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Biosynthesis of Griseofulvin

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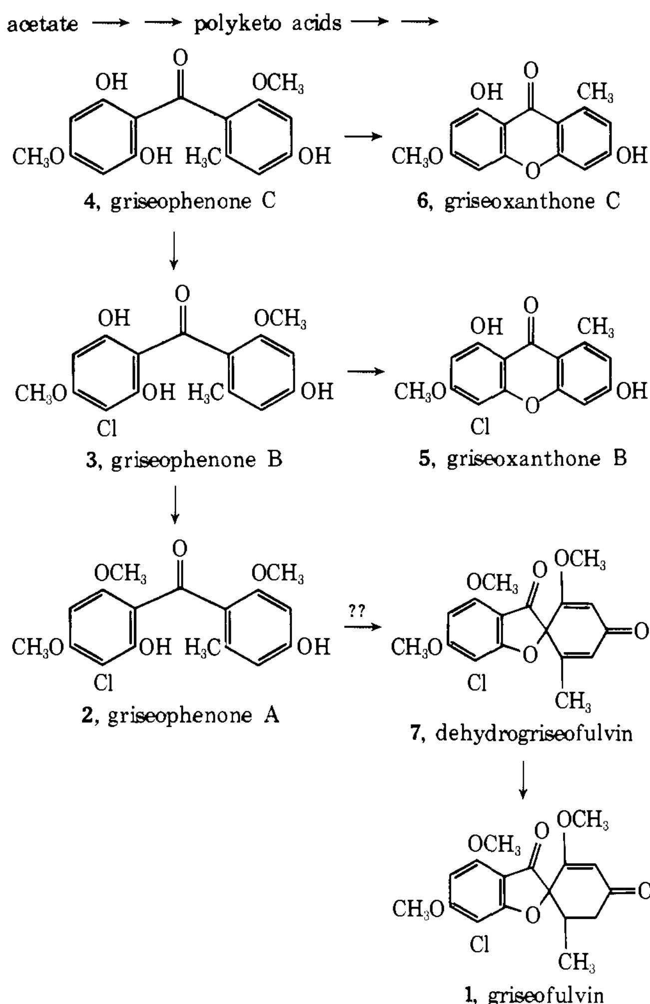
Abstract: The antifungal antibiotic griseofulvin (**1**) is a polyketide metabolite of *Penicillium griseofulvum* for which the present study has revealed that at least two and probably all three of the *O*-methyl groups are introduced after both carbocyclic rings have been formed. Benzophenone **11**, the monomethylated precursor predicted by earlier workers, could not be detected in cultures by carrier dilution experiments. Instead benzophenone **14** was shown to be a precursor of **1** by a feeding experiment in which **14** containing a tritium label in the *O*-methyl group was incorporated (14%) into **1**. Demethylation of labeled **1** first to **16** and then to **17** showed that less than 10% randomization of the label had occurred during biotransformation of **14** into **1**. The possibility that unmethylated benzophenone **18** was the precursor of **14** was considered, but synthetic **18** was found to be too unstable for use in carrier dilution or incorporation experiments, undergoing facile cyclization to xanthone **19**. The latter compound was, however, found to be a metabolite of *P. griseofulvum*, which lends support to the hypothesis that both **19** and **14** arise in the fungal culture from **18**. Earlier workers had postulated that the grisan ring was formed by oxidative cyclization of benzophenone **2** to give dehydrogriseofulvin but in vivo confirmation of this process had not been obtained. Another possible precursor to dehydrogriseofulvin, normethyldehydrogriseofulvin (**20**), has been synthesized and shown to be incorporated (44%) into **1**. These findings, in conjunction with those of previous studies, support the biosynthetic sequence: acetate → heptaacetic acid (**8**) → benzophenone **18** → benzophenone **14** → benzophenone **4** → benzophenone **3** → grisan **20** → dehydrogriseofulvin (**7**) → griseofulvin (**1**).

Griseofulvin (**1**), a chlorine-containing, antifungal antibiotic elaborated by *Penicillium griseofulvum* and related strains of *Penicillia*, has been the subject of numerous chemical and biological studies.[1] The polyketide origin of this compound was demonstrated in 1958 by Birch[2] using [1-^{14}C]acetate and later by Tanabe[3] with [2-^{13}C]acetate as the metabolic precursor. Cometabolites of **1** have provid considerable insight into its biosynthesis; compounds bearing a clear structural relationship include benzophenones **2–4** which have been given the trivial names griseophenones A, B, and C, respectively, xanthones **5** and **6**, known as griseoxanthones B and C, and dehydrogriseofulvin (**7**).[4] As presently understood, the biosynthesis of **1** involves cyclization of a polyketo acid to a benzophenone; the heterocyclic ring of the grisan structure is then formed by an intramolecular oxidative coupling.[5] The timing of methylations and chlorination is important. Sequential involvement of benzophenones **4** and **3** in the biosynthesis of **1** has been demonstrated.[6] Similar attempts to incorporate **2** have met with failure[6] but dehydrogriseofulvin (**7**) is efficiently transformed into **1**.[5b,7] The xanthones are apparently by-products rather than intermediates in the pathway. These relationships are summarized in Scheme I.

Benzophenone **4** is the most primitive compound in the pathway, other than acetate, thus far to be identified; in the present study a search has been made for aromatic precursors of **1**. In addition, the unresolved question of the stage at which formation of the grisan ring system occurs has been investigated and the paradoxical failure of **2** to be transformed into **7** and **1** can now be rationalized.

Aromatic Precursors of Griseophenone C (4). Heptaacetic acid (**8**) is a putative intermediate in the biosynthesis of griseofulvin and other metabolites derived from 7 acetate molecules.[5] The transformation of **8** into **4** requires (a) a Claisen cyclization, (b) an aldol cyclization, (c) dehydration,

Scheme I



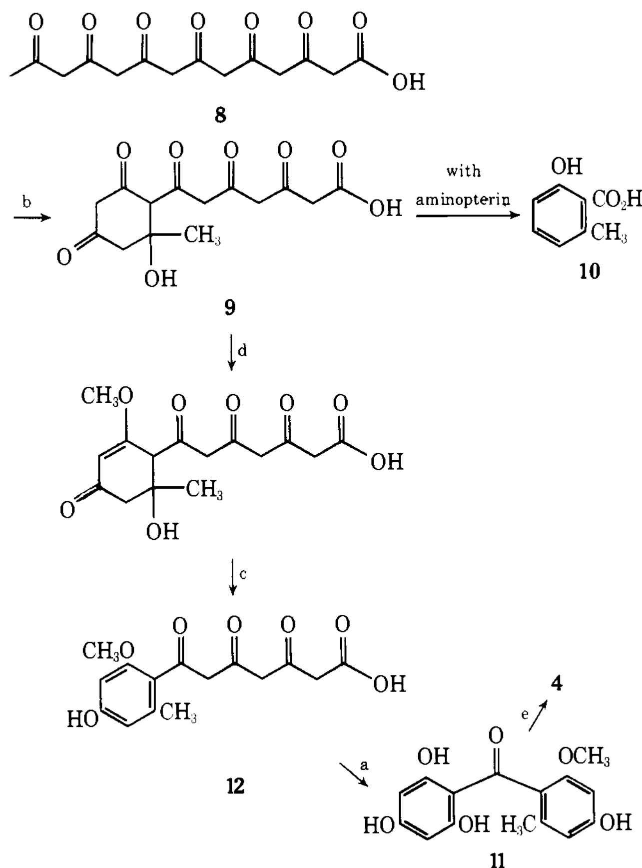
and (d and e) two methylations. Except that (c) must follow (b), no restrictions are placed upon the sequence of these steps. Birch has proposed that the sequence of steps is (b, d, c, a, e), as shown in Scheme II. The basis for this suggestion is the finding that aminopterin blocks formation of **4**.[4b] The inhibition does not lead to accumulation of a monomethylated or unmethylated benzophenone. Birch argued that aminopterin blocks the methylation (d) which is occurring prior to formation of either of the aromatic rings.[5b] Instead, intermediate **9** undergoes reduction, dehydration, and a retro-Claisen cleavage to give 6-methylsalicylic acid (**10**), which is a major metabolic product of inhibited cultures. On the basis of this scheme, **11** is the first benzophenone to be formed and should be isolable from cultures.

