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QUIESONE: AN INHIBITOR OF THE GERMINATION OF *PERONOSPORA TABACINA CONIDIA*

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Abstract—A potent germination inhibitor of *Peronospora tabacina* conidia has been isolated from tobacco leaves infected with this pathogen. From consideration of its spectral properties, it is suggested that the structure of this inhibitor is 5-isobutyroxy-β-ionone. The effects of this inhibitor on the germination of *P. tabacina* conidia have been investigated.

INTRODUCTION

THE EXISTENCE of inhibitors of the germination of *Peronospora tabacina* conidia in extracts of tobacco leaves infected with this pathogen was first demonstrated by Shepherd and Mandryk.[1] In this paper we describe the isolation and tentative identification of the major inhibitor from diseased tobacco plants. This inhibitor has been given the trivial name *quiesone* (Latin: quiescere, to sleep), since it is a potent, reversible inhibitor of *P. tabacina* conidial germination.

RESULTS

Isolation of Quiesone

Following inoculation of tobacco leaves with *P. tabacina*, acetone extracts were prepared from infected leaves harvested at daily intervals. Inhibitory activity, which was not detected in uninfected leaves, increased until the sixth day after inoculation and then remained constant. At this stage leaves were thoroughly infected with mycelium and would sporulate profusely if the plants were placed overnight at a humidity approaching saturation. In subsequent work, therefore, infection was allowed to develop for seven days before leaves were harvested and the inhibitory material extracted. As ethahol was found to be equally satisfactory for the initial extraction of inhibitory materials, this solvent, rather than acetone, was used during the large scale extraction of infected leaves.

Fractionation of the initial ethanol extract by counter-current distributed between light petroleum and aqueous methanol yielded material with an ED_{50} of 1 ppm when tested against the germination of *P. tabacina* conidia. This material, which lost activity if permitted to stand in diffuse sunlight for a week, was resolved into two inhibitory components by silica gel chromatography. Only the first of these (ED_{50} 0·1–0·01 ppm), which was 100× more active than the second component, was further purified by column chromatography on alumina and by preparative gas chromatography on SE 52 followed by Carbowax 20 M. Quiesone was obtained as one active fraction (ED_{50} approx. 0·0001 ppm) which, on re-chromatography on either Carbowax 20 M or SE 30 yielded a single peak only.

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[1] C. J. SHEPHERD and M. MANDRYK, *Trans. Br. Mycol. Soc.* **45**, 233 (1962).

Structure

The small amount of quiesone isolated (*ca.* 650 μg from 250 kg leaves) restricted the structural information to that obtained from spectral studies.

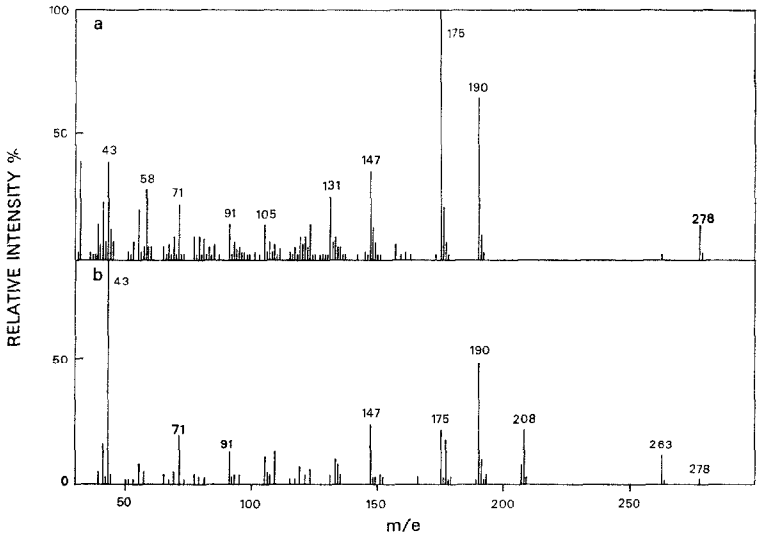


FIG. 1. MS OF, (a) QUIESONE, AND (b) 4-ISOBUTYROXY-β-IONONE.

