NOTE

TERPHENYLLIN, A NOVEL *p*-TERPHENYL METABOLITE FROM *ASPERGILLUS CANDIDUS**

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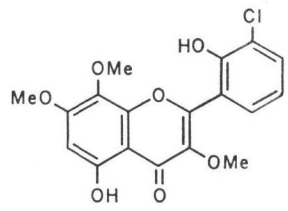
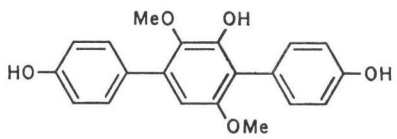
Aspergillus candidus is distinguished as the only fungus yet positively known to produce flavonoid metabolites[1~3]. The antibiotic chlorflavonin (**I**) together with a dechloro-analogue which can be metabolized to chlorflavonin and may, therefore, be a biosynthetic intermediate[3], have been isolated from two strains of the organism[4]. In the course of an investigation which established that these flavonoid compounds are biosynthesized *de novo* by the fungus and not by transformation of constituents in the medium[5] we recovered from cultures a new metabolite, terphenyllin, which we suggested might be 1,4-dimethoxy-2, 4′, 4″-trihydroxy-*p*-terphenyl (**II**)[6].

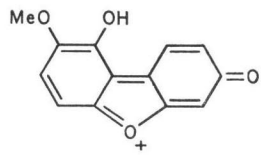
The compound, m.p. 239°C (decomp.), was extracted along with chlorflavonins from acidified cultures and was separated from them by chromatography on silicic acid. The molecular formular was determined by high resolution mass spectrometry to be $C_{20}H_{18}O_5$. Terphenyllin gave characteristic phenolic reactions with diazotized aromatic amines, showed benzenoid absorption in the ultraviolet region, and exhibited maxima associated with aromatic and hydroxyl groups in its infrared absorption spectrum. These properties were



I



II



III

accounted for, in part, by postulating two *p*-hydroxyphenyl substituents which appeared in the ^1H n.m.r. spectrum as separate AA′BB′ systems, along with temperature-dependent signals for the associated hydroxyl protons. Additional signals for two methoxyl groups, a third temperature-sensitive hydroxylic proton, and an aromatic proton weakly coupled to the protons in one of the methoxyl groups allowed the remaining $C_8H_8O_3$ portion of terphenyllin to be formulated as a dimethoxyhydroxybenzene. These assignments of signals in the n.m.r. spectrum were supported by the presence of fragment ions in the mass spectrum at *m/e* 323 (M^+-CH_3), 308 (M^+-2CH_3) and 292 ($M^+-CH_3-OCH_3$). A strong ion peak at *m/e* 229 was consistent with the loss of *p*-hydroxyphenyl and a hydrogen atom from the fragment at *m/e* 323 to give III or an equivalent structure. This

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Table 1. Incorporation of radioactivity from labeled supplements into terphenyllin

Supplement (μC_i/m mole)		Specific incorporation*
D-(*U*-^{14}C) Glucose	0.335	207
L-(*methyl*-^{14}C) Methionine	15.7	3.09
L-(*U*-^{14}C) Phenylalanine	16.5	7.47
L-(β-^{14}C) Phenylalanine	20.0	9.30
Sodium (α-^{14}C) cinnamate	41.3	0.151
Sodium (1-^{14}C) acetate	51.0	0.097

* 100×specific activity of metabolite/specific activity of precursor. No attempt has been made to correct for unlabeled precursors that may have been present in corn steep liquor.

evidence does not distinguish between several possible arrangements of the substituents on the central ring of terphenyllin but examination of the compound by X-ray crystallography has established the correct structure as **II**.[7]

Cultures of *Aspergillus candidus* supplemented with D-(*U*-^{14}C)glucose, L-(*methyl*-^{14}C) methionine, L-(*U*-^{14}C)phenylalanine or L-(β-^{14}C)phenylalanine yielded labeled **II** (Table 1). Since D-glucose was the main carbon source in the culture the high specific incorporation was not unexpected, but indicated that nutrients in the corn-steep liquor component of the medium did not compete strongly as precursors. Under these circumstances incorporations of labeled phenylalanine and methionine, although appreciably lower than those of labeled glucose, offer reasonable evidence that these compounds are biosynthetic precursors. The role of methionine is assumed to be supplying methyl groups to the methoxyl substituents, and phenylalanine, which in both uniformly and specifically labeled forms is more efficiently incorporated than methionine, is presumed to supply the *p*-terphenyl carbon skeleton. Previous studies on the biosynthesis of volucrisporin[8] and phlebiarubrone,[9] two terphenyl derivatives produced by other fungi, and on pulvinic acid[10], calycin[11], and related lactones biosynthesized by the mycobiont of lichens via terphenylquinones,[12] have shown that this ring system is derived by self-condensation of two phenylpropanoid units. The very low incorporation of (1-^{14}C)acetate and (α-^{14}C)cinnamate is consistent with studies on volucrisporin which indicated that neither of these compounds was an intermediate and that the terphenyl ring system is formed by self-condensation of phenylpyruvate derivatives[13].

