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Synthesis, Biological Activity and Metabolism of (*S*)-(+)-3'-Fluoroabscisic Acid

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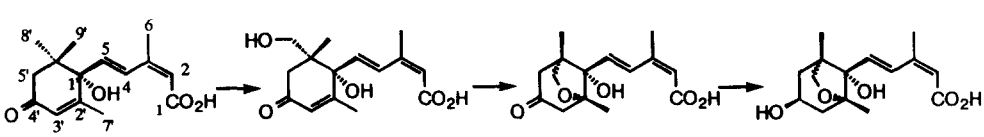
Abstract: (*S*)-(+)-3'-Fluoroabscisic acid (**5a**) was synthesized as an analogue of abscisic acid that can resist the metabolic cyclization of 8'-hydroxyabscisic acid to phaseic acid due to increased electron density at C-2'. The inhibitory activity of **5a** was slightly higher than that of abscisic acid in lettuce seed germination, and was almost equal to that of abscisic acid in rice seedling elongation. When **5a** was applied to bean shoots, 3'-fluoro-8'-hydroxyabscisic acid (**8a**), 3'α- and 3'β-fluorophaseic acids (**9a** and **10a**) and 3'α- and 3'β-fluorodihydrophaseic acids (**11a** and **12a**) were identified as the free metabolites. The methyl esters **8b**, **9b** and **10b** coexisted at equilibrium in the ratio of 7:6:1. This result indicates that **8a** is stable as back-isomerization of **9a** and **10a** occurs.

INTRODUCTION

The plant hormone (*S*)-(+)-abscisic acid (ABA, **1**) is a stress messenger that transduces environmental effects such as drought, freezing and wounding into biological responses.[1,2] The current commercial applications for ABA, however, are minimal owing to its rapid metabolism.[3] ABA is metabolized to 8'-hydroxyabscisic acid (8'-HOABA, **2**) by monooxygenase.[4] Ever since Milborrow identified this metabolite in 1969,[5] it has not been isolated from plant extracts because it spontaneously cyclizes *in vitro* to phaseic acid

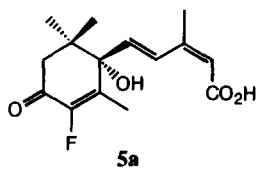


1 **2** **3** **4**

Fig. 1

(PA, **3**) (Fig. 1).[6] 8'-HOABA probably maintains its activity,[7] but PA is less active than ABA in most bioassays.[6] The cyclization of 8'-HOABA to PA is an intramolecular Michael addition which is initiated with the nucleophilic addition of the 8'-oxygen to the electron-deficient 2'-carbon and completed by subsequent protonation at C-3' in the intermediate enolate. This protonation *in vivo* occurs stereospecifically from the α-face of the cyclohexanone ring (originally the *si*-face of C-3' in ABA), so the cyclization to PA probably proceeds enzymatically *in vivo*.[8] PA *in vivo* is reduced to further inactive dihydrophaseic acid (DPA, **4**) (Fig. 1).[6] Another pathway of ABA metabolism involves conjugation with glucose at C-1 or C-1'.[6] The formation of the conjugate is less specific than the oxidation.[6]

We are developing highly active and long-lasting analogues of ABA that resist metabolic inactivation to PA, and described the highly potent (+)-8'-methoxyabscisic acid that was designed to lose the nucleophilicity of the 8'-oxygen by methylating the 8'-hydroxyl group and to prevent cyclization.[9] Here we designed a new analogue, (+)-3'-fluoroabscisic acid (**5a**) that can resist the cyclization by means of another mechanism. Substitution of the 3'-hydrogen by fluorine would increase the electron density of C-2' by pushing the π electron at C-3' toward C-2',[10] so **5a** can acquire resistance to the nucleophilic addition of the 8'-oxygen owing to the weakened electrophilicity at C-2'. The use of fluorine has the advantage of minimizing the steric change[11] which may lower the affinity for receptors or uptake proteins. In this paper, we describe the synthesis of **5a**, its inhibitory activity in lettuce seed germination and rice seedling elongation, and metabolism in bean shoots.