Quiesone has the molecular formula $C_{17}H_{26}O_3$ (derived from high resolution mass spectrometry (Fig. 1a and Tables 1 and 2)). The IR spectrum (Fig. 2a) suggested that it was an ester (ν_{max} 1735, 1185 cm^{-1}). Von Sydow[2] examined the mass spectra of 15 esters of monoterpene alcohols, and found that all had peaks corresponding to $[RCO]^+$ and $[P-RCO_2H]^+$, but few had a peak corresponding to $[RCO_2H]^+$. Quiesone had a strong peak

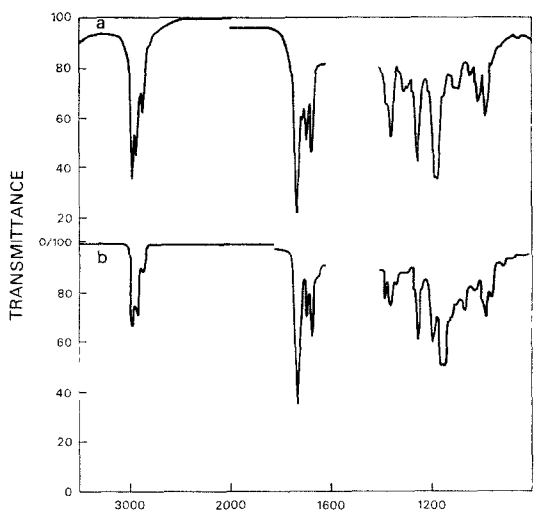


FIG. 2. IR SPECTRA (CS_2) OF, (a) QUIESONE, AND (b) 4-ISOBUTYROXY-β-IONONE.

[2] E. VON SYDOW, *Acta Chem. Scand.* **19**, 2083 (1965).

at *m/e* 190 ($C_{13}H_{18}O$, M-88), and a peak at *m/e* 71, which indicated that it was a butyrate. The absence of an M-28 peak argued against it being an *n*-butyrate.[3,4] A broad peak in the NMR spectrum (Fig. 3) at 5·1δ (1H) suggested that the ester group was attached to a tertiary carbon atom, and that the associated proton was extensively coupled.

TABLE 1. SOME MASS SPECTRAL TRANSITIONS FROM THE QUIESONE AND 4-ISOBUTYROXY-β-IONONE SPECTRA FOR WHICH METASTABLE PEAKS HAVE BEEN LOCATED

Transition	Metastable peak	Transition	Metastable peak
Quiesone		4-Isobutyroxy-β-ionone	
278 → 263	248·8	278 → 190	130·0
278 → 190	129·9	190 → 147	113·7
190 → 147	113·7	190 → 175	161·2
190 → 175	161·2	175 → 133	101·1
175 → 157	140·9		
175 → 131	98·1		

The third oxygen atom was involved in an α β, γ δ unsaturated ketone function (ν_{max} 1675 cm^{-1}; λ_{max}^{hexane} 277 nm with a shoulder at 214 nm). One of the double bonds was probably *trans* di-substituted (ν_{max} 1310 (w), 980 (m) cm^{-1}; 6·00 (d) and 7·00 (d) δ, J_{AB} 16 Hz),

TABLE 2. MOLECULAR FORMULAE OBTAINED FROM HIGH RESOLUTION MASS SPECTROMETRY OF QUIESONE AND 4-ISOBUTYROXY-β-IONONE VIA PEAK MATCHING WITH PERFLUOROKEROSENE

m/e	Formulae: Quiesone spectrum	Formulae: 4-isobutyroxy-β-ionone spectrum
278	$C_{17}H_{26}O_3$	$C_{17}H_{26}O_3$
190	$C_{13}H_{18}O$	$C_{13}H_{18}O$
175	$C_{12}H_{15}O$	$C_{12}H_{15}O$
147	$C_{11}H_{15}$ and $C_{10}H_{11}O$ (≃ 1:1)	$C_{11}H_{15}$ and $C_{10}H_{11}O$ (≃ 4:1)
133	Not determined	$C_{10}H_{13}$
131	$C_{10}H_{11}$	$C_{10}H_{11}$
71	Not determined	C_4H_7O

and the other tetra-substituted (no additional absorption 5–8δ). Use of the shielding increments derived by Matter *et al.*[5] indicated that the αβ bond was more likely to be the di-substituted one (calc. for αβ bond, αH: 6·33δ βH: 7·40δ; for γδ bond γH: 6·27δ, δH: 5·72δ; observed 6·00, 7·00δ). A slightly broadened singlet (1·79δ) and a sharp singlet (2·18δ) in the NMR spectrum indicated that methyl groups were attached to the tetra-substituted double bond and to the ketone function respectively.

