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AN $^{18}O_2$ STUDY OF THE BIOSYNTHESIS AND METABOLISM OF RISHITIN

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Key Word Index—*Solanum tuberosum*; Solanaceae; biosynthesis; metabolism; $^{18}O_2$; lubimin; rishitin; rishitin metabolites.

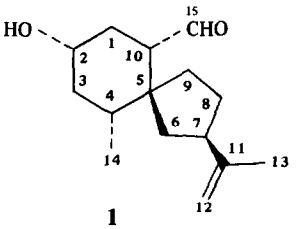
Abstract—An $^{18}O_2$ study has shown that the hydroxyl oxygen atoms of lubimin, rishitin and two metabolites of rishitin, 13-hydroxyrishitin and 11,12-dihydro-13 (or 12)-hydroxyrishitin, are derived from molecular oxygen. It could not be shown that the aldehyde oxygen of lubimin was derived from molecular oxygen, probably due to its exchange with the oxygen atom of water. These findings have established that mono-oxygenases are involved in all of the hydroxylation reactions and that, contrary to a previous proposal, 11,12-dihydro-13 (or 12)-hydroxyrishitin is not formed from rishitin by hydration.

INTRODUCTION

The accumulation of the terpenoid phytoalexins lubimin (**1**) and rishitin (**2**) is induced in potato (*Solanum tuberosum* L.) tuber tissue by numerous biotic (e.g. *Phytophthora infestans*) and abiotic (e.g. sodium arachidonate) elicitors [1]. These compounds, by virtue of their fungitoxic nature (ED_{50} 3.8×10^{-4} M for **2** against *P. infestans* [2]), constitute part of the chemical defence mechanism of white potato tubers. It has been suggested that the biosynthesis of rishitin from farnesyl pyrophosphate proceeds by the pathway: farnesyl pyrophosphate → germacrene – → solavetivone – → 15-dihydrolubimin → lubimin (**1**) → 3-hydroxylubimin (oxylubimin) → rishitin (**2**) [3–5], although other routes not involving vetispiranes may be possible [6]. The hydroxylation reactions are probably catalysed by microsomal, cytochrome P450 mono-oxygenases.

Healthy tuber tissue is able to metabolize exogenously supplied **2** to the more polar, less toxic compounds rishitin-M1 (**3**) and rishitin-M2 (**4**) [2]. Such metabolism may prevent the accumulation of injurious levels of **2** in healthy tuber tissues and in cells adjacent to infected tissues since, at 5×10^{-3} M, **2** is strongly phytotoxic [2].

The characterization [7] of the two metabolites as 13-hydroxyrishitin (**3**) and (11*R*)-11,12-dihydro-12-hydroxyrishitin (**4**, stereochemistry of side chain not shown) has shown that the detoxification involves hydroxylation of the isopropenyl side chain of **2**. It has been proposed that the formation of the second metabolite is by hydration of the isopropenyl double bond of **2** [7]. If this is the case, **3** (in which C-13 would be formed from C-12 of **2**) could be formed by dehydrogenation of **4** rather than by direct oxidation of C-13 of **2** or epoxidation of the 11,12-double bond of **2** followed by an allylic rearrangement. On the evidence, however, the second metabolite could equally well be 11,12-dihydro-13-hydroxyrishitin in which case it could be formed by reduction of **3**, this



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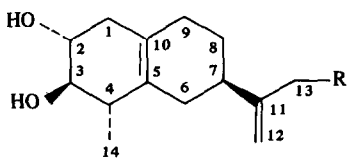
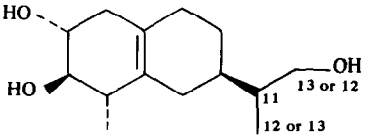


2 R = H
3 R = OH



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metabolite having been formed from **2** by one of the two oxidative routes just described through the mediation of a cytochrome P450 mono-oxygenase. An alternative route to these two metabolites is by reduction of **2** to form 11,12-dihydro-**2**. This compound could then be sequentially hydroxylated and dehydrogenated to form **4** and **3**, respectively.

In this study the origin of the oxygen atoms in compounds **1**–**4** has been investigated by means of $^{18}O_2$.

