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BIOSYNTHESIS OF ABSCISIC ACID FROM [1,2-$^{13}C_2$]ACETATE IN *CERCOSPORA ROSICOLA*

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Key Word Index—*Cercospora rosicola*; Deuteromycetes; fungus; biosynthesis; abscisic acid; ^{13}C NMR.

Abstract—[1,2-$^{13}C_2$]Sodium acetate was converted to abscisic acid (ABA) by *Cerospora rosicola*. The labelling pattern, determined by NMR spectroscopy, was in accord with biosynthesis via the isoprenoid pathway.

INTRODUCTION

The biosynthesis of the plant hormone abscisic acid (ABA) from mevalonic acid (MVA) has been demonstrated in higher plants, and the incorporation of tritium-labelled MVA into ABA has established the stereochemical course of this conversion [1–3]. However, the intermediates are unknown. The recent report that ABA is produced by a fungus, *Cercospora rosicola* Passerini [4], has raised the possibility that this organism may provide a better system for studying ABA biosynthesis than higher plants. A defined medium for growth of *C. rosicola* has been developed [5], and as a preliminary to more detailed biosynthetic studies we wished to confirm that ABA is synthesized by the fungus via the normal isoprenoid pathway. [1,2-$^{13}C_2$]Acetate was chosen as a precursor, because incorporation of intact acetate units is revealed by ^{13}C–^{13}C coupling in the NMR spectrum of the product [6]. We administered [1,2-$^{13}C_2$]acetate to *C. rosicola* and determined the labelling pattern in the ABA synthesized.

RESULTS AND DISCUSSION

Initially, mycelium was resuspended in the presence of various concentrations of unlabelled acetate in a growth-limiting medium to establish optimum levels for production of ABA. The standard medium contained glucose (2.2×10^{-2} M) as the major carbon source; when acetate was substituted for glucose, little ABA synthesis took place. In the presence of glucose, ABA synthesis was strongly affected by the acetate concentration, as follows. At 4×10^{-2} M acetate, synthesis was completely inhibited, while 1×10^{-3} and 5×10^{-4} M caused about 40% and 20% inhibition, respectively. At 1×10^{-4} M acetate a 20% enhancement of ABA synthesis was observed. A level of 7×10^{-4} M was chosen as a reasonable compromise between maximum synthetic rate and detectability of ^{13}C. Mycelia were resuspended in this concentration of [1,2-$^{13}C_2$]acetate for 2 days, and ABA was isolated and purified by HPLC.

In the proton noise-decoupled ^{13}C NMR spectrum, 12 of the 15 carbon resonances were accompanied by satellite doublets due to ^{13}C–^{13}C coupling from the incorporation of intact acetate units. The other three resonances were singlets, which showed that they had been derived from one carbon of an acetate unit which had been cleaved at some point on the biosynthetic pathway. The average incorporation of ^{13}C per carbon was 1.7%, calculated by the method of Leete and Yu [7]. Table 1 shows the coupling constants for those carbon atoms of ABA into which intact acetate had been incorporated, based upon assignments of the ^{13}C resonances previously made by Assante *et al.* [4]. The values of the coupling constants are in accord with those expected for the hybridization states of the carbons involved in coupling: 73 Hz for sp^2–sp^2, 40–46 Hz for sp^2–sp^3, and 36 Hz for sp^3–sp^3 [8]. Thus, six acetate units were incorporated intact into ABA, in the positions shown in Scheme 1. This pattern of labelling is in complete accord with biosynthesis of ABA through MVA via the isoprenoid pathway as in higher plants, as shown in Scheme 1. It has been suggested that in higher plants ABA may be derived by cleavage of a C_{40} carotenoid rather than directly from sesquiterpenoid precursors [3]. The present findings do not exclude either possibility in the case of *C. rosicola*.