Hay and Harris, while studying the cyclizations of esters related to **12**, found that the *o*-methoxyl group facilitated closure of the phloroglucinol ring; reactions of the corresponding *o*-hydroxyl compound followed another course.[8] If the aromatic rings are formed in the order suggested by Birch, introduction of the *O*-methyl group on the orcinol ring must occur before the second cyclization takes place, i.e., **12** and **11** would be precursors of **4**.

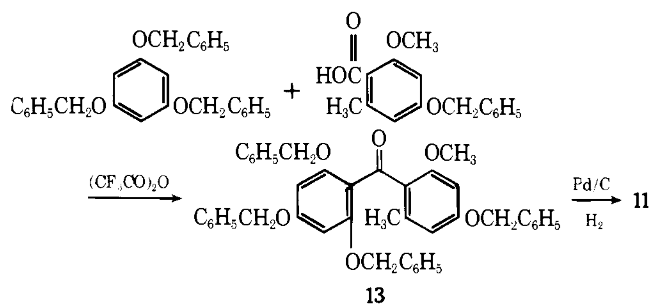
An authentic sample of **11** was required to facilitate a search for the compound among the metabolites of *P. griseofulvum*. Condensation of 2-methoxy-4-benzyloxy-*o*-toluic acid with the tribenzyl ether of phloroglucinol in the presence of trifluoroacetic anhydride[9] gave benzophenone **13** (88%) from which the benzyl groups were removed (72%) by hydrogenolysis to give **11** as a stable, crystalline solid (Scheme III).

The search for **11** among the fungal products was carried out by carrier dilution, which is one of the most sensitive ways

Scheme II



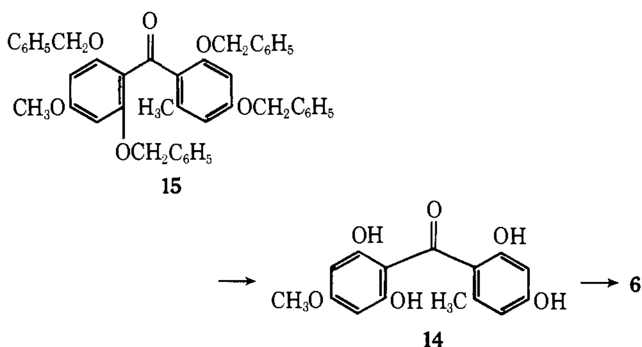
Scheme III



to detect trace components in a complex mixture. *P. griseofulvum* was grown in a rich germinating medium; in order to promote formation of nonchlorinated secondary metabolites, the resulting mycelial pad was transferred to a medium lacking both chloride ion and nitrogenous compounds. [1-^{14}C]Acetate was added and after 24 h, nonradioactive **11** was added to the culture, then reisolated, and purified exhaustively by recrystallizion and chromatography. After each step of purification the specific radioactivity was determined. The material, when pure, contained essentially no ^{14}C and it must be concluded that labeled benzophenone **11** was not present in the culture.

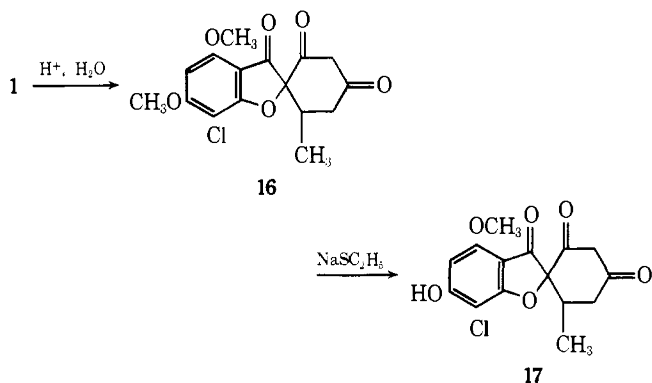
In view of this failure, attention was turned to the possibility that benzophenone **14**, rather than **11**, was the precursor of **4**. The synthesis of **14** involved condensation of 2,6-dibenzyloxy-4-methoxybenzoic acid with the dibenzyl ether of orcinol to give benzophenone **15** (69%). Nonequivalence of the aromatic protons on the orcinol ring of **15** established that acylation had occurred at the 4 position, rather than at the more hindered position between the benzyloxy groups. Hydrogenolysis gave benzophenone **14** (71%), which was somewhat unstable, invariably undergoing partial conversion to xanthone **6** during recrystallization or chromatography. Brief treatment

of **14** with sodium hydroxide gave efficient conversion to **6**. The stability of **14** was insufficient to permit its use in carrier dilution experiments.



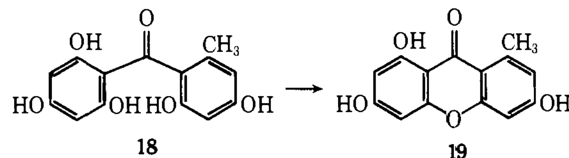
The possibility remained of demonstrating the intermediacy of **14** by incorporation of isotopically labeled **14** into griseofulvin. Partial conversion of **14** into xanthone **6** would not be seriously detrimental since **6** is unlikely to reenter the metabolic pathway. Benzophenone **14**, having a tritium label in the methoxyl group, was prepared by the route described above. Instability prevented exhaustive purification of radioactive **14**; but TLC showed that it was radiochemically homogeneous.