In several experiments **II** isolated separately from the mycelium and filtrate of cultures given radioactive supplements did not differ significantly in specific activity. However, **II** and chlorflavonins isolated from the same culture sometimes differed unexpectedly in isotopic content. Thus, the specific activities from cultures supplemented with L-(*methyl*-^{14}C)methionine and L-(*U*-^{14}C) phenylalanine were 0.475 and 1.24 μCi/m mole, respectively, for **II** but 5.12 and 4.50 μCi/m mole for chlorflavonin. On the other hand D-(*U*-^{14}C)glucose labeled **II** more efficiently (0.705 *vs* 0.512 μCi/m mole). These differences could be accounted for if the two metabolites were produced at different times during the growth of *A. candidus* cultures. Other explanations are possible, but further speculation does not appear to be warranted in view of present uncertainties in the biosynthesis of both metabolites. However, it is evident that *A. candidus* is unusual among the microfungi in its metabolism of phenylpropanoid amino acids.

Experimental

Culture of *Aspergillus candidus*:

The strains used were obtained from the Commonwealth Mycological Institute, Kew, England (CMI 16046), and the American Type Culture Collection, Rockville, Maryland (ATCC 20022). Both produced terphenyllin,

but strain CMI 164046 was used in all biosynthetic experiments. Cultures were maintained on potato-dextrose agar slants. A vegetative inoculum was prepared by transferring spores and mycelium to a 500-ml Erlenmeyer flask containing 100 ml of glucose-corn steep liquor medium. The culture was incubated in the dark at 25°C for 2 days on a rotary shaker (200 rpm, 1.5 in eccentricity) and used in 4-ml portions to inoculate cultures for the production of terphenyllin. Production cultures were grown under the same conditions and on the same medium. The latter consisted of: D-glucose (50 g), corn steep liquor (50 ml, supplied by Canada Starch Company, Cardinal, Ontario), and calcium carbonate (2 g), in distilled water (1 liter). The pH was adjusted to 6.6 before sterilization.

Isolation:

Cultures harvested at 6 days were adjusted to pH 4.5 and filtered. The filtrate was extracted with chloroform and the mycelium in a Soxhlet apparatus with acetone. The extracts were evaporated and the residual solids were leached with petroleum ether (b.p. 30~80°C). The insoluble material was then extracted with chloroform and the extracts were chromatographed, either combined or separately, on a column of silicic acid (No. 2847, 100 mesh, Mallinkrodt Chemical Works, St. Louis, Missouri). Terphenyllin was collected, as a colorless zone following the yellow zone containing chlorflavonins, by elution with ethyl acetate. It was crystallized as needles from aqueous methanol and sublimed in high vacuum at 170°C to a constant m.p. of 239°C (decomp.); yield 10~20 mg/liter of culture, approximately half of which was in the mycelium.

Properties:

In the ultraviolet region absorption maxima were observed in an ethanolic solution at 225 (inflexion) and 275 nm (both of log ε 4.09). The infrared absorption spectrum (KBr) had maxima at 3300 (O-H stretching) 3070, 3040~3010 (aromatic C-H stretching), 1605, 1595 (aromatic C=C stretching) 895 and 825 cm^{-1} (aromatic C-H bending). The mass spectrum contained peaks at *m/e* 338 (100 %), 323 (40 %), 308 (19 %), 292 (22 %), 291 (11 %), 263 (8 %), 229 (17 %), 223 (6 %), 168 (6 %), 93 (2 %) and 77 (19 %). Accurate mass measurement of the molecular ion by peak matching against perfluorokerosene gave a value of 338.1168 (calculated for $C_{20}H_{18}O_5$: 338.1154). The ^1H nuclear magnetic resonance spectrum, recorded in acetone-d_6 at 100 MHz with tetramethylsilane as the internal reference, showed temperature-dependent signals at δ 8.37 br (s, 1H), 8.17 br (s, 1H), and 7.42 (s, 1H) each assigned to hydroxyl groups; signals for one AA'BB' system of aromatic protons at δ 7.5 (d, J 8.7 Hz, 2H) and 6.92 (d, J 8.7 Hz, 2H), and a second at δ 7.24 (d, J 8.8 Hz, 2H) and 6.83 (d, J 8.8 Hz, 2H); an aromatic proton signal at δ 6.67 (s, 1H): and two aromatic methoxyl signals at δ 3.68 (s, 3H) and 3.37 (s, 3H). The methoxyl group responsible for the δ 3.68 signal was weakly coupled (J 0.5 Hz) to the aromatic proton at δ 6.67.

Terphenyllin could be distinguished from the chlorflavonins by thin-layer chromatography on silica gel HF_{254} (E. Merck, Darmstadt). With benzene-acetic acid - water (100 : 30 : 1) the Rf values were 0.18 and 0.57; with chloroform-ethyl acetate (3 : 1) they were 0.21 and 0.50, respectively. These substances were detected as quenching zones under ultraviolet light of 254 nm, and by spraying with an aqueous solution of Fast Bordeaux Salt BD (Irwin Dyestuff Division, Chemical Developments of Canada Ltd., Montreal, Quebec) followed by 1 % sodium carbonate solution. After the latter treatment terphenyllin gave an orange zone whereas the chlorflavonin zone was orange-red.

Radiotracer experiments:

Labeled compounds were sterilized as aqueous solutions by autoclaving, and each was dispensed into 1 liter of culture medium immediately after inoculation. Except for D-(U-^{14}C)glucose, which was effectively diluted by the carbon source of the nutrient medium, all radioactive supplements were mixed with carrier so that their concentration in the culture was approximately millimolar. Cultures were incubated, harvested, and extracted as described above. Terphenyllin was separated from the chlorflavonins by chromatography on a column of silicic acid and purified to constant specific activity. Pro-

cedures for the separation and purification of chlorflavonins have been described elsewhere.[8] The radiochemical purity of all products was monitored by thin-layer radiochromatography. Specific activities were measured by counting samples to 1 % standard deviation with a liquid scintillation spectrometer.

Acknowledgements

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