Trichodiene Accumulation by Ancymidol Treatment of *Gibberella pulicaris*

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Treatment of *Gibberella pulicaris* with ancymidol (**6**) causes an inhibition of diacetoxyscirpenol (**4**) formation and a concomitant accumulation of the trace intermediate trichodiene (**1**); the isolation and identification of (**1**) were facilitated by comparison with totally synthetic material.

The trichothecene mycotoxins have been associated with a variety of human and animal health problems which occur after the ingestion of fungally contaminated grain.[1] Specific inhibitors have been useful both in the control of other mycotoxins and in the elucidation of their biosynthetic pathways.[2] Herein we report our finding of the first known inhibitor to block trichothecene biosynthesis with the concomitant build-up of trichodiene (**1**).[3]

The first committed step in the fungal production of trichothecenes (Scheme 1) is the isomerization–cyclization of *trans,trans*-farnesylpyrophosphate, (**2**), to trichodiene (**1**).

Examination of this transformation[4] and subsequent steps in trichothecene biosynthesis[5] has been hampered by the unreliable and minute yields of trichodiene available fron natural sources. The synthetic routes devised for racemic trichodiene are intricate and time consuming; and, to date, chiral trichodiene has not yielded to synthesis.

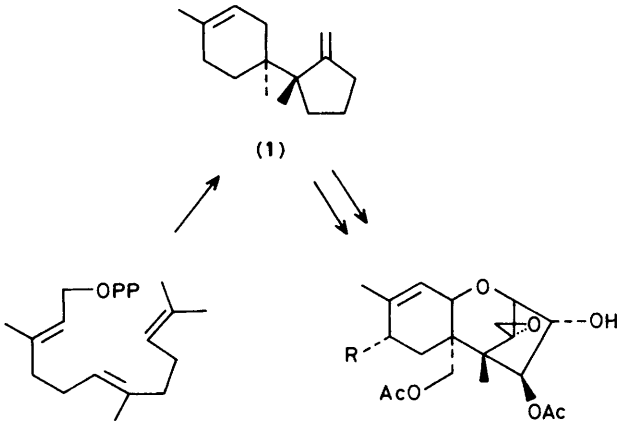
From our initial studies on trichothecene biosynthesis,[6] it was determined that in *Fusarium* each of the biological oxidations from trichodiene to T-2 toxin (**3**) requires molecular O_2 as a substrate. If the biosynthetic pathway is linear, then intermediates should accumulate upon specific inhibition of each of the six or more postulated mono-oxygenases.

In an attempt to accumulate trichothecene intermediates by inhibition of the fungal biosynthetic pathway, an inhibitor screen was undertaken. Twenty-five compounds which are postulated to inhibit cytochrome P-450 mono-oxygenases were tested for their ability to suppress diacetoxyscirpenol (DAS), (**4**), formation using liquid cultures of *Gibberella pulicaris* (Fries) Saccardo (anamorph: *Fusarium sambucinum*

Table 1. Accumulation of trichodiene in ancymidol-treated fermentations.

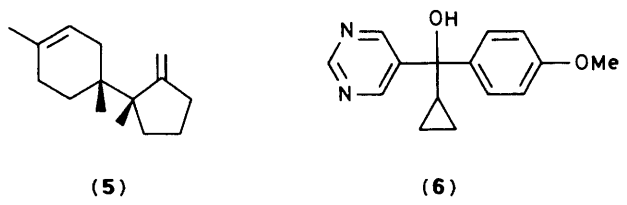
	Liquid culture control /mg dm^{-3}	Liquid culture plus ancymidol (516 p.p.m.)[a] /mg dm^{-3}	Solid culture control/ mg kg^{-1}	Solid culture plus ancymidol (516 p.p.m.)/ mg kg^{-1}
[DAS]	53	4.9[b]	250	143[b]
[Trichodiene]	[c]	2.2	8	202

[a] Ancymidol was added in two equal portions of 258 p.p.m. at 24 and 48 h after inoculation. [b] Total DAS produced including toxin formed prior to the addition of ancymidol. [c] Not detected (<0.01 mg dm^{-3}).



OPP = orthopyrophosphate

Scheme 1



Fuckel).[7] The culture extracts, when examined by t.l.c. and g.c.–mass spectrometry (m.s.), proved exceptionally complex showing the presence of several compounds which seemed to be sesquiterpenoid in origin. Owing to this complexity, it was essential to obtain authentic standards from which the chromatographic properties and spectral fingerprints could be obtained. Therefore, a sample of (±)-trichodiene, the only proven trichothecene biosynthetic intermediate, was synthesized by the convergent method of Gilbert and Weichman.[8] The 40:60 diastereoisomeric mixture of (±)-(**1**) and (±)-bazzanene, (**5**), obtained from this synthesis was quite suitable for our identifications, since (**1**) and (**5**) exhibit identical chromatographic properties but are differentiable by ^{1}H n.m.r. spectroscopy.

With a trichodiene standard in hand, scrutiny of the more promising inhibitor-treated fungal extracts revealed the presence of (**1**) [t.l.c. (SiO_2): R_f 0.50 in hexane; g.c.–m.s. (electron impact): *m/z* 204 (M^+, 0.5%), 109(100), 108(90), 67(62)]. Ancymidol (**6**) was one of the best inhibitors of DAS production by *G. pulicaris* and cultures treated with (**6**) provided the largest titres of trichodiene. Ancymidol has been shown to be a specific inhibitor of kaurene oxidase in the gibberellic acid pathway of *Marah macrocarpus*,[9] and as such has found commercial utility as a plant growth regulator.†

Experiments were next undertaken to develop a practical process for obtaining substantial quantities of trichodiene. Various environmental factors as well as the carbon source used in fungal fermentations are known to affect the nature and quantity of trichothecene toxins that are produced.[10] A dramatic increase in the accumulation of trichodiene was observed when ancymidol-treated solid fermentations employing rice as the substrate were used in place of liquid fermentations with glucose as the carbon source[11] (Table 1).

The following procedure was used to obtain 850 mg of optically pure {$[\alpha]_D^{25}$ + 30.1° (c 1.26 in $CHCl_3$)}trichodiene. A surface culture of *G. pulicaris* NRRL 13455 (R-6380[7] obtained from the Fusarium Research Center, Pennsylvania State University) was used to prepare 500 ml of an aqueous conidial suspension (2 × 10^7 conidia ml^{-1}). Fourteen 2.8 dm^3 Fernbach flasks containing 135 ml distilled water and 300 g of converted rice (presoaked overnight and autoclaved) were each inoculated with 40 ml of the above suspension. After four days, ancymidol (0.6 mmol, 6 ml of a 100 mM solution in dimethyl sulphoxide) was added to each flask and the incubation was continued at 28 °C with daily manual shaking. Ten days after addition of ancymidol, the contents of each flask were extracted by blending with EtOAc. Trichodiene (202 mg kg^{-1} rice), which exhibited chromatographic (t.l.c. and capillary g.c.) behaviour and spectral (m.s., i.r., n.m.r.) properties identical to those of the synthetic trichodiene, was purified by gravity chromatography (SiO_2) using hexane as eluant.

Thus, the combination of treatment of *G. pulicaris* with ancymidol and growth on solid rice cultures served to provide ready access to quantities of natural trichodiene. The effect of ancymidol on other trichothecene-producing organisms, as well as the identification of other intermediates in inhibitor-treated cultures, will be of interest.

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† Ancymidol was developed by Eli Lilly and Co., and is synonymous with EL-531 and the trademark A-Rest.

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