RESULTS

Synthesis de novo *of lubimin* (**1**) *and rishitin* (**2**) *in the presence of* $^{18}O_2$

Compounds **1** (31 μg) and **2** (95 μg) were isolated from aged potato discs which had been treated with sodium arachidonate (an abiotic elicitor of phytoalexin synthesis) and incubated in $^{18}O_2/N_2$ (see Experimental). Their identities were confirmed by their co-chromatography with authentic standards (TLC and GC). The mass spectral data on **1** and **2** (Table 1) showed the presence of one and two ^{18}O atoms, respectively.

Fate of applied ^{14}C-*labelled* **1** *and* **2** *in the presence of* $^{18}O_2$

In the first experiment, ^{14}C-labelled **1** (100 μg, 33 × 10^3 cpm) was applied to aged potato discs which were then incubated in either air or $^{18}O_2/N_2$ for 24 hr. Radio-TLC (ethyl acetate–cyclohexane, 1:1) and radio-GC of the ethyl acetate-soluble lipids from the incubation performed in air indicated that the radioactivity was associated with unmetabolized **1** (33%, R_f 0.30, R_t 17.5 min), **2** (30, 0.22, 10.5), 3-hydroxylubimin (12, 0.07, 60.5) and two polar metabolites, A (16, 0.08, 83.5) and B (9, 0.03, 73.5), later identified as **3** and **4**. In the extract from the $^{18}O_2/N_2$ incubation, however, no 3-hydroxylubimin and very little **2** were formed. GC/MS analysis of TLC purified samples showed that the molecular ion of **2** isolated from the discs incubated in $^{18}O_2/N_2$ was 2 a.m.u. higher than that from the discs incubated in air (Table 1).

In a comparable experiment using ^{14}C-labelled **2** (1 mg, 112 × 10^3 cpm), radio-TLC (ethyl acetate) suggested that, during the 24 hr incubation period, 50% of ^{14}C-**2** had been converted into the two metabolites, A and B, in the ratio 4:1. The polarities of these metabolites were shown to be greater than **2** by TLC (R_f: **2**, 0.46; A, 0.32; B 0.27) and by GC [R_t (min): **2**, 10.5; A, 83.5; B, 73.5]. These results were consistent with the metabolites being hydroxylated derivatives of **2**.

The poor resolution of the metabolites in ethyl acetate resulted in their combined elution for GC/MS analysis. The *m/z* values for the molecular ions of the metabolites [determined by CI(NH_3)GC/MS because water was lost too readily from each during EIMS] established that one ^{18}O atom was incorporated into each metabolite (Table 1). They were also indicative of A being a monohydroxy derivative of **2** and B being a dihydro derivative of A.

Subsequent to GC/MS analysis A and B were separated on TLC by double development with ethyl acetate–cyclohexane (9:1). Additional structural information was then obtained by ^{1}H NMR spectroscopy. The spectral data for A were consistent with this metabolite being 13-hydroxyrishitin (**3**) [360 MHz, acetone-d_6, TMS as int. reference δ1.13 (3H, *d*, C$\underline{H}_3$-14), 4.77 and 5.06 (each 1H, *s*, C$\underline{H}_2$-12), 4.08 (2H, *s*, C$\underline{H}_2$OH-13), 3.10 (1H, *t*, C$\underline{H}$OH-3), 3.52 (1H, *m*, C$\underline{H}$OH-2) cf. ref. [7]]. The mass spectral data had suggested that metabolite B was the 11,12-dihydro derivative (**4**) of **3**. This was borne out by ^{1}H NMR spectroscopy which showed that the signals for the propenyl double bond present in the spectrum of **3** had been replaced by those for an extra methyl group (δ0.92, 3H, *d*, C$\underline{H}_3$-12 cf. ref. [7]).