The feeding experiment was conducted by growing the fungus in a rich germinating medium and then transferring the mycelial pad to nitrate-free Czapek-Dox medium to which tritiated **14** had been added. After 7 days, griseofulvin was isolated and found to contain 14% of the added radioactivity. A degradation was performed to ascertain whether **14** had been incorporated as a single unit rather than undergoing degradation to small molecules which were subsequently metabolized. If turnover of this type did occur, the isotopic label would probably reappear distributed among the three methoxyl groups. Acidic hydrolysis of griseofulvin formed from labeled **14** gave griseofulvic acid (**16**).[10] Treatment of the latter with sodium ethanethiolate removed the 6-*O*-methyl group to give grisan **17**.[11] Barring redistribution of label from the 6-*O*-methyl position, the specific activity of **16** should be the same as that of the griseofulvin but **17** should be free of radioactivity. The degradation showed that less than 10% randomization had occurred. This small amount may merely reflect catabolism of griseofulvin; demethylation of griseofulvin by other microorganisms is well documented.[12,13]

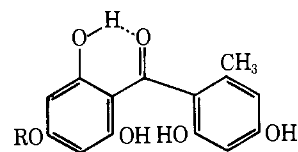


Examination of the other radioactive metabolites formed from labeled **14** revealed that the major one was xanthone **6**. Benzophenone **4** was present along with trace quantities of several unidentified compounds. Undoubtedly, much of **6** was formed by a direct, nonenzymic cyclization of **14**; in a control experiment using the nutrient solution without fungus, nonenzymic conversion of **14** into **6** was largely complete within 3 days. Enzymic formation of **6** directly from **14** or via benzophenone **4** may also occur. There is no information bearing directly upon this question; however, Rhodes has observed that conditions leading to high concentrations of benzophenone **4** in cultures (i.e., deficiency of chloride ion) lead also to accumulation of xanthone **6**.[4b,6] If formation of **6** is nonenzymic while the steps leading to griseofulvin are all enzyme-mediated, it might be possible to increase the efficiency of the latter process by using lower concentrations of **14**.

The possibility that **18** was the precursor of **14** received attention next. The pentahydroxybenzophenone was prepared (61%) by condensation of the tribenzyl ether of phloroglucinol with the dibenzyl ether of orsellinic acid followed by hydrogenolysis. The structure of **18** was established spectroscopically but all attempts to purify the compound for elemental analysis led to formation of xanthone **19**. The cyclization occurred in aqueous and organic solvents, even below room temperature. Xanthone **19** is a metabolite of the lichen *Lecanora reuteri*.[14]



The instability of benzophenones **14** and **18** was unexpected.[8,15] Benzophenones **4** and **11** can also be converted to xanthones but these cyclizations are much slower and are carried out with base catalysis. With **4** and **11**, cyclization must involve nucleophilic attack on the orcinol ring, whereas with **14** and **18** either the orcinol or the phloroglucinol ring could be the site of attack. With the latter compounds nucleophilic attack on the phloroglucinol ring would be facilitated by intramolecular hydrogen bonding of one of the *o*-hydroxyl groups to the carbonyl group, thus holding the carbonyl group planar with the phloroglucinol ring.



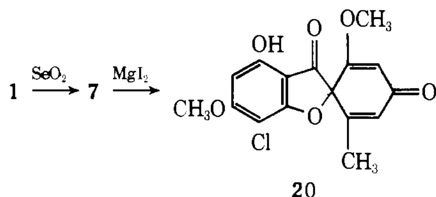
Facilitation of xanthone formation by multiple *o*- and *p*-hydroxyl groups might also result from conversion of the aromatic rings of the benzophenones into nonaromatic ions or tautomers. Under acidic and basic conditions rapid exchange of ortho and para protons of phloroglucinol and resorcinol has been observed. Acidic exchange is mediated by σ complexes; the σ complexes can be intercepted with nucleophiles.[16] Refluxing methanolic HCl, for example, converts phloroglucinol to its dimethyl ether.[17] Base-catalyzed exchange has been ascribed to anions of keto tautomers.[18] The cyclizations of **14** and **18** occurred without addition of catalysts, but the presence of trace acidic or basic impurities cannot be ruled out.

The instability of **18** made isolation and incorporation experiments with the compound impracticable. On the other hand, if **18** were present in fungal cultures in a free state, cyclization to xanthone **19** would undoubtedly compete with O-methylation. A search for **19** by carrier dilution showed that it was, in fact, being produced in the cultures. The quantity of **19** accumulated by the fungus in a chloride-deficient medium was sufficient to permit direct spectroscopic characterization. The presence of **19** does not *prove* that benzophenone **18** is also present, but it does give indirect support to the hypothetical role of benzophenone **18** as precursor of **14**.

Grisan Formation. The details of the biosynthesis of the grisan ring system have been obscured by the observation[6] that, although benzophenones **3** and **4** are transformed into griseofulvin, benzophenone **2** is not. Rhodes has offered the suggestion that the conversion of **3** into griseofulvin is brought about by a multienzyme complex within which more or less

simultaneous methylation, oxidation, and reduction occur; intermediates would be enzyme-bound and not released into the medium as long as the three steps were perfectly synchronized.[6] By this argument benzophenone **2** and dehydrogriseofulvin (**7**) would both be abnormal metabolites. A major difference betweeen them is that **7** is able to reenter the pathway but **2**, perhaps for steric reasons, cannot. Rhodes may have attached undue significance to the presence of benzophenone **2**, in view of its failure to undergo further in vivo transformation. An alternative hypothesis is that **2** is only a by-product which cannot participate in the formation of griseofulvin. If this were the case, the pathway must then involve oxidative cyclization of benzophenone **3**. Methylation of the cyclization product, i.e., grisan **20**, would give **7** which would be reduced to griseofulvin.

To test this possibility an authentic sample of **20** was synthesized. A procedure based on griseofulvin seemed preferable to oxidative cyclization of **3** since it would ensure that **20** had the correct absolute configuration. Dehydrogriseofulvin (**7**), prepared from griseofulvin (**1**) by dehydrogenation with selenium dioxide,[19] was demethylated selectively (47%) with magnesium iodide to give **20**. An examination of fermentation broth by carrier dilution indicated that **20** was present. Radioactivity (introduced via [1-^{14}C]acetate) remained with **20** through several recrystallizations, TLC in two solvent systems, and two fractionations by HPLC, but traces of persistent contaminants made it difficult to obtain a constant specific activity.



