Phytochemistry, Vol 23, No 9, pp. 1913–1915, 1984.
Printed in Great Britain.

0031-9422/84 $3.00 + 0.00
Pergamon Press Ltd.

BIOSYNTHESIS OF ABSCISIC ACID FROM FARNESOL DERIVATIVES IN *CERCOSPORA ROSICOLA*

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(*Revised received 7 February* 1984)

Key Word Index—*Cercospora rosicola*; Deuteromycetes; biosynthesis; sesquiterpenes; farnesol; farnesyl pyrophosphate; farnesyl phosphate; farnesic acid; abscisic acid.

Abstract—(*E,E*)-[1-^{14}C]Farnesyl phosphate and (*E,E*)-[1-^{14}C]farnesyl pyrophosphate were both converted to abscisic acid by *Cercospora rosicola* resuspensions. (*E,E*)-[1-^{14}C]Farnesol, (*E,Z*)-[1-^{14}C]farnesol, (*E,Z*)-[1-^{14}C]farnesyl pyrophosphate, (*E,E*)-[1-^{14}C]farnesic acid, and (*E,Z*)-[1-^{14}C]farnesic acid were not converted to abscisic acid by the fungus. These findings provide information on the sequence of the reactions involved in converting farnesyl pyrophosphate to abscisic acid. Specifically, they suggest that the transformations involving the three terminal carbons in the side chain occur after one or more changes elsewhere in the molecule.

INTRODUCTION

The finding of Assante *et al.* [1] that the fungus *Cercospora rosicola* produced large amounts of the plant hormone abscisic acid (ABA) (**1**) has led to the use of this micro-organism as a system for studying ABA biosynthesis. The normal terpenoid precursors acetate [2], mevalonate [3] and farnesyl pyrophosphate (FPP) (**3**) [4] are converted to ABA by the fungus. Evidence has been presented that the last step on the pathway is the conversion of 1'-deoxy-ABA (**2**) to ABA [3], and **2** is probably produced by oxidation of a 4'-hydroxy intermediate [3, 5]. Two other compounds, (*E,Z*)-ionylideneethanol and (*E,Z*)-ionylideneacetic acid, have also been shown to be converted to ABA [3, 5], but since they have not yet been shown to be endogenous constituents, they cannot be considered as proven intermediates. Here we report an investigation of the steps immediately following FPP on the pathway. After this work was completed, a paper appeared in which two of the compounds used in this work, (*E,E*)-farnesol (**5**) and (*E,Z*)-farnesol (**8**) were shown not to be converted to ABA by the fungus [6]. Our work confirms these findings. Furthermore, we show that (*E,E*)-farnesyl phosphate (**4**) is converted to ABA, whereas (*E,Z*)-farnesyl pyrophosphate (**7**), (*E,E*)-farnesic acid (**6**) and (*E,Z*)-farnesic acid (**9**) are not.

RESULTS AND DISCUSSION

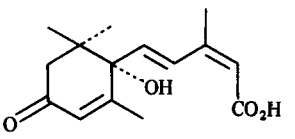
Resuspended mycelia of *C. rosicola* were used as reported previously to study ABA biosynthesis [2, 4]. In some runs, the incorporation of (*E,E*)-[1-^{14}C]FPP into ABA was much lower than that normally observed. In these cases, significant amounts of radioactive farnesol were detected in the medium. Samples analysed after short incubation periods showed that much of the [1-^{14}C]FPP had been converted to farnesyl phosphate (FP) (**4**) and farnesol. When the mycelium was removed by filtration several hours after resuspension and [1-^{14}C]FPP added to the filtrate, hydrolysis to FP and farnesol was again observed. Thus, the fungus must excrete a phosphatase into the medium. More recent work has produced evidence that two different enzymes may be present, one causing conversion to FP and the other to farnesol [S. Hasegawa, personal communication]. This could account for the low yields obtained in some runs, if high levels of phosphatase activity were present and if farnesol was not a precursor of ABA.