DISCUSSION

Through the use of [^{14}C]lubimin (**1**) and [^{14}C]rishitin (**2**), it has been confirmed that **1** can act as a precursor of **2** [3–5] and that **2** is metabolized to **3** and **4** [7] in aged potato tuber discs. In discs in which phytoalexin accumulation was induced by sodium arachidonate in the presence of $^{18}O_2/N_2$, **1** and **2** were both labelled with the heavy isotope. The mass spectral data (Table 1) showed that, in the case of **2**, the vicinal hydroxyl groups were labelled with ^{18}O whereas, in the case of **1**, only one of the

Table 1. Diagnostic mass spectral ions of compounds **1**–**4** biosynthesized in the presence of either air or $^{18}O_2/N_2$

Compound	Incubation atmosphere	*m/z* (rel. abundance) EI	CI(NH_3)
(a) *Synthesized* de novo			
1	Air	236 $[M]^+$ (9), 218 $[M-H_2O]^+$ (5), 193 (20), 175 $[193-H_2O]$ (19), 161 (12)	254*
1	$^{18}O_2/N_2$	238 $[M]^+$ (3), 218 $[M-H_2\,{}^{18}O]$ (7), 195 (17), 193 (6), 175 $[195-H_2\,{}^{18}O$ and $193-H_2O]^+$ (23), 161 (20)	256
2	Air	222 $[M]^+$ (5), 204 $[M-H_2O]^+$ (32), 189 (11), 161 (39)	240
2	$^{18}O_2/N_2$	226 $[M]^+$ (6), 206 $[M-H_2\,{}^{18}O]^+$ (27), 191 (9), 163 (30)	244
(b) *Synthesized from* ^{14}C-**2**			
3†	Air	220 $[M-H_2O]^+$ (24), 202 $[M-2H_2O]^+$ (8)	256
3	$^{18}O_2/N_2$	222 $[M-H_2O]^+$ (20), 202 $[M-H_2O, H_2\,{}^{18}O]^+$ (5)	258
4‡	Air	222 $[M-H_2O]^+$ (100)	258
4	$^{18}O_2/N_2$	224 $[M-H_2O]^+$ (100)	260

*$[M+NH_4]^+$ (100).

†Metabolite A, 13-hydroxyrishitin.

‡Metabolite B, 11,12-dihydro-13 (or 12)-hydroxyrishitin.

two oxygen atoms in the molecule was labelled. This labelled atom would almost certainly be that of the oxygen atom of the OH-2 group since **1** is a precursor of **2**, in which both hydroxyl groups are labelled from $^{18}O_2$. Furthermore, any $^{18}O_2$ incorporated into the aldehyde group of **1** would have exchanged readily with that of water via the formation of gem-diol intermediate [8] during the course of the experiment and the extraction procedure.

When ^{14}C-labelled **2** was metabolized by aged tuber tissue in the presence of $^{18}O_2/N_2$ the isolated metabolites (**3** and **4**) were each shown to contain one ^{18}O atom. This demonstrates that oxygenation of the isopropenyl side chain of **2** is mediated by a mono-oxygenase for the formation of both **3** and **4**. Hydroxylation of **2** to form **3** proceeds, almost certainly, by direct attack on C-13 to form the 13-hydroxy derivative (**3**) of **2** without any involvement of an epoxide or allylic rearrangement. ^{13}C NMR spectroscopy has been used to demonstrate that this mechanism operates in the case of the formation of 13-hydroxycapsidiol from capsidiol [9]. This sesquiterpenoid phytoalexin of pepper is closely related to **2** and is similarly hydroxylated in its isoprenyl side chain by healthy pepper tissue. The most obvious explanation for the origin of **4** lies in the reduction of **3** to form its 11,12-dihydro derivative. Hydration of the isopropenyl double bond, as suggested in ref. [7], is evidently not the mechanism by which **4** was formed in this study. Alternative mechanisms for the oxidative metabolism of **2** to **3** can be advanced (see Introduction). However, the true mechanisms can only be established by a ^{13}C NMR study of the type described in ref. [9].

This study establishes that the oxygen atoms of the hydroxyl groups of the potato phytoalexins, **1** and **2**, are indeed derived from molecular oxygen, as are those of the OH-13 groups of the metabolites **3** and **4**. The hydroxylation reactions are presumably catalysed by microsomal, NADPH-dependent, cytochrome P450 mono-oxygenases. Support for this assertion is provided by the observations that: (a) the synthesis of lubimin from IPP in cell-free systems prepared from elicitor-treated tuber discs is NADPH-dependent; and (b) the synthesis of rishitin and lubimin from MVA in elicitor-treated tuber discs is inhibited by the cytochrome P450 inhibitor metyrapone [T. Coolbear, unpublished work].