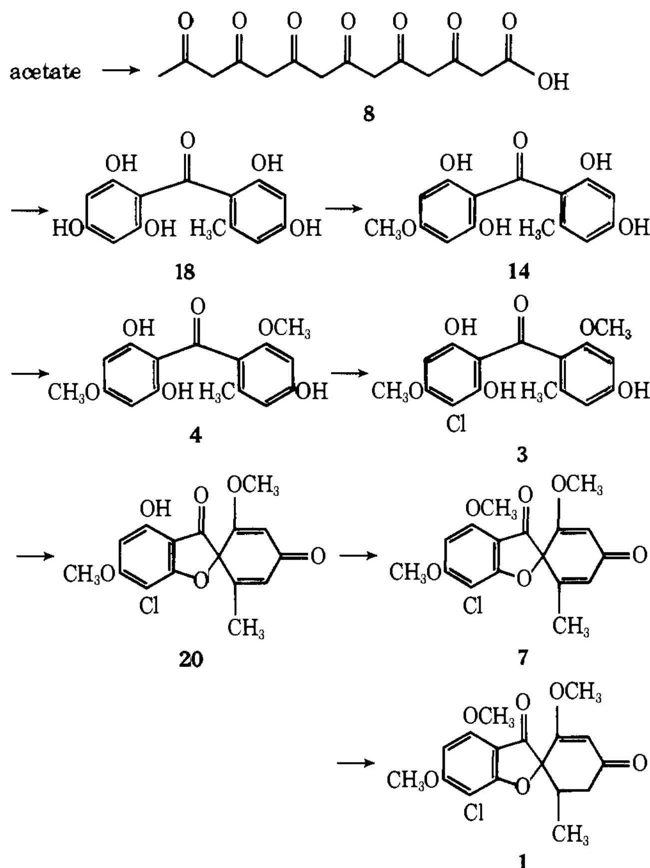
Consequently, the transformation of **20** into griseofulvin was investigated. Radioactively labeled **20** was prepared from griseofulvin which had been biosynthesized from [1-^{14}C]-acetate. A 7-day fermentation in the presence of labeled **20** gave ether-extractable compounds containing 81% of the original radioactivity. Griseofulvin, after purification by alumina chromatography, contained 44% of the original radioactivity; its specific activity was unchanged by recrystallization. Unaltered **20** was the major radioactive component in the base-soluble fraction; in addition, low levels of radioactivity were found in acid **10** and benzophenone **4** (less than 3% in each) indicating minor degradation to acetate and reincorporation had occurred. The high level of incorporation into griseofulvin leaves little doubt that **20** is being transform into griseofulvin by the route proposed.

Discussion

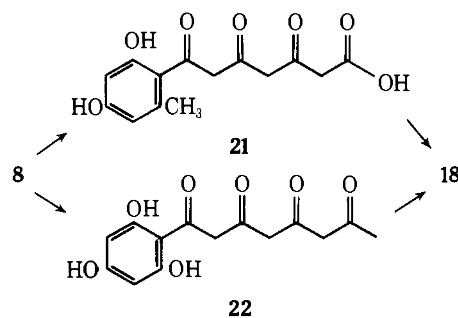
The present experiments coupled with results of the earlier studies now establish that griseofulvin (**1**) arises from the monomethylated benzophenone **14** by O-methylation to give **4** followed by chlorination to give **3**. An oxidative cyclization of benzophenone **3** to grisan **20** followed by an additional O-methylation gives dehydrogriseofulvin (**7**), from which griseofulvin is formed by reduction. From the presence of xanthone **19**, benzophenone **18** is inferred to be the precursor of **14** (see Scheme IV).

No light is cast upon the formation of benzophenone **18** from polycarbonyl intermediates. Chemical model studies[8] by Harris and Hay have indicated that, if hexaketo acid **8** undergoes aldol cyclization to orcinol derivative **21**, methylation of the *o*-hydroxy group of **21** must take place prior to closure of the phloroglucinol ring. By this pathway, **11** would be the first benzophenone formed. This conclusion must be

Scheme IV



reconsidered, in view of the fact that **14** and apparently **18** are involved in griseofulvin formation rather than **11**. Enzymic catalysts undoubtedly control the conformations of polycarbonyl compounds better than the simple catalysts used in the biomimetic studies and might be able to catalyze closure of the phloroglucinol ring with **21** without prior O-methylation, but an attractive alternative is that Claisen-type cyclization of **8** occurs first, giving phloroglucinol **22**. Benzophenone **18** is formed by a subsequent aldol cyclization. Information on this point will be difficult to obtain because of the anticipated lability of **21** and **22**.



Experimental Section

Melting points were taken with a Thomas-Hoover or a Mel-Temp apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Inc. Knoxville, Tenn. Ultraviolet spectra of solutions in 95% EtOH were determined using a Cary spectrophotometer. Infrared spectra were recorded with a Beckmann IR-10 spectrophotometer. NMR spectra were obtained with a JEOL MH-100 spectrometer; chemical shifts are reported in parts per million (ppm) (δ) employing tetramethylsilane as an internal standard. Low-resolution mass spectra were obtained with an LKB 9000 mass spectrometer with the samples introduced by direct insertion. Radioactivity of ^{14}C and ^{3}H was assayed with a Beckmann LS-100 scintillation counter. Toluene containing 0.5% of 2,5-diphenyloxazole

was used as the scintillation medium. Samples were counted to ±2% and internal standards were used to correct for quenching. Efficiencies of 57% for ^3H and 95% for ^{14}C were obtained on unquenched samples. High-pressure liquid chromatography (HPLC) was carried out on a Waters ALC 202 instrument. Silica gel plates containing a fluorescent indicator were used for thin-layer chromatography. Plates were examined by ultraviolet light and visualized with I_2 or diazotized benzidine.

1,3,5-Tris(benzyloxy)benzene. A mixture of phloroglucinol dihydrate (5.0 g, 31 mmol), $C_6H_5CH_2Cl$ (16 g, 126 mmol), and K_2CO_3 (24 g, 174 mmol) in 75 ml of hexamethylphosphoramide was heated at 80° under nitrogen for 3 days. Workup by addition to water and extraction into hexane gave, after washing the hexane solution with aqueous KOH, 9.7 g (79%) of the product, which after recrystallizations from Et_2O–hexane and from EtOH gave fine, white needles: mp 86–87°;[20] ir (KBr) 1600 cm^{-1} (aromatic), no hydroxyl in 3000-cm^{-1} region; NMR ($CDCl_3$) 5.02 (s, 6, CH_2's) 6.32 (s, 3, aromatic), 7.42 (m, 15, phenyls); uv 270 nm (ϵ 12 400). Anal. Calcd for $C_{27}H_{43}O_3$: C, 81.79; H, 6.10. Found: C, 82.03; H, 5.99.

Methyl 4-Benzyloxy-6-hydroxy-*o*-toluate. Methyl orsellinate (0.25 g, 2 mmol, prepared by treatment of orsellinic acid[21] with CH_2N_2), $C_6H_5CH_2Cl$ (0.27 g, 3 mmol), and anhydrous K_2CO_3 (1.7 g, 12 mmol) were heated at reflux in 40 ml of 3-pentanone for 15 h. Isolation by chromatography on silica gel (hexane–Et_2O elution) gave 0.28 g (75%) of the benzylated ester which crystallized from cyclohexane: mp 68.5–69°; ir (KBr) 1620 cm^{-1} (carbonyl); NMR ($CDCl_3$) 2.45 (s, 3, CH_3), 3.85 (s, 3, OCH_3), 4.97 (s, 2, CH_2), 6.36 (s, 2, aromatic), 7.35 (s, 5, phenyl), 13.46 (s, 1, OH); MS *m/e* (rel intensity) 272 (58, parent ion), 257 (29), 240 (100), 212 (36); uv 216 nm (ϵ 29 100), 264 (19 900), sh 290 (7330). Anal. Calcd for $C_{16}H_{16}O_4$: C, 70.58; H, 5.92. Found: C, 70.83; H, 6.00.