[3] H. BUDZIKIEWICZ, C. DJERASSI and D. H. WILLIAMS, *Mass Spectrometry of Organic Compounds*, p. 176, Holden-Day, San Francisco (1967).

[4] E. STENHAGAN, S. ABRAHAMSSON and F. W. MCLAFFERTY, *Atlas of Mass Spectral Data*, Vol. 1, pp. 275–277, Interscience, New York (1969).

[5] U. E. MATTER, C. PASCUAL, E. PRETSCH, A. PROSS, W. SIMON and S. STERNHELL, *Tetrahedron* **25**, 691 (1969).

One unit of unsaturation remained to be accounted for. If it is assumed that the C_{13} skeleton is terpenoid then the remaining unit of unsaturation must be a ring as a straight chain terpene could not contain a tetrasubstituted double bond.

The most likely structure to fit the spectral data is that of a β-ionone skeleton (I) substituted with an isobutyroxy group in either the 4, 5, or 6 position. Comparison of the spectra of quiesone with those of 4-isobutyroxy-β-ionone* (Figs. 1–3, Tables 1 and 2);

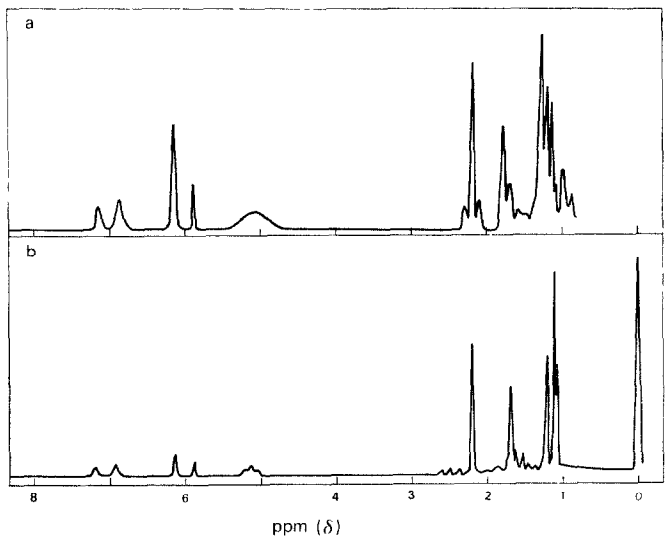


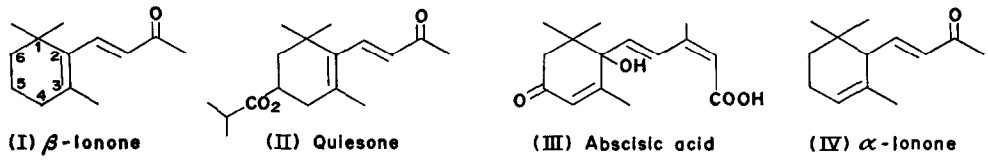
FIG. 3. NMR SPECTRA (CCl_4, 60MHz) OF, (a) QUIESONE, AND (b) 4-ISOBUTYROXY-β-IONONE. The quiesone spectrum was obtained by locking the signal for 0 with TMS and using a multi-scanning device coupled with a time averaging computer. 8–3δ: 100 scans, 3–1δ: 27 scans.