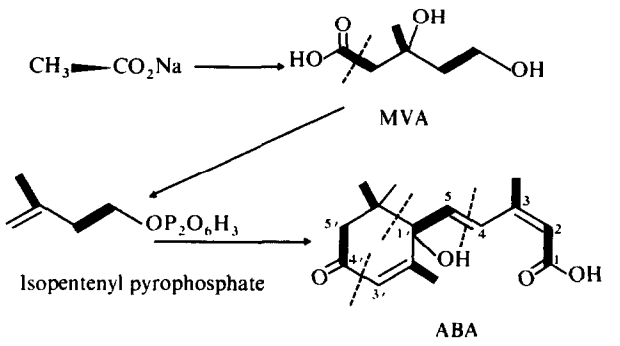
EXPERIMENTAL

HPLC. Analyses of ABA were carried out on a Partisil PXS 5/25 ODS column (Whatman Inc.) isocratically with 50% MeOH in 4 mM H_3PO_4 [9]. Semipreparative separations were made on a Magnum 9 ODS reversed-phase column (Whatman Inc.) with a 40-min linear gradient from 30% MeOH in 3 mM H_3PO_4 to 98% MeOH in 1 M H_3PO_4 at a flow rate of 6 ml/min. Detection was by UV absorbance at 268 nm.

Table 1. Chemical shifts and coupling constants of carbon atoms of ABA biosynthesized from sodium [1,2-$^{13}C_2$]acetate

Carbon No*	$J^{13}C$–^{13}C (Hz)
1 (170.6)–2 (118.1)	73
3 (163.0)–3-Me (21.4)	42
4 (128.5)	—
5 (136.9)–1′ (79.9)	46
2′ (151.5)–2′-Me (19.1)	42
3′ (127.1)	—
4′ (198.3)–5′ (49.7)	40
6′ (41.7)–6′-Me (24.3)	36
6′-Me (23.1)	—

* Values in parentheses are chemical shifts in ppm.



Scheme 1. Biosynthesis of ABA from [1,2-$^{13}C_2$]acetate. Dotted line in MVA formula shows which C–C bond is broken in forming isopentenyl pyrophosphate, and those in ABA formula delineate the three isoprene units.

Growth conditions. C. rosicola (strain no. 138.35, Central Bureau voor Schimmelcultures, Baarn, The Netherlands) was cultured on potato dextrose agar. A chemically defined liquid medium [5] was used for seed and growth cultures. It contained (per liter): 20.0 g glucose, 0.2 g $MgSO_4$, 0.5 g KCl, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.8 g KH_2PO_4, 0.001 g thiamine, 3.0 g monosodium glutamate, and 1 ml of a trace metal solution (0.05 g $FeSO_4 \cdot 7H_2O$, 0.033 g $MnCl_2$, 0.25 g $ZnSO_4$, 0.40 g $CuSO_4 \cdot 4H_2O$, and 0.00005 g H_3BO_3 in 100 ml H_2O). Autoclaved liquid media (30 ml) in 125-ml flasks were inoculated with 2 ml of mycelia, from petri dish cultures blended in sterile H_2O. After 7 days incubation, medium (100 ml) in 500-ml flasks was inoculated with the blended seed culture (2 ml). Cultures were incubated on reciprocating shakers at 23–24° under continuous fluorescent lighting.

Administration of precursor. A 7-day-old culture, which had produced 16 μg of ABA/ml, was filtered, washed twice with H_2O, and resuspended at 350 mg cell dry wt/100 ml autoclaved 20% defined growth media in 500-ml flasks containing 6 mg of filter-sterilized [1,2-^{13}C]sodium acetate (90% ^{13}C, Merck) in 1 ml H_2O. Twenty-seven such flasks were incubated 48 hr under the conditions used for growth. ABA production was 0.18, 5.95 and 13.61 μg/ml for 0, 24 and 48 hr, respectively, after resuspension.

Isolation of ABA. The pH of the combined filtrates was adjusted to 2.5 with 5 N HCl. The filtrate was passed through four C_{18} reversed-phase Sep-Pak cartridges (Waters Associates) in series. The cartridges were washed with H_2O and eluted with MeOH. The combined MeOH eluates were chromatographed on the semiprep. HPLC column. The ABA (R_t 24–26 min) was collected, the MeOH was evapd and the aq. phase was passed through a C_{18} Sep-Pak cartridge. The ABA was eluted with MeOH and rechromatographed (27 mg). The ^{13}C NMR spectrum was run with proton noise-decoupling in $CDCl_3$ at 15 MHz, with TMS as standard. A pulse width of 18° with a pulse interval of 1 sec gave approx equal intensities of the resonances.

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