Methyl 4-Benzyloxy-6-methoxy-*o*-toluate. Methyl 4-benzyloxy-6-hydroxy-*o*-toluate (8 g, 29 mmol), Me_2SO_4 (5.56 g, 44 mmol), and anhydrous K_2CO_3 (15 g, 108 mmol) were refluxed in acetone for 15 h. Isolation by chromatography on silica gel (hexane–Et_2O elution) gave 6.4 g (76%) of the product. An analytical sample was prepared by repeated chromatography and microdistillation. Prolonged standing at −20° gave crystals: mp 37.5–38.5°; ir ($CHCl_3$) 1710 cm^{-1} (carbonyl); NMR ($CDCl_3$) 2.25 (s, 3, CH_3), 3.78 (s, 3, OCH_3), 3.92 (s, 3, OCH_3), 5.10 (s, 2, CH_2), 6.50 (s, 2, aromatic), 7.50 (m, 5, phenyl); uv 256 nm (ϵ 8500), sh 275 (7600). Anal. Calcd for $C_{17}H_{18}O_4$: C, 71.31; H, 6.34. Found, C, 71.12; H, 6.50.

4-Benzyloxy-6-methoxy-*o*-toluic Acid. A solution of the above ester (1.02 g, 2.6 mmol) and KOH (1 g) in MeOH was refluxed for 4 days to give 0.86 g (89%) of the acid as a white solid: mp 145–145.5° after recrystallization from cyclohexane; ir (KBr) 2700–3000 (carboxyl OH), 1700 cm^{-1} (carbonyl); NMR ($CDCl_3$) 2.58 (s, 3, CH_3), 3.95 (s, 3, OCH_3), 5.13 (s, 2, CH_2), 6.54 (d, 2, aromatic), 7.46 (m, 5, phenyl); uv 276 nm (ϵ 7500); MS *m/e* (rel intensity) 272 (100, parent), 257 (10). Anal. Calcd for $C_{16}H_{16}O_4$: C, 70.58; H, 5.92. Found: C, 70.19; H, 5.66.

2,4,4′,6-Tetrakis(benzyloxy)-2′-methoxy-6′-methylbenzophenone (13). A mixture of 4-benzyloxy-6-methoxy-*o*-toluic acid (0.3 g, 1.1 mmol) and 1,3,5-tris(benzyloxy)benzene (1.75 g, 4.5 mmol) in 15 ml of CH_2Cl_2 was treated with 0.75 ml (5.3 mmol) of $(CF_3CO)_2O$ for 4 min at ambient temperature.[9] After removal of the solvent in vacuo, the residue was dissolved in Et_2O; the solution was washed with $NaHCO_3$ and with water, dried ($MgSO_4$), and evaporated. Chromatography of the residue on silica gel (hexane–Et_2O elution) gave 0.64 g (88%) of **13** as an oil which crystallized on standing: mp 105–106° after recrystallization from EtOH; ir (KBr) 1660 cm^{-1} (carbonyl); NMR ($CDCl_3$) 2.08 (s, 3, CH_3), 3.32 (s, 3, OCH_3), 4.72 (s, 4, $2CH_2$), 4.78 (s, 2, CH_2), 4.82 (s, 2, CH_2), 6.02 (s, 2, CH), 6.08 (s, 2, CH), 6.9–7.2 (m, 20, phenyls); uv 241 nm (ϵ 27 000), 273 (38 000); MS *m/e* (rel intensity) 650 (10, parent), 619 (5), 559 (10), 91 (100). Anal. Calcd for $C_{43}H_{38}O_6 \cdot 0.5H_2O$: C, 78.28; H, 5.96. Found: C, 78.56; H, 5.90.

2,4,4′,6-Tetrahydroxy-2′-methoxy-6′-methylbenzophenone (11). A mixture of benzophenone **13** (0.426 g, 0.65 mmol) and 10% Pd/charcoal (0.12 g) in 35 ml of absolute EtOH was treated with H_2 in a Brown[2] hydrogenator until uptake of H_2 ceased. The product (0.137 g, 72%) crystallized from CH_2Cl_2 at 5°. Recrystallization from H_2O gave yellow prisms: mp 177–178°; ir (KBr) 2800–3500 (broad OH), 1560–1650 cm^{-1} (carbonyl and aromatic); NMR (CD_3COCD_3) 2.08 (s, 3, CH_3), 3.62 (s, 3, OCH_3), 5.88 (s, 2, aromatic), 6.32 (s, 2, aromatic), 10.4–10.7 (hydroxyls); uv sh 226 nm (ϵ 17 200), 294 (22 800), sh 333 (7300); MS *m/e* (rel intensity) 290 (17, parent), 275 (26), 259 (98), 153 (100), 137 (74). Anal. Calcd for $C_{15}H_{14}O_6 \cdot 0.25H_2O$: C, 61.12; H, 4.96. Found: C, 61.22; H, 4.97.

Methyl 2,6-Bis(benzyloxy)-4-methoxybenzoate. Methyl 4-methoxy-γ-resorcylate[22] (0.69 g, 3.5 mmol) was treated with benzyl chloride (1.1 g, 8.7 mmol) and anhydrous K_2CO_3 (5 g, 36 mmol) in 25 ml of hexamethylphosphoramide for 2 h at 100°. Isolation by chromatography on silica gel (hexane–Et_2O elution) gave 0.93 g (71%) of ester: mp 108–109° after recrystallization from Et_2O–hexane; ir (KBr) 1725 cm^{-1} (carbonyl); NMR ($CDCl_3$) 3.82 (s, 3, OCH_3), 3.98 (s, 3, OCH_3), 5.22 (s, 4, CH_2's), 6.28 (s, 2, aromatic), 7.52 (m, 10, phenyls); uv 270 nm (ϵ 22 800). Anal. Calcd for $C_{23}H_{22}O_5$: C, 73.00; H, 5.86. Found: C, 72.78; H, 6.02.

2,6-Bis(benzyloxy)-4-methoxybenzoic Acid. Saponification of the above ester (4.5 g, 11.9 mmol) with KOH (7.5 g), $HOCH_2CH_2OH$ (40 ml), and H_2O (300 ml) at reflux for 7.5 days gave 3.16 g (73%) of the acid: mp 122–123.5° after recrystallization from Et_2O–hexane; ir ($CHCl_3$) 1705 cm^{-1} (carbonyl); NMR ($CDCl_3$) 3.82 (s, 3, OCH_3), 5.22 (s, 4, CH_2's), 6.28 (s, 2, aromatic), 7.3–7.6 (m, 10, phenyls); uv 270 nm (ϵ 16 500). Anal. Calcd for $C_{22}H_{20}O_5$: C, 72.51; H, 5.53. Found: C, 72.23; H, 5.63.

