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7′-HYDROXY (−)-*R*-ABSCISIC ACID: A METABOLITE OF FEEDING (−)-*R*-ABSCISIC ACID TO *XANTHIUM STRUMARIUM*

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Key Word Index—*Xanthium strumarium*; Ambrosiaceae; metabolism; abscisic acid; 7′-hydroxy (−)-*R*-abscisic acid; mass spectrometry; ORD.

Abstract—Feeding of (±)-abscisic acid to leaves of *Xanthium strumarium* resulted in formation of a new metabolite. The compound was identified as 7′-hydroxy (−)-*R*-abscisic acid by high resolution mass spectrometry of its methyl ester and monoacetate, and by optical rotary dispersion. The numbering system for abscisic acid has been extended to include the exocyclic methyl groups. Feeding racemic [2-^{14}C]abscisic acid to *Xanthium* leaves resulted in *ca* 20% conversion of the radiolabelled compound into the new metabolite. Evidence is presented that, in *Xanthium*, only the synthetic (−)-*R*-enantiomer of abscisic acid is hydroxylated at the 7′-position.

INTRODUCTION

In *Xanthium strumarium*, ABA (1) is metabolized via two major pathways. One route involves conjugation of the free acid to form ABA-glucose ester [1]. A second pathway consists of oxidation at the 8′-methyl group followed by cyclization to form phaseic acid [2, 3]. During the course of our experiments, we observed that a significant fraction of fed racemic [2-^{14}C]ABA was metabolized via a third route to a previously unidentified compound [4]. Recently, Lehmann *et al.* [5, 6] have reported the formation of a new metabolite, 2′-hydroxymethyl-ABA, after feeding [2-^{14}C]ABA (presumably as a racemic mixture) to cell suspension cultures of various plant species.

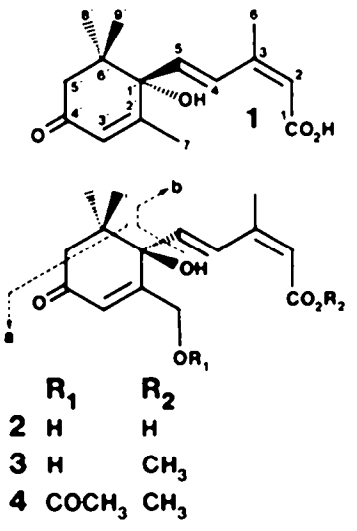
We report here the structural determination of our unidentified metabolite in *Xanthium* as 7′-hydroxy (−)-*R*-ABA (2). Isolation of the 1′-epimer of 2 was claimed by Lehmann *et al.* [5]. We present evidence that, in *Xanthium*, 7′-hydroxy (−)-*R*-ABA is not a metabolite of endogenous (+)-*S*-ABA, but rather occurs as an artifact of feeding the unnatural (−)-*R*-isomer of ABA. In addition, we propose to extend the numbering system for ABA to include the previously unnumbered exocyclic methyl groups.

RESULTS AND DISCUSSION

The new metabolite (2) was isolated from *Xanthium* leaves after feeding (±)-[2-^{14}C]ABA via the petioles. The leaves were extracted with acetone followed by purification by reverse phase HPLC. The metabolite was converted to its methyl ester with ethereal diazomethane and then chromatographed using normal phase HPLC to give pure 3.

Although the use of ^{1}H NMR was limited due to the small amount of 3 available, the spectrum showed only four of the five methyl resonances normally present in ABA methyl ester. Resonances for the 8′ and 9′ methyl groups (δ1.08 and 1.17) and methoxy protons (δ3.72) were similar to those observed for ABA methyl ester. However, only one vinylic methyl signal (δ2.06) was observed in 3, strongly suggesting that one of the vinylic methyl groups of ABA (either the 7′ or 6 methyl) had been modified.