To determine if the latter was the case, (*E,E*)-[1-^{14}C]-farnesol was administered to a resuspension of *C. rosicola*. The farnesol was extensively metabolized, less than 1% being recovered. However, only about 25% of the administered radioactivity could be accounted for in the medium and the mycelial extract. Apparently most of the metabolism results in loss of C-1, perhaps as CO_2. The ABA produced by the fungus was isolated by reversed-phase column chromatography. It was then subjected to the procedures recommended by Milborrow and Noddle [7] for demonstrating the incorporation of radioactive precursors into ABA. Less than 1% of the farnesol was converted to ABA, a very low yield compared to a control in which (*E,E*)-[1-^{14}C]FPP was used as the substrate under the same conditions (Table 1). These results in-

Table 1. Incorporation of labelled substrates into ABA

[1-^{14}C] Labelled compound	Incorporation (%)	
	Control*	Substrate
(*E,E*)-Farnesyl phosphate (**4**)	11.3	10.8
(*E,E*)-Farnesol (**5**)	16.7	0.7
(*E,Z*)-Farnesol (**8**)	16.7	< 0.03
(*E,Z*)-Farnesyl pyrophosphate (**7**)	9.6	< 0.12
(*E,E*)-Farnesic acid (**6**)	12.6	< 0.4
(*E,Z*)-Farnesic acid (**9**)	12.6	< 0.05

*Controls were run using (*E,E*)-[1-^{14}C]farnesyl pyrophosphate as the substrate, simultaneously under the same conditions.



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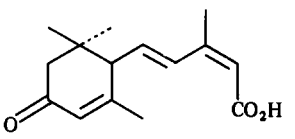

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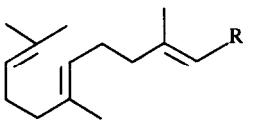
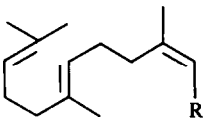


3 R = CH_2OPP
4 R = CH_2OP
5 R = CH_2OH
6 R = CO_2H



7 R = CH_2OPP
8 R = CH_2OH
9 R = CO_2H

dicate that (*E,E*)-farnesol is not on the biosynthetic pathway to ABA. The small amount of incorporation observed probably resulted from phosphorylation of some of the administered farnesol by the mould.

The possible role of the other hydrolysis product, FP, was then investigated. When (*E,E*)-[1-^{14}C]FP was administered to resuspended *C. rosicola*, it was incorporated into ABA almost as efficiently as the control (Table 1). The possibility that the FP was first converted to FPP cannot be ruled out, but this seems unlikely considering that almost complete phosphorylation of the FP would have been required. In any case, hydrolysis of FPP to FP by the phosphatase in the medium would not significantly affect the incorporation of radioactivity into ABA, but further hydrolysis to farnesol would result in a low incorporation. It was found that this undesirable reaction could be suppressed in part by the addition of 0.3 mM inorganic pyrophosphate to the medium. At higher concentrations of pyrophosphate, ABA biosynthesis was also inhibited, being completely blocked by 10 mM pyrophosphate. Therefore, 0.3 mM pyrophosphate is now added routinely to the medium, and the incorporation of FPP into ABA has been consistently in the range of 10–15%.

The conversion of (*E,E*)-FPP to ABA requires transformations at several positions of the basic C_{15} carbon skeleton. Nothing is known about the sequence of these changes, except that the first is not hydrolysis of (*E,E*)-FPP to (*E,E*)-farnesol and the last is probably the insertion of the hydroxyl group [3]. Among the required changes is isomerization of the 2,3-double bond from *trans* to *cis*. Therefore, the *cis* isomers, (*E,Z*)-[1-^{14}C]farnesol and (*E,Z*)-[1-^{14}C]farnesyl pyrophosphate (**7**) were synthesized and administered to *C. rosicola* resuspensions. In neither case was significant incorporation of radioactivity into ABA observed (Table 1). Thus, the isomerization must take place at a later stage on the pathway.

Two other possible ABA precursors, (*E,E*)-[1-^{14}C]-farnesic acid (**6**) and (*E,Z*)-[1-^{14}C]farnesic acid (**9**) were also synthesized and administered to *C. rosicola* resuspensions. Again, no significant incorporation of radioactivity was observed in either case (Table 1), indicating that conversion of C-1 to a carboxyl group also occurs at a later stage on the pathway.

These findings have narrowed the range of possible steps on the biosynthetic pathway. The transformations involving C-1, C-2 and C-3 are removal of the pyrophosphate group, conversion of C-1 to a carboxyl group, and isomerization of the 2,3-double bond. This work shows that all three of these reactions occur after one or more changes elsewhere in the molecule. Other possible intermediates are now being synthesized in radioactive form for further studies of ABA biosynthesis.