2,2′,4′,6-Tetrakis(benzyloxy)-4-methoxy-6′-methylbenzophenone (15). A solution of the above acid (0.8 g, 3.7 mmol) and 3,5-bis(benzyloxy)toluene (2.65 g, 8.7 mmol) in CH_2Cl_2 (25 ml) was treated with $(CF_3CO)_2O$ (2.5 ml, 18 mmol) for 5 min at ambient temperature.[9] Isolation by the procedure followed with **13** gave 0.99 g (69%) of **15** as an oil which slowly crystallized: mp 107–108° after recrystallization from Et_2O–hexane; ir ($CHCl_3$) 1650 cm^{-1} (carbonyl); NMR ($CDCl_3$) 2.20 (s, 3, CH_3), 3.71 (s, 3, OCH_3), 4.67 (s, 2, CH_2), 4.74 (s, 4, CH_2's), 5.00 (s, 2, CH_2), 6.02 (s, 2, phloroglucinol aromatic), 6.32 (s, 1, orcinol aromatic), 6.9–7.5 (m, 20, phenyls); uv 284 nm (ϵ 17 300). Anal. Calcd for $C_{43}H_{38}O_6$: C, 79.37; H, 5.89. Found: C, 79.60; H, 6.04.

2,2′,4′,6-Tetrahydroxy-4-methoxy-6′-methylbenzophenone (14). A mixture of benzophenone **15** (0.997 g, 1.5 mmol) and 10% Pd/charcoal (350 mg) in EtOAc (15 ml) and EtOH (75 ml) was treated with H_2 at room temperature in a Brown[2] hydrogenator. After uptake of H_2 ceased, the catalyst was removed by filtration. After evaporation of the filtrate, the residue was triturated with H_2O to give 0.311 g (71%) of benzophenone **14** as a yellow solid, which melted at 115°, solidified again, and remelted at 250–251°: ir (Nujol) 3300 (OH), 1620 (carbonyl), 1600, 1575 cm^{-1}; NMR ($CDCl_3$–CD_3COCD_3) 2.12 (s, 3, CH_3), 3.80 (s, 3, OCH_3), 6.0 (s, 2, phloroglucinol aromatic), 6.36 (broad s, 2, orcinol aromatic), 12.9 (v broad s, OH); uv 294 nm (ϵ 16 700), 340 (6000); MS *m/e* (rel intensity) 290 (4, parent), 272 (64), 243 (33), 167 (78), 151 (89), 125 (75), 123 (80), 115 (100). Anal. Calcd for $C_{15}H_{14}O_6 \cdot 3H_2O$: C, 52.33; H, 5.85. Found: C, 52.80; H, 5.80.

Attempts to purify **14** further by recrystallization or by chromatography on silica gel or HPLC on Corasil II led invariably to material contaminated with xanthone **6**. Brief treatment of **14** with aqueous NaOH caused quantitative conversion to **6**: mp55–256° after recrystallization from 95% EtOH (lit.upc4a mp 253–255°); ir (THF) 2800–3500 (broad OH), 1550–1650 cm^{-1} (carbonyl and aromatic); NMR (CD_3COCD_3) 2.82 (s, 3, CH_3), 3.94 (s, 3, OCH_3), 6.26 (d, 1, J = 3 Hz, aromatic), 6.41 (d, 1, J = 3 Hz, aromatic), 6.72 (s, 2, aromatic), 7.36 (s, OH), 13.50 (s, OH); uv 240 nm (ϵ 37 700), sh 264 (10 600), 310 (20 200), sh 340 (11 500); MS *m/e* (rel intensity) 272 (100, parent), 243 (46).

Benzophenone 14 Containing Tritium in the 4-Methoxyl Group. Tritiated CH_2N_2 was prepared from *N*-methyl-*N*-nitrosotoluenesulfonamide (2.15 g, 0.01 mol), KOH (0.5 g), EtOH (2.5 ml), ^3H_2O (0.8 ml, 10 mCi), and Et_2O. The ethereal [^3H]CH_2N_2 was added to methyl 2,4,6-trihydroxybenzoate (1.0 g, 5.4 mmol) in EtOAc. After 16 h methyl 4-methoxy-γ-resorcylate (0.956 g, 91%) was isolated by chromatography on silica gel (hexane–Et_2O elution), mp 108–111°. Recrystallization from cyclohexane ge 0.73 g, mp 117–118.5° (1.16 × 10^{11} dpm/mol). This material was carried through the sequence of reactions described above.

In the final step, tritiated **15** (0.086 g, 0.132 mmol, mp 104.5–105.5°, 1.02 × 10^{11} dpm/mol) was hydrogenolyzed by treatment in EtOAc (3.5 ml) and absolute EtOH (15 ml) with Pd/charcoal (35 mg, 10%) and H_2 at atmospheric pressure. Isolation as before by trituration with H_2O gave 35.6 mg (93%) of **14** (6.45 × 10^{10} dpm/mol).[23] Upon standing the aqueous filtrate deposited a few milligrams of xanthone **6** (ca. 6.0 × 10^{10} dpm/mol).

Methyl 4,6-Bis(benzyloxy)-*o*-toluate. Methyl orsellinate (8.5 g, 47 mmol), $C_6H_5CH_2Cl$ (15.2 g, 120 mmol), and anhydrous K_2CO_3 (20 g, 144 mmol) were heated at 135° for 1 h in hexamethylphosphoramide (60 ml). The cooled reaction mixture was diluted with water and extracted with Et_2O. The ethereal solution, after washing with aqueous base, yielded 16 g (95%) of the ester: mp 68-69° after recrystallization from Et_2O–hexane; ir (KBr) 1710 cm^{-1} (carbonyl); NMR ($CDCl_3$) 2.24 (s, 3, CH_3), 3.82 (s, 3, OCH_3), 4.92 (s, 2, CH_2), 4.97 (s, 2, CH_2), 6.38 (s, 2, aromatic), 7.30 (m, 10, phenyls); uv sh 249 nm (ϵ 6000), sh 282 (2400); MS *m/e* (rel intensity) 362 (100, parent), 331 (42). Anal. Calcd for $C_{23}H_{22}O_4$: C, 76.22; H, 6.12. Found: C, 76.42; H, 6.26.

4,6-Bis(benzyloxy)-*o*-toluic Acid. Treatment of the above ester (0.5 g, 1.4 mmol) with KOH (0.7 g, 12.5 mmol), glycerol (8 ml), and H_2O (35 ml) at reflux for 12 days gave the corresponding acid (0.386 g, 80%), mp 101-102°, after recrystallization from cyclohexane (lit.[9] mp 100-101°).

2,2′,4,4′,6-Pentakis(benzyloxy)-6′-methylbenzophenone. Treatment of a mixture of the above acid (0.263 g, 0.76 mmol) and 1,3,5-tris-(benzyloxy)benzene (1.07 g, 2.7 mmol) with $(CF_3CO)_2O$ (0.8 ml) in $CHCl_3$–CH_2Cl_2 (15 ml) for 5 min at ambient temperature gave, after chromatography on silica gel (hexane–Et_2O elution), 0.335 g (61%) of the condensation product as a colorless oil. Further chromatography failed to give material of analytical purity, but spectroscopic characterization supported the assigned structure: ir ($CHCl_3$) 1650 cm^{-1} (carbonyl); NMR ($CDCl_3$) 2.18 (s, 3, CH_3), 4.62 (s, 2, CH_2), 4.70 (s, 4, CH_2's), 4.95 (s, 2, CH_2), 4.98 (s, 2, CH_2), 6.13 (s, 2, aromatic), 6.30 (s, 1, aromatic), 6.34 (s, 1, aromatic), 6.9–7.4 (m, 25, phenyls); uv sh 235 nm (ϵ 21 500), 294 (11 400); MS *m/e* (rel intensity) 726 (11, parent), 635 (12), 396 (71), 91 (100).