The high resolution mass spectrum of 3 gave a weak molecular ion at *m/z* 294 ($C_{16}H_{22}O_5$) showing that 3 contained an additional oxygen compared to MeABA. The principal fragmentation pattern is the cleavage of C_4H_8 from the six-membered ring (*a* [7]) to give *m/z* 238 and the subsequent loss of methanol and water to give strong fragment ions at *m/z* 206 (88%) and *m/z* 188 (100%). Both these fragments still contain an additional



	R_1	R_2
2	H	H
3	H	CH_3
4	$COCH_3$	CH_3

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oxygen when compared to the corresponding fragments in MeABA, indicating that the 6'-*gem*-dimethyl groups are not modified in **3**. Similarly, the strong fragment ion at *m/z* 125 (*b*, 52%) indicates that the side chain in **3** is unchanged from ABA methyl ester, eliminating modification of the 6-methyl group. Combined with the NMR data, this strongly suggests hydroxylation at the 2'-methyl position. To confirm this, the monoacetate **4** was synthesized by treating **3** with acetic anhydride–pyridine using conditions that were known not to acetylate the more highly hindered 1'-hydroxyl group [G. Boyer, unpublished results]. After purification by normal phase HPLC, **4** gave a molecular ion in the low resolution mass spectrum at *m/z* 336 indicating the addition of one acetoxy group to ABA methyl ester. Although it was not possible to obtain a molecular ion, high resolution mass spectroscopy clearly delineated the entire fragmentation pattern for **4**. As was observed for **3**, the principal fragmentation pattern is the cleavage of C_4H_8 from the parent ion (*a*) followed by losses of methanol, water and acetic acid. Ions at *m/z* 125 (*b*, 44%) and *m/z* 280 (5%) confirm that the side chain and 6'-*gem*-dimethyl groups remain unmodified. A key fragment ion at *m/z* 263 (3%) corresponding to the loss of CH_2OAc from the parent ion confirms the location of the new hydroxyl group at the 2'-methyl position.

Metabolite **2** showed a dramatic shift in chromatographic behaviour on HPLC and TLC after treatment with diazomethane, indicating that it exists as the free acid. Therefore, the structure of **2** is 3-methyl-5-(1'-hydroxy-2'-hydroxymethyl-6'-dimethyl-4'-oxo-cyclohex-2'-enyl)-penta-2*Z*,4*E*-dienoic acid as characterized by its methyl ester **3**. A similar compound, i.e. its 1'-epimer, has been reported as a novel metabolite of ABA (racemic mixture ?) fed to cell suspension cultures of various plant species [5, 6].

It should be pointed out that the trivial name 2'-hydroxymethyl-ABA [4], is an incorrect usage of chemical nomenclature, since the parent compound ABA already contains a methyl group at the 2'-position. A correct nomenclature (but also more cumbersome) would be 2'-hydroxymethyl-2'-demethyl-ABA. Instead, we would like to propose an extended numbering system for ABA [8] as shown in **1**. This includes the exocyclic methyl groups. Using this system, **2** is named 7'-hydroxy (−)-*R*-ABA and the compound previously referred to as 6'-hydroxymethyl-ABA [9] would be named 8'-hydroxy ABA.

The ORD spectrum of **3** strongly suggests the origin of **2** in higher plants. The rotation maxima of **3** are of similar magnitude as those for (+)-ABA, but with opposite sign (Table 1). This indicates that **2** results from metabolism of the (−)-isomer of ABA rather than from the naturally occurring (+)-isomer. To confirm that **2** is indeed an artifact of feeding the unnatural (−)-isomer of ABA, the following feeding experiment was done: (a) 0.0148 mg (±)-[2-^{14}C]ABA as a radiotracer; (b) 2 mg (±)-ABA; (c) 1 mg (+)-ABA; and (d) a 5% ethanol control without added exogenous ABA. The four samples were purified separately by HPLC using treatment (a) as a guide to locate the active fractions. The amount of metabolite **2** was estimated in the various treatments after methylation by the relative peak heights on normal phase HPLC (using ABA methyl ester as the standard) or, in the case of treatment (b), by GC (with a flame ionization detector) of the acylated derivative **4**. The results of this experiment are summarized in Table 2. *Ca* 20% of the fed (±)-ABA was converted to **2**, while less than 1% of the (+)-ABA was similarly metabolized. Since (+)-ABA is commercially obtained by the chemical separation of (+)- from (−)-ABA, it is possible that a slight contamination of (−)-ABA in the (+)-ABA may have gone undetected. Therefore, the actual conversion of (+)-ABA to **2** may have been even less. If all of **2** formed in treatment (b) came from the (−)-isomer, this would indicate a 46% conversion of (−)-*R*-ABA via the 7'-hydroxylation pathway. Similar results were obtained when (−)- and (+)-[2-^{14}C]ABA were fed separately to *Xanthium* leaves. Whereas phaseic acid was the major metabolite (44%) of the (+)-isomer, 68% of the (−)-isomer was conjugated to form the glucose ester and 24% was metabolized to 7'-hydroxy (−)-*R*-ABA. Other metabolites of (+)-ABA included: ABA-glucose ester (12%), dihydrophaseic acid (7%), and conjugates of phaseic and dihydrophaseic acids (24%).