EXPERIMENTAL

The preparation of (*E,E*)-[1-^{14}C]farnesol and (*E,E*)-[1-^{14}C]-FPP has been described previously [4]. Silica gel G plates were used for TLC, unless otherwise specified. TLC plates were scanned for radioactivity and integrated with a Berthold LB 2832 Linear Analyzer, and radioactivity in column chromatographic eluates was detected with a Berthold LB 503 Radioactivity Monitor, equipped with a solid scintillator (cerium-activated glass) detector cell. Liquid scintillation counting was used to accurately determine the radioactivity of column fractions.

Administration of radioactive substrates. *C. rosicola* growth cultures and resuspensions were prepared as described previously [4]. The resuspensions were made in 10 ml of standard medium contained in 50 ml flasks, which were shaken at 120 rpm under continuous fluorescent lighting. The ABA content of the medium was monitored by HPLC [4], and when it began to increase rapidly (usually 12–24 hr) the radioactive substrates were added, in 100–200 μl of H_2O if water-soluble, otherwise in 20 μl of DMSO (control experiments showed that this level of DMSO had no effect upon ABA biosynthesis). Sodium pyrophosphate was added simultaneously to a concentration of 0.3 mM. After 16–24 hr the medium was separated from the mycelium by filtration. In some cases the mycelium was then extracted by grinding in a tissue grinder with 90% EtOH.

Isolation and purification of ABA. The filtrate was passed through a C_{18} Sep-Pak cartridge (Waters Associates). The Sep-Pak was washed with H_2O and eluted with 1.5 ml MeOH. The eluate was evaporated to dryness, taken up in 0.5 ml 0.05 M Pi buffer, pH 6, and chromatographed on a 6 × 65 mm column of PRP-1 resin (Hamilton). The column was eluted with a linear gradient (20% to 90% MeOH). The ABA fraction was methylated (CH_2N_2) and examined by TLC (hexane–EtOAc, 1:1). If the ABA methyl ester was not radiochromatographically pure, it was then chromatographed on a 6 × 65 mm column of Sepralyte

diol (Analytichem). The column was eluted with a linear gradient (trichlorotrifluoroethane (TTE) to TTE–EtOAc, 17:3). The ABA methyl ester fraction was then reduced with $NaBH_4$ to a mixture of two diol isomers [7]. If TLC (hexane–EtOAc, 2:3) showed that the diols were not radiochemically pure, they were isolated by chromatography on a 6 × 65 mm Sepralyte diol column, eluted with a linear gradient (TTE to TTE–EtOAc, 3:1).

(E,E)-[1-^{14}C]*Farnesyl phosphate.* (*E,E*)-[1-^{14}C]Farnesol [4] was phosphorylated by the method used for synthesis of farnesyl pyrophosphate, with tetrabutylammonium phosphate being substituted for tetrabutylammonium pyrophosphate. The product was isolated by chromatography on a 6 × 65 mm colum of DEAE-Sephacel (Pharmacia), eluting with 0.2 M $(NH_4)_2HPO_4$, and it was further purified by chromatography on a 6 × 65 mm column of Sepralyte C_{18} (Analytichem), eluting with a gradient of 20% to 80% MeOH. The material obtained was shown to be radiochemically pure by TLC (silica gel H, iso-PrOH–NH_4OH–H_2O, 7:2:1).

(E,Z)-[1-^{14}C]*Farnesol.* The methyl (*E,Z*)-[1-^{14}C]farnesate obtained previously in the synthesis of (*E,E*)-[1-^{14}C]farnesol [4] was reduced as described for the (*E,E*)-isomer.

(E,Z)-[1-^{14}C]*Farnesyl pyrophosphate.* The (*E,Z*)-[1-^{14}C]farnesol from above was phosphorylated as described for the (*E,E*)-isomer [4].

(E,E)- *and* (E,Z)-[1-^{14}C]*Farnesic acid.* A mixture of methyl (*E,E*)- and (*E,Z*)-[1-^{14}C]farnesate, obtained previously as a column fraction during purification of the (*E,E*)-isomer [4], was hydrolyzed with 0.5 N KOH in 90% MeOH, overnight at 25°. The mixture of farnesic acids was separated by chromatography on a 6 × 65 mm column of silica gel (Polygosil 60-2540, Machery-Nagel), eluting with C_6H_6–EtOAc–HOAc (97:3:0.3). Fractions were obtained containing each of the isomers in radiochemically pure form, as shown by TLC ($CHCl_3$–EtOAc–HOAc, 90:10:1).

Acknowledgements—We thank Mary Nelson for expert technical assistance and Shin Hasegawa for helpful discussions. Reference to a product name or company does not imply endorsement of that product or company by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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