2,2′,4,4′,6-Pentahydroxy-6′-methylbenzophenone (18). A mixture of the above benzophenone (0.335 g, 0.46 mmol) and Pd/charcoal (10%, 200 mg) in EtOAc (5 ml) and EtOH (40 ml) was treated with H_2 at atmospheric pressure. After uptake of H_2 had ceased, the catalyst was removed by filtration and the solution was evaporated in vacuo and at room temperature to give **18**, essentially quantitatively as a viscous, pale yellow oil: ir (neat) 3400-3300 (OH), 1640 sh (carbonyl), 1630-1620 cm^{-1} (aromatic); NMR (CD_3COCD_3) 2.11 (s, 3, CH_3), 5.91 (s, 2, aromatic), 6.28 (broad s, 2, aromatic), 9.0-11.0 (v broad, OH's); uv 225 nm (ϵ 11 800), 298 (11 600), 335 (5700); MS *m/e* (rel intensity) 258 (100), 229 (14). The mass spectrum was identical with that of xanthone **19**; dehydration to form **19** probably occurred in the ion source.

On TLC (80:20:2 Et_2O–hexane–CH_3CO_2H), **18** appeared as a slow-moving, tailing spot which gave a cherry red color with diazotized benzidine. Attempts to crystallize **18** led to formation of xanthone **19** as did open column chromatography on silica gel and HPLC on Corasil II. Gradual cyclization occurred in the ethanolic solution used for the uv determination. A rapid and quantitative cyclization occurred in aqueous sodium hydroxide to give, after acidification, xanthone **19**: mp 285-290° dec after recrystallization from EtOH–H_2O (lit.[14] mp 272-275°); ir (KBr) 3600-3200 (OH), 1670-1600 cm^{-1} (carbonyl and aromatic); NMR (CD_3COCD_3) 2.80 (s, 3, CH_3), 6.26 (d, 1, J = 3 Hz, aromatic), 6.36 (d, 1, J = 3 Hz, aromatic), 6.74 (s, 2, aromatic), 13.5 (3, hydroxyl); uv 242 nm (36 200), sh 250 (26 300), sh 265 (18 100), 310 (18 500), sh 340 (10 300); MS *m/e* (rel intensity) 258 (100, parent), 229 (14).

4-Normethyldehydrogriseofulvin (20). Dehydrogriseofulvin (**7**) was prepared by dehydrogenation of griseofulvin with SeO_2 as described by Taub et al.[19] Dehydrogriseofulvin (0.270 g, 0.77 mmol) was treated with MgI_2 [prepared from Mg (0.04 g), I_2 (0.2 g), Et_2O (0.3 ml), and C_6H_6 (5 ml)] in 10 ml of C_6H_6 for 3 h at reflux.[24] The cooled mixture was washed with dilute HCl and then extracted with 2 N Na_2CO_3. Acidification of the extract gave a tan powder which was chromatographed on silica gel (hexane–$CHCl_3$ elution) to give 0.123 g (47%) of grisan **20** as a white solid: mp 229-231° after recrystallization from $CHCl_3$–Et_2O and drying in vacuo at 58°; ir (KBr) 1688 (carbonyl), 1610 cm^{-1}; NMR ($CDCl_3$) 1.80 (s, 3, CH_3), 3.64 (3, s, 2′-OCH_3), 3.98 (s, 3, 6-OCH_3), 5.68 (s, 1, vinyl), 6.23 (s, 1, vinyl), 6.25 (s, 1, aromatic), 8.0 (broad s, 1, 4-OH); uv 370 nm (ϵ 2000), 330 (4300), 292 (31 300), 223 (31 200); $[\alpha]^{26}D$ −33.1° (acetone); MS *m/e* (rel intensity) 338 (33, parent), 336 (71, parent), 295 (19), 293 (57), 157 (38), 155 (100). Anal. Calcd for $C_{16}H_{13}O_6Cl$: C, 57.06; H, 3.89. Found: C, 57.30; H, 4.15.

[^{14}C]-4-Normethyldehydrogriseofulvin. [^{14}C]Griseofulvin (0.136 g), biosynthesized by *P. griseofulvum* from sodium [1-^{14}C]acetate, was purified to constant specific activity (1.99 × 10^7 dpm/mol) by chromatography and recrystallization from benzene. Conversion to **20** by the procedures described above gave after chromatography and recrystallization 14.6 mg (11%) of material, mp 224-228° (1.79 × 10^7 dpm/mol). Care was taken to ensure that the sample of radioactive **20** was free of griseofulvin, dehydrogriseofulvin, and 4-normethylgriseofulvin.

Fungal Experiments. Cultures of *P. griseofulvum* Dierckx, ATC 11885, were maintained on malt agar slants which had the following composition (in g/l.): malt extract (20), peptone (1), glucose (20), agar (20), and distilled water. The germinating medium (medium 1) contained (in g/l.) peptone (20), malt extract (10), glucose (40), soluble starch (20), $NaNO_3$ (3.0), KH_2PO_4 (1.0), $MgSO_4{\cdot}7H_2O$ (0.5), KCl (0.5), $FeSO_4{\cdot}7H_2O$ (0.02), and distilled water.[25] Medium 2 contained (in g/l.) K_2HPO_4 (1.0), $MgSO_4{\cdot}7H_2O$ (0.5), KCl (0.5), $FeSO_4{\cdot}7H_2O$ (0.01), glucose (30), and distilled water. Medium 3 was modeled on that of Bayan[25] and contained (in g/l.) glucose (75), KH_2PO_4 (1.0), $MgSO_4{\cdot}7H_2O$ (0.5), $FeSO_4{\cdot}7H_2O$ (0.001), $CuSO_4{\cdot}5H_2O$ (0.00015), $ZnSO_4{\cdot}7H_2O$ (0.001), $MnSO_4{\cdot}H_2O$ (0.0001), $Na_2MoO_4{\cdot}4H_2O$ (0.0001), and distilled water. All media were sterilized with steam at 2 atm of pressure for 30 min.

Carrier Dilution with Benzophenone 11. A 500-ml Erlenmeyer flask containing 125 ml of medium 1 was inoculated with *P. griseofulvum* spores. After being shaken at 25° for 40 h, the mycelium was separated by filtration and divided approximately equally between two 500-ml Erlenmeyer flasks containing 125 ml of medium 3. To each flask was added 20 μCi of sodium [1-^{14}C]acetate in 0.04 ml of water. The stationary cultures were allowed to ferment at 25° for 24 h. The contents of the flasks were combined; the mycelium was separated from the broth by filtration and continuously extracted with acetone in a Soxhlet apparatus for 2 h. The acetone was evaporated and the residue was combined with the broth. Nonradioactive benzophenone **11** (0.097 g) was added to the aqueous broth as the sodium salt. The solution was acidified to pH 3 with dilute H_2SO_4 and extracted with six 100-ml portions of Et_2O. The extracts were combined and extracted with six 100-ml portions of dilute NaOH. These extracts were combined, acidified with dilute HCl, and extracted with six 100-ml portions of Et_2O. The pooled ethereal extracts were dried ($MgSO_4$) and evaporated in vacuo to leave 0.119 g of residue, which was chromatographed on silica gel (hexane–Et_2O elution) to give 0.079 g of benzophenone **11** (1393 dpm/mg). Six sequential recrystallizations from water gave specific activities of 564, 95, 81, 77, 46, and 36 dpm/mg, respectively. From the failure to achieve constant specific activity and the low level of activity remaining, the conclusion was reached that **11** itself was not radioactive.