It is interesting to speculate at this point on a biochemical mechanism for the hydroxylation of ABA at the 7'-position. If one considers the three point model of Ogston [10] for the attachment of an optically active substrate to the enzyme surface, and postulates that the oxygenase that forms 8'-hydroxy ABA from (+)-*S*-ABA in the phaseic acid pathway recognizes the 1'-hydroxyl and 4'-ketone functionalities as two points of attachment (see Fig. 1), then molecular models suggest that due to inversion of the cyclohexane ring, the same enzyme may hydroxylate the 7'-methyl position in the unnatural (−)-*R*-isomer. Thus, we may be observing the response of an enzyme system to a synthetic compound that it was never intended to encounter. Whether this is the case, or if there is a second enzyme system capable of hydroxylating ABA at the 7'-position, remains to be established.

Table 1. Optical rotary dispersion of (+)-*S*-ABA (**1**) and the methyl ester of 7'-hydroxy (−)-*R*-ABA (**3**)

1*	3
$[\alpha]_{287}$ + 24 000°	$[\alpha]_{291}$ − 21 600°
$[\alpha]_{269}$ 0°	$[\alpha]_{268}$ 0°
$[\alpha]_{245}$ − 69 000°	$[\alpha]_{244}$ + 55 000°
$[\alpha]_{225}$ 0°	$[\alpha]_{226}$ 0°

*Reference [11].

EXPERIMENTAL

Xanthium strumarium L., Chicago strain, was grown in a greenhouse as previously described [1, 3]. (±)-[2-^{14}C]ABA

Table 2. Conversion of different stereoisomers of ABA fed to *Xanthium* leaves to **2**

Treatment	2 (mg)	Conversion (%)
a. (±)-[^{14}C]ABA, 0.0148 mg	0.0024	16
b. (±)-ABA, 2.0 mg	0.46	23
c. (+)-ABA, 1.0 mg	0.038	0.4
d. Control, 0 mg	—	--

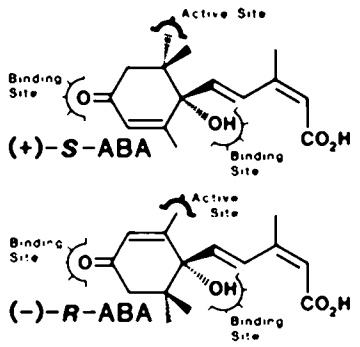

Fig. 1. Hypothetical binding of (+)-*S*-ABA (upper) and (−)-*R*-ABA (lower) to the ABA oxygenase, using the three-point attachment model of Ogston [10]. If the oxygenase that catalyses the conversion of (+)-ABA to phaseic acid recognizes the 1'-hydroxyl and 4'-keto groups as points of attachment, and if (−)-ABA binds to the same active site, then due to inversion of the cyclohexane ring, the same enzyme might hydroxylate the 7'-methyl position of the (−)-isomer.

