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BIOSYNTHESIS OF THE PHYTOALEXIN WYERONE IN *VICIA FABA*

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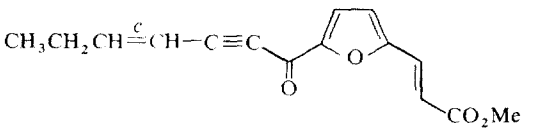
Key Word Index—*Vicia faba*; Leguminosae; *Botrytis cinerea*; wyerone; biosynthesis.

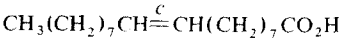
Abstract—In contrast to earlier results [1-^{14}C] acetate, [2-^{14}C] malonate and [*n* 9,10-^{3}H] oleate show significant incorporations into wyerone and related *Vicia faba* phytoalexins, following infection by *Botrytis cinerea*.

INTRODUCTION

Vicia faba represents an anomaly amongst the Leguminosae in that, on infection with various strains of *Botrytis*, it produces furanoacetylenic phytoalexins [1] such as wyerone (**1**) instead of the isoflavonoid phytoalexins more usually associated with this family [2]. Wyerone itself is structurally anomalous in that if its biosynthesis occurs via oleate (**2**) and crepenynate (**3**) by the conventionally accepted route [3]; then fairly extensive bond



1 Wyerone



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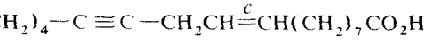

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reorganization must occur prior to the cyclization of the furan ring. This anomaly has prompted Jones and coworkers [4] to examine the incorporation of [1-^{14}C] acetate, [9,10-^{3}H] [9-^{14}C] oleate, and [9,10-^{3}H][9-^{14}C] crepenynate into wyerone, but the low incorporation figures (0.03, 0.05 and 0.02%, respectively) were not considered to be sufficiently meaningful to throw any light on the biosynthetic pathway leading to wyerone.

During their feeding experiments, the Oxford group applied their labelled precursors directly to healthy *Vicia faba* tissue, and under these conditions there is no reason why *de novo* synthesis of wyerone should occur since wyerone is a true phytoalexin [5] and is not formed in healthy tissue. Phytoalexin levels should only become significant when the bean tissue is combating pathogenic species, and undoubtedly the fact that any incorporation of labelled precursors was observed at all, was due to *Mucor* type infections which were reported to be in evidence during the course of their work.

RESULTS AND DISCUSSION

Initially we set out to determine the optimum conditions for wyerone induction in *Vicia faba* and Table 1 indicates the various methods employed. Wyerone concentration was determined by the increase in UV absorption at 350 nm. The two methods which showed greatest promise were treatment with 10^{-2}M mercuric chloride solution and infection with *Botrytis cinerea* and the latter method was employed in all subsequent feeding studies.

Time course studies using [1-^{14}C] acetate indicated that the incorporations steadily increase from 0.02% after 24 hr, to peak at 0.14% five days after the administration of the labelled substrate. After five days the incorporation level starts to fall, probably due to wyerone degradation. A small variation in the incorporation levels was also observed depending upon the time of the application of the inducer relative to the feeding of the labelled substrate. When [1-^{14}C] acetate was fed 15 hr prior to infection an incorporation of 0.13% was observed, whereas application of the labelled substrate 15 hr after infection results in an incorporation of 0.16%.

Using the conditions under which the highest incorporation of [1-^{14}C] acetate was observed, feeding experiments were carried out using [2-^{14}C] malonate and [*n* 9,10-^{3}H] oleate giving incorporation figures of 0.45 and 0.70%, respectively. Comparison of these incorporation values with those of Ross [4] shows a significant improvement (between 5 and 15 times the incorporation levels) clearly demonstrating the role of the pathogen in the induction of *de novo* wyerone biosynthesis. Furthermore the trend in the incorporations in progressing from acetate, through malonate to oleate, showing increasing incorporation levels is consistent with an acetate plus *n* malonate pathway leading to oleate during the early stages of wyerone biosynthesis.

Table 1. Wyerone induction in *Vicia faba* cotyledons*

Method	Wyerone level (μg/g fr. wt cotyledon)
Freeze branding†	52
Slicing‡	62
$HgCl_2$ (10^{-4} m)§	16
$HgCl_2$ (10^{-2} m)	337
Botrytis cinerea‖	386

* 3 days after induction.

† Cell necrosis induced by pressing a spatula at −170° onto tissue.

‡ Cotyledons sliced with a scalpel.

§ Applied as an aq. solution direct to cotyledon tissue.