although revealing many similarities, indicated that the two compounds were not identical. While adding strong support to the structural derivation above, this rules out attachment of the isobutyroxy group at the 4 position. Attachment of the isobutyroxy group at the 5 position would seem more likely than attachment at the 6 position. If the isobutyroxy group were attached to the 6 position, a doublet of doublets (or a triplet if two peaks overlap) might be expected in the NMR spectrum from the hydrogen at the 6 position (as is found in the spectrum of 4-isobutyroxy-β-ionone), In fact, a broad peak (5·1δ) was found indicating more extensive coupling, as would be expected if attachment were at the 5 position. It is perhaps of interest to note that an oxygen function at the 5 position is common in related naturally occurring compounds (e.g. abscisic acid (III),[6,7] phaseic acid,[8] theaspirone,[9] an allenic compound found in grasshopper secretions,[10] and xanthoxin[11]). By analogy,

* 4-Isobutyroxy-β-ionone was kindly provided by Professor J. D. Bu'lock of the University of Manchester, England.

[6] K. OHKUMA, J. L. LYON, F. T. ADDICOTT and O. E. SMITH, *Science* **142,** 1592 (1963).
[7] J. W. CORNFORTH, B. V. MILBORROW and G. RYBACK, *Nature, Lond.* **206,** 715 (1965).
[8] J. MACMILLAN and R. J. PRYCE, *Tetrahedron* **25,** 5893 (1969).
[9] K. INA and Y. SAKATO, *Tetrahedron Letters* 2777 (1968).
[10] J. MEINWALD, K. ERICKSON, M. HARTSHORN, Y. C. MEINWALD and T. EISNER, *Tetrahedron Letters* 2959 (1968).
[11] H. F. TAYLOR and R. S. BURDEN, *Nature, Lond.* **227,** 302 (1970).

therefore, and from the NMR spectral properties, the isobutyroxy group is assigned to the 5 position.



Biological Activity

The effect of quiesone and several related compounds on the germination of *P. tabacina* conidia is shown in Table 3. Although considerably less active than quiesone, only β-ionone (I) inhibited germination (ED_{50} 0·15 ppm): all other compounds tested, including 4-isobutyroxy-β-ionone and α-ionone (IV), were without observable effects at the concentrations

TABLE 3. EFFECT OF QUIESONE AND RELATED COMPOUNDS ON THE GERMINATION OF *Peronospora tabacina* CONIDIA

Compound	Range of concentrations (ppm) tested	% Germination at maximum concentration tested
None	—	95
Abscisic acid	2–20	95
β-Carotene	0·1–1	96
4,4-Dimethyl-1-cyclohexene	0·1–1	95
β-Ionone	0·01–1	2 (ED_{50} 0·15 ppm)
Quiesone	0·0001–1	0 (ED_{50} 0·0001 ppm approx.)
4-Isobutyroxy-β-ionone*	0·01–1	96
4-Acetoxy-β-ionone*	0·01–1	95
α-Ionone	0·01–1	94
Phytol	0·01–1	93
Vitamin A	0·1–1	96

Conidia were prepared and incubated as described previously.[12] Each substance was added at the start of incubation and the percentage germination was assessed after 4 hr.

* Kindly supplied by Professor J. D. Bu'lock of the University of Manchester, England.

used. Although germ-tubes do not begin to appear until after 60 min incubation,[12] both quiesone and β-ionone only inhibit germination if added within 30 min of the start of

[12] D. W. HOLLOMON, *J. Gen. Microbiol.* **62**, 75 (1970).

incubation (Fig. 4). Addition after this time is without effect on either germination or subsequent germ-tube elongation. If conidia incubated for 4 hr in the presence of either quiesone or β-ionone are washed by centrifugation with distilled water, they subsequently

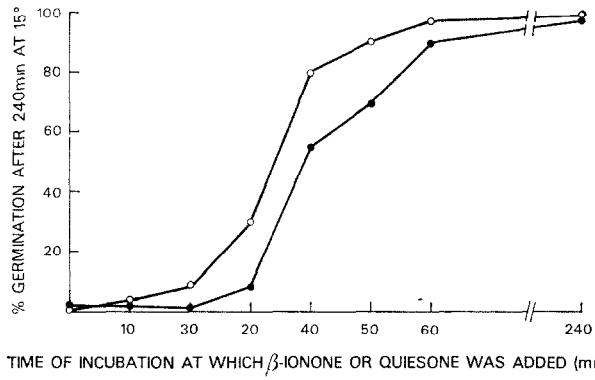


FIG. 4. EFFECT OF THE TIME OF ADDITION OF β-IONONE OR QUIESONE ON THE GERMINATION OF *Peronospora tabacina* CONIDA.
Conidia were prepared and incubated as described previously[12] and germination was assessed in all cases after 4 hr incubation. ○—quiesone; ●—β-ionone.

germinate normally indicating that neither compound is fungicidal (Table 4). By contrast, conidia incubated in the presence of cycloheximide (actidione) for 4 hr fail to germinate when washed and incubated in its absence.