(New England, 35.9 mCi/mmol), (+)- and (−)-[2-^{14}C]ABA (a gift from Dr. D. C. Walton, SUNY, Syracuse, NY; each 4 mCi/mmol), (±)-ABA (Sigma), or (+)-ABA (Fluka) in 5% EtOH were fed to detached *Xanthium* leaves and allowed to metabolize for 2 days. Leaves were pulverized in liquid N_2 and extracted with Me_2CO. Me_2CO was removed *in vacuo* and the material purified by HPLC. The initial step involved reverse phase chromatography on four Bondapak C_{18}/Porasil B columns (0.65 × 60 cm, 9.9 ml/min) using a 30 min gradient of 20 to 50% EtOH in 1% HOAc. Fractions containing **2** (22–24 min) were lyophilized and rechromatographed using a 0–60% gradient of MeOH in 1% HOAc (Waters, μ Bondapak C_{18} column, 0.34 × 30 cm, 40 min gradient, flow rate 2.0 ml/min). Radioactive fractions (35–37 min) were again lyophilized and methylated with CH_2N_2 to give **3**. Normal phase chromatography (Waters, μ Porasil column, 0.34 × 30 cm, 30–70% EtOAc in hexane, 40 min gradient, 2.5 ml/min) gave pure **3** eluting at 35–37 min. In the feeding experiment with (+)- and (−)-ABA, reverse phase HPLC was performed using a μ Bondapak C_{18} column (0.78 × 30 cm) with a 40 min gradient from 10 to 35% EtOH in 1% HOAc at a flow rate of 2.5 ml/min. Radioactivity in the column effluent was detected on-line with a Flo-One radioactive flow detector (Radiomatic Instruments & Chemical Co., Inc., Tampa, FL) connected to a Waters HPLC system.

Compound **3**. UV λ_{max}^{MeOH} 266 nm (ε 21 600); ORD: $[\alpha]_{291}$ = −21 600°, $[\alpha]_{268}$ = 0°, $[\alpha]_{244}$ = +55 000°, $[\alpha]_{226}$ = 0°; ^{1}H NMR (400 MHz, *ca* 65 μg in $CDCl_3$), methyl signals at 1.08, 1.17, 2.06 and 3.72 ppm; EIMS (probe) 80 eV, *m/z* (rel. int.): 294.1467 [M]$^+$ (0) (calc. for $C_{16}H_{22}O_5$: 294.1467], 276.1358 [M − H_2O]$^+$ (9) (calc. for $C_{16}H_{20}O_4$: 276.1362), 263.1292 [M − OMe]$^+$ (7) (calc. for $C_{15}H_{19}O_4$: 263.1283), 262.1220 (2) (calc. for $C_{15}H_{18}O_4$: 262.1205), 258.1244 (M − $2H_2O$]$^+$ (2) (calc. for $C_{16}H_{18}O_3$: 258.1256), 238.0846 [M − C_4H_8]$^+$ *a* (14) (calc. for $C_{12}H_{14}O_5$: 238.0841), 221.080 (10) (calc. for $C_{12}H_{13}O_4$: 221.0814), 220.0731 [*a* − H_2O]$^+$ (18) (calc. for $C_{12}H_{12}O_4$: 220.0735), 206.0584 [*a* − MeOH]$^+$ *c* (88) (calc. for $C_{11}H_{10}O_4$: 206.0580), 188.0481 [*c* − H_2O]$^+$ (100) (calc. for $C_{11}H_8O_3$: 188.0474), 178.0615 [*c* − CO]$^+$ (6) (calc. for $C_{10}H_{10}O_3$: 178.0615], 160.0530 (32) (calc. for $C_{10}H_8O_2$: 160.0524), 152.0841 (52) (calc. for $C_9H_{12}O_2$: 152.0838), 149.0599 (9) (calc. for $C_9H_9O_2$: 149.0603, 132.0576 (13) (calc. for C_9H_8O: 132.0575), 125.0606 [*b*]$^+$ (52) (calc. for $C_7H_9O_2$: 125.0602), 94.0413 [*b* − OMe]$^+$ (5) (calc. for C_6H_6O: 94.0409), 91.0554 (14) (calc. for C_7H_7: 91.0548).