‖ 20 μl of a solution containing 2.5×10^{6} conidia/ml applied directly to each cotyledon.

EXPERIMENTAL

Radiochemicals. Sodium [1-^{14}C] acetate (sp. act. 59.3 mCi/mM), sodium [2-^{14}C] malonate (17 mCi/mM) and [*n* 9, 10] oleic acid (2.4 Ci/mM) were purchased (Amersham). Counting was carried out [4] on an Intertechnique scintillation counter LS20.

Plant materials and feeding techniques. Seeds of *Vicia faba* L. (cv Aquadulcia claudia) were purchased (Suttons). The seeds were placed in plastic trays between layers of moist tissue paper and left to germinate and imbibe for 3 days. After this period the outer seed coat and the developing stem and root were removed and discarded. *Botrytis cinerea* was cultivated on standard MX media or V8 agar for 20 days. Spore concns were usually in the order of 5×10^6 conidia/ml. Spores were centrifuged and washed with sterile distilled H_2O prior to use.

Prepared cotyledons were placed on moist tissues in sandwich boxes and infected with spores from mature sporulating cultures of *Botrytis cinerea.* The prepared seeds were then allowed to stand for 15 hr during which time the spore suspension soaked into the bean tissue. After this time the labelled substrates were applied as aq. solns, the boxes closed and the seeds incubated at room temp. for 5 days. After this period the necrotic lesions were removed, frozen in liquid N_2 and ground in a pestle and mortar. The finely ground lesions were soaked overnight in Et_2O, filtered, washed with Et_2O, the Et_2O extracts dried, filtered and concd. The conc soln was then subjected to PLC on 20 × 20 cm Si gel plates (Merck GF_{254}) in hexane/Me_2CO (2:1) followed by $CHCl_3$/petrol (2:1). The wyerone (R_f *ca* 0.6) was removed from the plate and eluted with Et_2O. The crude wyerone was diluted with carrier (*ca* 20 mg) and crystallized to constant mp and specific activity. TLC fails to separate wyerone from dihydrowyerone. However, we have previously shown that repeated crystallization of wyerone from cyclohexane removes all traces of the dihydro derivative as evidenced by the disappearance of the M + 2 peak in the MS of wyerone purified in this manner.

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ANEMONIN, PROTOANEMONIN AND RANUNCULIN FROM *KNOWLTONIA CAPENSIS*

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Key Word Index—*Knowltonia capensis*; Ranunculaceae; anemonin; protoanemonin; ranunculin; lactones.

The genus *Knowltonia* (Ranunculaceae), is endemic to South Africa and there are a large number of reported uses of these plants in folk medicine [1]. Prior to this report, no detailed chemical studies had been carried out on members of this genus.

Aqueous and ethanol extracts of fresh *Knowltonia capensis* exhibited significant antibacterial activity against *Staphylococcus aureus*, while extraction of the dried ground plant material according to the preliminary fractionation procedure recommended by the National Cancer Institute [2] gave a chloroform fraction which exhibited significant *in vivo* antileukemic activity in the P388 lymphocytic leukemia test system [3]. Extraction of dried ground plant material with ethanol, followed by partitioning of the extract between ether and aqueous acid and extraction of the neutralized aqueous fraction with chloroform, gave a fraction which accounted for most of the observed antibacterial activity. Chromatography of this fraction on silica gave anemonin (**1**). Steam distillation of fresh plant material and extraction of the steam distillate with chloroform afforded protoanemonin (**2**) [4], which rapidly polymerized to anemonin. Extraction of fresh plant material with dilute HCl according to the procedure of Hill and van Heyningen [5] yielded the stable glucoside, ranunculin (**3**).

Ranunculin has been postulated as the precursor of protoanemonin [5], which has been shown to occur exclusively in members of one tribe of the Ranunculaceae, the Anemoneae and in the genus *Helleborus* [6]. However, in 1972 Carl Tschesche and coworkers found that, whereas extraction of *Ranunculus repens* and *Helleborus foetidus* according to the method of Hill and van Heyningen yielded ranunculin and a new isomer isoranunculin (**4**), when the plant material was carefully extracted with aqueous acetone, no trace of ranunculin could be found and two new glycosides, ranuncoside (**5**) and ranunculoside (**6**) were isolated [7]. These workers therefore concluded that ranunculin is an artifact generated during the acid extraction procedure, and that the genuine precursor of protoanemonin has yet to be isolated.

The aqueous acetone extraction of fresh *Knowltonia capensis*, however, afforded ranunculin as the major component and we were unable to detect the presence