TABLE 4. GERMINATION OF *Peronospora tabacina* CONIDA FOLLOWING TREATMENT WITH THREE GERMINATION INHIBITORS

Inhibitor	% Germination at the end of 1st incubation	% Germination at the end of 2nd incubation
β-Ionone (1·0 μg/ml)	1	94
Quiesone (0·001 μg/ml)	2	96
Cycloheximide (0·56 μg/ml)	1	8

Conidia were prepared as described previously[12] and incubated in the presence of either β-ionone, quiesone, or cycloheximide (1st incubation). After 4 hr at 15° conidia were collected and washed three times by centrifugation in distilled water. Conidia were finally incubated for a further 4 hr in the absence of any inhibitors (2nd incubation).

Early in the germination of *P. tabacina* conidia there appears to be a change in the protein synthesizing system which results in an increase in the rate of protein synthesis.[13] However, when conidia are incubated in the presence of either quiesone or β-ionone their

[13] D. W. HOLLOMON, *Arch. Biochem. Biophys.* **145**, 643 (1971).

protein synthesizing activity remains constant (Fig. 5). Yet neither compound inhibits the amino acid incorporating activity of cell free systems prepared from conidia (Table 5), indicating that their effect on *in vivo* protein synthesis is not a direct one. By contrast, this activation of protein synthesis early in germination occurs normally when conidia are incubated in the presence of either α-ionone or abscisic acid.

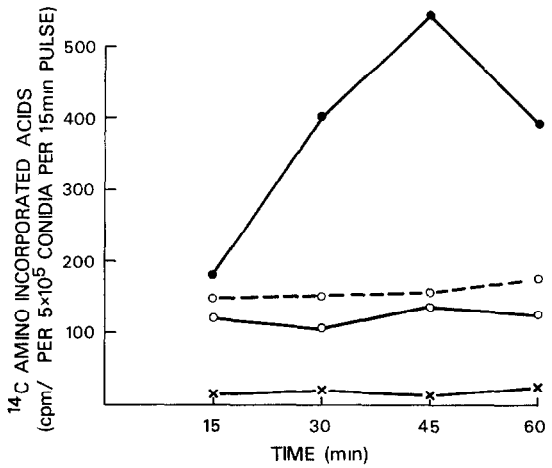


FIG. 5. EFFECT OF β-IONONE AND QUIESONE ON THE RATE OF INCORPORATION OF ^{14}C-AMINO ACIDS INTO PROTEIN BY WHOLE *Peronospora tabacina* CONIDIA DURING GERMINATION.
Conidia were prepared and incubated as described previously,[12] and labelled with 15 min pulses of ^{14}C-amino acids (0·16 μC/ml) given at 15 min intervals after the start of incubation. Incorporation was stopped by the addition of 0·5 ml cold 30% TCA and conidia were then treated as described previously.[16] Incorporation during each 15 min period is represented in the figure as the end point of the time interval. Quiesone, β-ionone and cyclohexemide were added at the start of the incubation. ●—no addition; ○—quiesone (0·001 μg/ml); ○- - β-ionone (1·0 μg/ml); ×—cyclohexemide (0·56 μg/ml).