Incorporation of Labeled Benzophenone 14. The mycelium, obtained from a 100-ml culture of *P. griseofulvum* which had grown in medium 1 for 64 h, was isolated by filtration, washed with medium 2, and then added to a 250-ml Erlenmeyer flask containing 80 ml of medium 2, pH 6.3, to which 2.6 mg (1.016 × 10^6 total dpm) of labeled **14** had been added. At 3-day intervals, 3-ml aliquots were removed from the stationary culture and extracted with $CHCl_3$. The extract was washed with 5% NaOH, dried, and examined by TLC. Griseofulvin was the only compound visible in the neutral fraction. After 7 days, the pH reached 5.0 and the mycelial pad was separated by filtration and washed with water. The filtrate was acidified (pH 2) and extracted continuously for 6 h with Et_2O. The ethereal extract was washed with NaOH after which 145 000 dpm (14%) remained in the ether layer, essentially all of the counts being in griseofulvin. The basic solution contained 137 000 dpm. The ethereal solution was evaporated and the residue placed on a 5 × 50 mm alumina (Grade V) column. Elution with hexane, C_6H_6, and finally C_6H_6–EtOH (50:1) gave a fraction containing 2.47 mg of griseofulvin, mp 217-219° (lit.[26] mp 220-221°), to which nonradioactive griseofulvin was added to bring the total weight to 100.8 mg (121 000 total dpm, 1200 dpm/mg). The specific activities after two successive recrystallizations from benzene were 1189 and 1200 dpm/mg, respectively.

After recrystallization, the labeled griseofulvin (4.23 × 10^8 dpm/mol) was degraded by treating 39.7 mg (0.113 mmol) with 0.04 ml of 2 N H_2SO_4 in 0.21 ml of HOAc at 100° for 45 min, during which time crystals appeared. The mixture was cooled and the crystals were collected by filtration, washed with Et_2O and MeOH, and air-dried to give 33.6 mg (88%) of griseofulvic acid (**16**), mp 263-266° (lit.[26] mp 255-258°), 3.98 × 10^8 dpm/mol. On account of insolubility, 0.005 ml of hyamine hydroxide (1 M in MeOH) had to be added to the scintillation mixture. Treatment of **16** (29.3 mg, 0.086 mmol) with

86% KOH (0.06 ml), CH_3CH_2SH (0.03 ml), Et_3N (0.03 ml), and *N,N*-dimethylacetamide (1 ml) for 7 days at room temperature under N_2 followed by addition of more CH_3CH_2SH (0.03 ml) and standing an additional 4 days (TLC indicated no **16** remained) gave, after acidification with HCl, removal of the solvents in vacuo, addition of H_2O, and storing at 5°, 11.2 mg (40%) of normethylgriseofulvic acid (**17**), mp 256–257° dec and 257–258° dec after recrystallization from EtOH (lit.[26] mp 262°), 0.16×10^8 dpm/mol. Thus, 90.3% of the radioactivity was localized at the predicted site.

The acidic components, obtained by extraction of the ethereal extract of the culture broth with NaOH, were examined by HPLC [Corasil II, with elution by pentane–EtOAc–MeOH–HOAc (75:25:1:0.1)] which showed the presence of xanthone **6** and benzophenone **4**. The mycelial pad was extracted with acetone in a Soxhlet extractor and the extract (78 900 total dpm) was examined by TLC and found to contain xanthone **6** and griseofulvin. The two spots were removed and eluted; they contained 43 and 24%, respectively, of the radioactivity in this fraction.

Identification of Xanthone 19. The carrier-dilution experiment was identical with that used for isolation of benzophenone **11** except that EtOAc was used as the extraction solvent. From 0.101 g of nonradioactive **19** added to the broth, 0.088 g was recovered by chromatography on silica gel. Recrystallization from aqueous EtOH gave poor recovery and little purification. A 5-mg portion of the material was fractionated by HPLC on a Waters Carbowax-400 Durapack column (3 m × 2.3 mm) using 1:1 EtOAc–pentane for elution. The center of the major peak was collected; on rechromatography, small fractions were collected and counted. The radioactivity was contained primarily within the fractions containing **19**; the average specific activity of the fractions was 479 dpm/mg.

In a similar experiment, both xanthones **6** and **19** were isolated by carrier dilution from a culture grown as described above; 12 mg of **6** and 11 mg of **19** were added to the culture broth prior to isolation of the metabolites by extraction and separation by chromatography on silica gel. Purification by HPLC gave xanthones **6** and **19** with activities of 740 and 609 dpm/mg, respectively.

In yet another experiment, xanthone **19** was isolated without the aid of carrier dilution. Three 500-ml flasks containing 150 ml of medium 2 plus KNO_3 (2.3 g/l.) and "Proflo" cottonseed flour (10 g/l.) were inoculated with *P. griseofulvum* and grown as stationary cultures for 24 days. After removal of the mycelium, the broth was extracted repetitively with Et_2O and the extracts were combined and evaporated. The residue was chromatographed on silica gel. The fractions eluted with 10–50% Et_2O–hexane were combined, concentrated, and further separated by HPLC on Corasil II with EtOAc–pentane (1:3) eluent. The peak with retention time corresponding to authentic **19** was collected. The material had the same R_f on TLC, color with diazotized benzidine, uv spectrum, and mass spectrum as authentic material.

Incorporation of 20 into Griseofulvin. The fungus was grown in a shaken culture in medium 1 for 48 h; the mycelium (3.5 g, wet) was collected by filtration, washed with medium 2, divided into two parts, and placed in two flasks containing 75 ml of medium 2. To each flask was added half of a solution containing 5.3 mg of labeled **20** (5300 dpm/mg), 0.1 M NaOH (0.05 ml), and medium 2 (10 ml). After 7 days the stationary cultures were combined, acidified with dilute HCl, and extracted exhaustively with Et_2O. The ethereal extract contained 81% of the added radioactivity; extraction with base lowered this value to 53%. Nonradioactive griseofulvin (28 mg) was added to the neutral fraction and was purified by chromatography on alumina (specific activity 299 dpm/mg, total incorporation 44%) and then by three recrystallizations from benzene (final specific activity 289 dpm/mg).

The base-soluble fraction was examined by TLC and column chromatography on silica gel. The main radioactive component was unaltered grisan **20**. Labeled benzophenone **4** and 6-methylsalicylic acid (**10**) were also present; each represented less than 3% incorporation.

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