark at 25° for 18 hr. and then extracted with chloroform. The chloroform extract was then concentrated to about 100 ml. and washed twice with 10 ml. of cold 2% KOH to remove 50 mg. of unreacted I. The chloroform solution was then washed with water until neutral and evaporated to dryness. The residue (40 mg.) was dissolved in acetone and streaked on three thick (0.5 mm.) TLC plates which

[1] Celite marketed by Johns-Manville Products Corp., New York, N. Y.




*Scheme II*

were developed with solvent system *b*. The zone corresponding in location to V was eluted with chloroform–methanol (10%) and evaporation of solvent yielded 29 mg. of a white powder. Recrystallization from benzene yielded 7 mg. of white crystals, m.p. 287–289°. [Lit. (4) m.p. 288–290°.] $[\alpha]_D^{25}$ 0° (c 0.5). The infrared, NMR, and ultraviolet spectra of (±)-V and (−)-V were congruent and (±)-V and (−)-V had identical mobilities by co-chromatography on TLC plates with solvent systems *a* and *b*, (±)-V exhibiting a single spot by ultraviolet irradiation and permanganate oxidation.

## RESULTS

(+)-Griseofulvin (VII) was converted to (−)-V by selenium dioxide dehydrogenation and (−)-V was converted to I by reductive cleavage in acetic acid using powdered zinc. Griseophenone A (I) was then converted to (±)-V in a phosphate buffer (pH 7.6) containing horseradish peroxidase and hydrogen peroxide. The reaction did not take place in the absence of peroxidase and/or hydrogen peroxide. The infrared, NMR, and ultraviolet spectra of (−)-V, prepared by chemical means from (+)-VII, and (±)-V, prepared enzymatically from I, were congruent and their mobilities were identical on TLC.

## DISCUSSION

The formation of (±)-V was not unexpected as Fales (12) has reported the horseradish peroxidase catalyzed conversion of methylacetylphloroglucinol (VII) (Scheme II), to the racemates, benzofuran derivative (IX), and usnic acid (X). Fales in fact reports the formation only of racemates from oxidative couplings in peroxidase systems.

The formation of racemic dehydrogriseofulvin (V) suggests the possibility that another enzyme(s) (including other peroxidases) could be involved in the *in vivo* conversion of I to (−)-V. On the other hand, it is possible that *in vivo*, peroxidase catalyzes the conversion of II to an optically active VI *via* IV, and that VI is then chlorinated to form (+)-VII. While considerable evidence exists that chlorination of II occurs in the biosynthesis of (+)-VII (15), nevertheless it has been shown that chlorination of a spiran can occur as in the biosynthesis of geodin (16).

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*Keyphrases*

Griseophenone A—conversion to (±)-dehydrogriseofulvin
Peroxidase, horseradish, hydrogen peroxide—catalyzed conversion
Chromatography, TLC, column—separation, identity
IR spectrophotometry—structure
UV spectrophotometry—structure
NMR spectroscopy—structure
Optical rotation—identity