DISCUSSION

Compounds containing the ionone skeleton represent an important group of biologically active compounds, many of which are known to control developmental changes in organisms. Perhaps the best known is abscisic acid (III) which is not only involved in the control of abscission but appears also to play an important role in maintaining bud and seed dormancy.[14] Xanthoxin, a compound with growth inhibiting properties similar to those of abscisic acid, has recently isolated from seedlings of both dwarf bean and wheat.[11] The production of carotenoids by certain Phycomycetous fungi is controlled by trisporic acids which, at least in one case, appear to regulate the synthesis of enzymes involved in carotenogenesis.[15]

Although quiesone is absent from healthy tobacco and appears only after infection and spread of the pathogen throughout the leaf, because *P. tadacina* is an obligate parasite,

[14] P. F. WAREING and G. RYBACK, *Endeavour* **29**, 84 (1970).
[15] D. M. THOMAS, R. C. HARRIS, J. T. O. KIRK and T. W. GOODWIN, *Phytochem.* **6**, 361 (1967).
[16] D. W. HOLLOMON, *J. Gen. Microbiol.* **55**, 267 (1969).

TABLE 5. EFFECT OF QUIESONE AND β-IONONE ON THE AMINO ACID INCORPORATION BY TWO FRACTIONS PREPARED FROM GERMINATED *Peronospora tabacina* CONIDIA*

Additions	^{14}C-amino acids incorporated as cpm/assay	
	20 000 *g* fraction	117 000 *g* fraction
None (5 μl acetone)	3060	2520
Quiesone 0·0005 μg	2840	2650
0·005	3080	2820
0·05	3240	2530
β-Ionone 0·1	3230	2410
1·0	3150	2400
10·0	3080	2370

* Both fractions were prepared as described previously[13] from conidia incubated for 30 min at 15°. The reaction mixture for the assay of amino acid incorporating activity contained (in a final vol. of 0·12 ml) 1·2 μmol. Tris, pH 7·8; 1·2 μmol magnesium acetate; 7·2 μmol KCl; 0·72 μmol 2-mercaptoethanol; 0·4 μmol ATP (adjusted to pH 7·8); 0·08 μmol GTP; 3·0 μmol phosphoenol pyruvate (sodium salt); 2·5 μg pyruvate kinase; 5·0 μg chloramphenicol; 0·5 μC ^{14}C-amino acid mixture; 300 μg of protein from 20 000 *g* fraction or 200 μg protein from 117 000 *g* fraction. Quiesone or β-ionone were added in 5 μl acetone. The reaction mixture was incubated at 25° and incorporation was stopped after 30 min in the case of the 20 000 *g* fraction and after 45 min in the case of the 117 000 *g* fraction by the addition of 5% trichloroacetic acid +0·1% casamino acids and 1 mg of bovine serum albumin. Proteins were sedimented and prepared for counting by liquid scintillation spectrometry as described previously.[13]

direct evidence is not yet available as to whether quiesone is a product of either host or fungus. Preliminary experiments suggest, however, that inhibitory material exists within conidia and that this material possesses similar biological properties to those expressed by quiesone. Examination of an acetone extract of conidia by silica gel TLC (solvent: 30% ethylacetate/light petroleum) revealed one inhibitory region with the same R_f as quiesone (0·6).

Quiesone exhibits considerable specificity, not only in relation to the effect of closely related compounds (Table 3) on *P. tabacina* conidial germination, but also in relation to its effect on the germination of spores of other species. Both quiesone and β-ionone failed to inhibit the germination of spores of five unrelated species (*Alternaria tenuis*, *Aspergillus nidulans*, *Monilinia fructicola*, *Neurospora crassa*, and *Puccinia graminis*).

EXPERIMENTAL

MS were determined on an AEI-MS-902 double focusing mass spectrometer. IR spectra were recorded in CS_2 solution, NMR spectra were obtained at 60 Mc/s in CCl_4, using tetramethylsilane as an internal reference, with a C-1024 time averaging computer. UV spectra were recorded in hexane. All GLC separations were carried out using a Shimadzu GC-IC chromatograph fitted with a flame ionization detector. The light petroleum used had a boiling range of 60–80° unless stated otherwise, and all solvents used in the isolation after the counter-current distribution stage were redistilled.