EXPERIMENTAL

Chemicals. ^{14}C-labelled **1** (333 cpm/μg) and **2** (112 cpm/μg) were extracted from the pooled $CHCl_3$-soluble lipid extracts from expts in which potato tuber tissue had been infected with *Phytophthora infestans* and then incubated with either [^{14}C]acetate or [^{14}C]MVA. After separation from each other by TLC on silica gel G developed with EtOAc–cyclohexane (1:1. R_f: **1**, 0.30; **2**, 0.22), **1** and **2** were purified by TLC in the following systems: $CHCl_3$–MeOH–HOAc (85:13:2; R_f: **1** 0.71; **2**, 0.56); EtOAc (R_f: **1**, 0.56; **2**, 0.46). Visualization of marker spots of the labelled compounds was by spraying with vanillin–H_2SO_4 reagent [10] and heating to 100°. Sodium arachidonate was from Sigma. $^{18}O_2$, 99 atom %, was from Amersham International. All solvents were re-distilled before use.

Plant material. Tubers of *Solanum tuberosum* cv. Kennebec (R1) were obtained from a commercial grower in Aberdeen, Scotland. They were stored at 4°, in darkness, until required. Discs (15 mm diam. × 5 mm) of tuber tissue were prepared from tubers that had been equilibrated to room temp. and surface sterilized with 15% Everchlor for 15 min. The discs were rinsed (× 3) in sterile distilled water and aged for 24 hr on 1% H_2O–agar at room temp. in darkness, prior to treatment.

Treatment of tuber tissue. In the arachidonate expt, 40 μl freshly prepared soln of sodium arachidonate (5 mM) was spread over the top surface of each disc (10 per expt). In the radiochemical expts, tuber slices (0.5 mm thick) prepared aseptically from the top surfaces of aged tuber discs were placed in small glass Petri dishes and treated with either 100 μg ^{14}C-**1** (12 discs) or 1 mg ^{14}C-**2** (60 discs). The substrates were dissolved in a minimum vol. of Me_2CO and then made up to either 1 ml (^{14}C-**1**) or 10 ml (^{14}C-**2**) with 0.01 M Pi buffer, pH 7.2.

The Petri dishes containing the treated slices were placed on top of a mixture of glass marbles and H_2O contained in a sterile, gas tight reaction vessel the lid of which was fitted with three inlet–outlet ports. Two of the ports led to the gas phase (400 ml), the third down into the H_2O and opening close to the bottom of the chamber. The chamber was purged with He (15 min) followed by N_2 (15 min). After closing the gas ports, H_2O (100 ml) was aspirated from inside the chamber and the resultant vacuum used to draw in 100 ml $^{18}O_2$, thus creating a physiological mixture of N_2 and O_2. The chamber was then left for 24 hr at room temp. in the dark. Control expts were performed in which sterile air was allowed to replace the 100 ml H_2O evacuated from the chamber.

Extraction and purification of **1**–**4**. In the sodium arachidonate expts, **1** and **2** were extracted from the first millimetre of the treated discs by the method outlined in ref. [11].

In the rest of the expts, the tissue slices plus the bathing soln were exhaustively extracted with $CHCl_3$–MeOH (2:1). After filtration, the solvent was removed from the extract by rotary evaporation *in vacuo* and the residue extracted (× 3) with EtOAc. The EtOAc extracts were combined, reduced in vol., and subjected to TLC developed with EtOAc. The ^{14}C-labelled metabolites and unmetabolized [^{14}C]phytoalexin were located using a Panax TLC radio scanner and then eluted with dry Me_2CO.

Analytical methods. GC/MS (EI- and CI-NH_3): Finnegan 4500 Quadrapole Mass Spectrometer using the following chromatographic conditions: glass column (2 mm × 2 m) packed with 3% OV 225 on Gas Chrom Q, He 25 ml/min, injection temp. 225°, column temp. 180°. Analytical GC: FID (GCD chromatograph, Pye Unicam) rest of conditions similar to those just described. Quantification of the phytoalexins was relative to methyl arachidonate. Radio-GC: conditions as for analytical GC, stream splitter 15% to FID, samples for radioassay collected at 1 min intervals in capillary tubes maintained at 0°.

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