Compound **4**. Compound **3** was treated with Ac_2O–C_5H_5N (2:1) for 2 hr at 25° and purified by normal phase HPLC using conditions described above. Pure **4** eluted between 10 and 12 min. EIMS: (probe) 80 eV, *m/z* (rel. int.): 336 [M]$^+$ (3), 305.1408 [M − OMe]$^+$ (5) (calc. for $C_{17}H_{21}O_5$: 305.1389), 280.0949 [M − C_4H_8]$^+$ *a* (5) (calc. for $C_{14}H_{16}O_6$: 280.0947), 276.1372 [M − HOAc]$^+$ (12) (calc. for $C_{16}H_{20}O_4$: 276.1361), 263.1296 [M − CH_2OAc]$^+$ (3) (calc. for $C_{15}H_{19}O_4$: 263.1283), 248.0674 [*a* − MeOH]$^+$ (21) (calc. for $C_{13}H_{12}O_5$: 248.0685), 245.1150 [M − CH_2OAc − H_2O]$^+$ (6) (calc. for $C_{15}H_{17}O_3$: 245.1178), 238.0838 [M − $Me_2CH_2COCH_2$]$^+$ *d* (41) (calc. for $C_{12}H_{14}O_5$: 238.0841), 220.0730 [*a* − MeOH − CO]$^+$ *c* (98) (calc. for $C_{12}H_{12}O_4$: 220.0736), 192.0805 [*c* − CO]$^+$ (9) (calc. for $C_{11}H_{12}O_3$: 192.0786), 188.0539 [*a* − MeOH − HOAc]$^+$ (93) (calc. for $C_{11}H_8O_3$: 188.0474), 178.0620 [*d* − HOAc]$^+$ (9) (calc. for $C_{10}H_{10}O_3$: 178.0630), 165.0554 [*d* − CH_2OAc]$^+$ (35) (calc. for $C_9H_9O_3$: 165.0552), 164.0847 (3) (calc. for $C_{10}H_{12}O_2$: 164.0837), 161.0593 [*c* − OAc]$^+$ (100) (calc. for $C_{10}H_9O_2$: 161.0602], 160.0503 [*c* − HOAc]$^+$ (38) (calc. for $C_{10}H_8O_2$: 160.0525), 132.0611 (15) (calc. for C_9H_8O: 132.0576), 125.0606 [*b*]$^+$ (44) (calc. for $C_7H_9O_2$: 125.0602), 94.0413 [*b* − OMe]$^+$ (5) (calc. for C_6H_6O: 94.0409), 91.0547 (15) (calc. for C_7H_7: 91.0547).

Chromatography. GC (180 × 0.2 cm, 3% SE-30, 165–240° at 5°/min, 25 ml N_2/min); TLC (silica gel, EtOAc–hexane, 2:1) R_f: MeABA, 0.76; **3**, 0.35; **4**, 0.67. The MS were recorded on an AEI MS-50 instrument, electron energy 80 eV, probe temp. 150°.

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REFERENCES

1. Boyer, G. L. and Zeevaart, J. A. D. (1982) *Plant Physiol.* **70**, 227.
2. Zeevaart, J. A. D. and Boyer, G. L. (1982) in *Plant Growth Substances* 1982 (Wareing, P. F., ed.) p. 335. Academic Press, New York.
3. Zeevaart, J. A. D. (1980) *Plant Physiol.* **66**, 672.
4. Boyer, G. L. and Zeevaart, J. A. D. (1984) in *Plant Research '83*. Ann. Rep. MSU-DOE Plant Res. Lab., pp. 160–161. East Lansing, Mich. State Univ.
5. Lehmann, H., Preiss, A. and Schmidt, J. (1983) *Phytochemistry* **22**, 1277.
6. Lehmann, H., Böhm, H. and Schütte, H.-R. (1983) *Z. Pflanzenphysiol.* **109**, 423.
7. Gray, R. T., Mallaby, R., Ryback, G. and Williams, V. P. (1974) *J. Chem. Soc. Perkin Trans.* 2, 919.
8. Addicott, F. T., Lyon, J. L., Ohkuma, K., Thiessen, W. E., Carns, H. R., Smith, O. E., Cornforth, J. W., Milborrow, B. V., Ryback, G. and Wareing, P. F. (1968) *Science* **159**, 1493.
9. Milborrow, B. V. (1969) *Chem. Commun.* 966.
10. Ogston, A. G. (1948) *Nature* **162**, 963.
11. Cornforth, J. W., Milborrow, B. V. and Ryback, G. (1966) *Nature* **205**, 1269.