Assessment of germination during isolation of inhibitory material. Conidia were collected and prepared as described previously.[12] Fractions obtained during the isolation were dissolved in acetone (1 mg/ml) and a portion diluted with water to give a final acetone concentration of 2%. 1:10 serial dilutions in water were prepared from this 2% acetone solution and each dilution assayed for germination inhibitory material using the method of Shepherd and Mandryk.[1] One drop from each dilution to be tested, together with one drop of conidial suspension, were incubated on a 2% Agar disc (dia. 10 mm) in a Petri-dish. Germination was stopped after 4 hr at 15° by the addition of 1 drop of 10% formalin to each disc and the % germination assessed from counts of 100 conidia. Each concentration was assayed in triplicate and, at the levels used, acetone had no apparent effect on germination. In all other experiments, conidia were germinated in Petri-dishes (2·5 cm dia.) as described previously.[12]

Extraction of inhibitory material. Every fortnight, twelve 12-week-old *Nicotiana tabacum* plants, c.v. Virginia Gold (grown as described by Shepherd and Mandryk[1]), were foliage inoculated with a suspension of *P. tabacina* conidia. After 7 days the leaves were harvested and extracted with hot EtOH (approx. 35 l.). The filtered extract was evaporated to about 2 l. and extracted with light petroleum (b.p. 40–70°, 1 × 2 l., 2 × 500 ml). The light petroleum extract was evaporated down to 1 l., and extracted with 85% MEOH (1 × 1 l., 2 × 500 ml, 1 × 380 ml), The first two MeOH extracts were evaporated and the residue redissolved in the remaining MeOH. Water (120 ml) was added to give 1 l. of 75% MeOH. This was subjected to a simple counter-current procedure between petrol and 75% MeOH and significant activity was obtained in the petrol fractions. The active fractions from a number of counter-current distributions were normally pooled after evaporation to obtain greater reproducibility in subsequent work. The average yield of crude extract from 12 plants at each harvest was 2·4 g.

Crude extract (3·5 g) was dissolved in 5% EtOAc/light petroleum (40 ml) and applied to a column of silica gel (350 g, Merck Kieselgel 0·05–0·20 mm) equilibrated and packed in 2% EtOAc in light petroleum. The column was eluted with 300 ml of each of 5, 10, 15, 18, 20, 22, 25, 30, 35, 40% EtOAc in light petroleum. After evaporating the solvents, inhibitory material was found in fractions eluted between 1900–2100 ml (ED_{50} 0·1–0·01 ppm) and between 2600 and 2800 ml (ED_{50} 10–1 ppm). The first of these fractions from two silica gel columns was dissolved in 20 ml light petroleum and applied to a column of alumina (110 g Woelm basic alumina containing 15% water) and eluted with 100 ml of each of 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 80, 100% benzene in light petroleum. Removal of the solvents left activity in the fractions eluting between 800 and 1000 ml (ED_{50} 0·01 ppm).

Active material from several alumina columns (200 mg) was dissolved in light petroleum (0·8 ml) and injected (40 μl/injection) into a glass column (3·4 m × 6 mm i.d.) containing 4% SE 52 on Chromosorb G (AW-DMCS 60–70 mesh). The temperature programme was 200–210° at a rate of 4°/min, 210–220° at 2°/min, 220–235° at 1°/min, 235–255° at 4°/min. The carrier gas (N_2) pressure was 1·0 kg/cm². Activity was recovered in one fraction with a retention time of 20·5 min, which corresponded to a single peak on the chromatogram trace. This material (4·5 mg) was dissolved in acetone (0·2 ml) and was injected onto 1% Carbowax 20 M on Chromosorb G(AW-DMCS 60–70 mesh) in 50 μl aliquots. The column (3·4 m × 4 mm i.d.) was operated isothermally at 200° at a N_2 pressure at 1·0 kg/cm². Collection of the one active peak yielded 650 μg (estimated by comparison with peak areas of phytol which had the same retention time on Carbowax 20M). This yield was obtained from leaves of 700 infected plants (250 kg). Rechromatography of a portion of the active material (in CS_2) on either 1% Carbowax 20 M or 0·5% SE-30 revealed only a single peak.

Radioisotope. ^{14}C-amino acid mixture (CFB 104) was purchased from the Radiochemical Centre, Amersham, England and contained a mixture of 14 *l.*-amino acids.

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Key Word Index—Peronospora tabacina; Fungi; Phycomycetes; spore germination inhibitor; quiesone; 5-*iso*butyroxy-β-ionone; tobacco leaves.