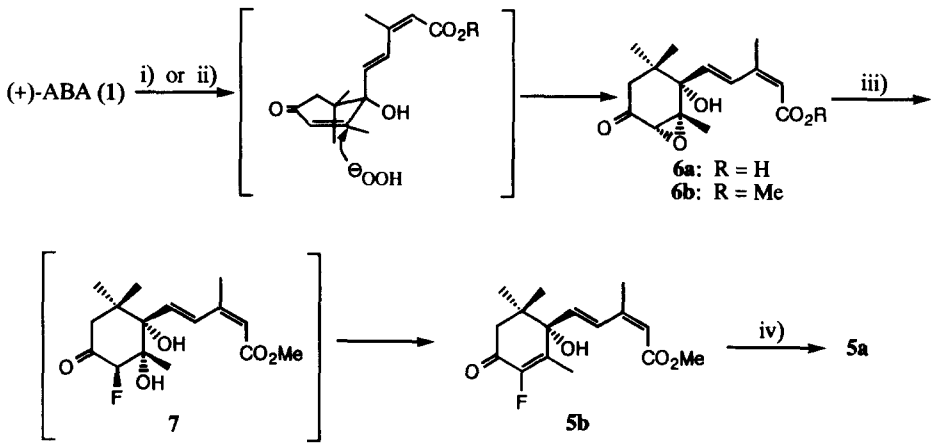
5a

RESULTS AND DISCUSSION

Synthesis

Optically pure **5a** was synthesized from (*S*)-(+)-ABA (Fig. 2). The methyl ester **6b** was synthesized stereospecifically by two methods. First, epoxidation of (+)-ABA with alkaline hydrogen peroxide[12] afforded only the oxirane **6a**, which was converted to the corresponding methyl ester (**6b**). Second, treating the methyl ester of ABA with hydrogen peroxide and tetrabutylammonium fluoride[13] gave **6b** with the same configuration at C-2' and C-3' as that of the **6b** synthesized first. In the NOESY of **6b**, there was an NOE between the 5- and 7'-protons. This finding suggested that C-7' in **6b** was *cis* to the side chain; **6b** was the

α-oxirane. This stereoselective epoxidation may be caused by adding hydrogen peroxide from the less hindered α-face, that is, the opposite side of the side chain (Fig. 2). The reaction of **6b** with a complex of hydrogen fluoride and *N*-ethyldiisopropylamine[14] afforded the fluoro-olefin **5b**, probably via the fluorohydrin **7**. Hydrolysis of **5b** with alkali gave the free acid **5a**.



i) a) H_2O_2, NaOH b) CH_2N_2 ii) a) CH_2N_2 b) H_2O_2, Bu_4NF iii) Et(*i*-Pr)$_2$N(HF)$_3$ iv) NaOH

Fig. 2. Synthesis of **5a**

The existence of a fluorine at C-3' in **5a** was proven by the disappearance of the 3'-proton in ^{1}H NMR and the appearance of ^{19}F signal and ^{13}C signals split by coupling with fluorine. The optical purity of **5a** was confirmed by HPLC with a chiral column that gave only one peak under the conditions that give two separate peaks for the racemic **5a**. The absolute configuration at C-1' in **5a** was determined to be *S*, as it had the same Cotton effects in the circular dichroism (CD) spectrum as (+)-ABA.[9,15]

The ^{13}C signal of the 2'-carbon in **5a** appeared in a field higher by 24.7 ppm than that of ABA.[16] The ^{13}C chemical shifts correlates to electron density in a carbon atom,[10] so this result showed that the electron density of C-2' in **5a** is higher than that in ABA as expected. In contrast to C-2', C-3' in **5a** shifted toward a low field by 23.9 ppm compared to that in ABA by repulsion of the π-electron and the electrons of the outermost shell of fluorine atom.

Biological activity

The inhibitory activities of **5a** in the lettuce seed germination and the elongation of the second leaf sheath of rice seedling were tested (Fig. 3). The activity of analogue **5a** was slightly higher than that of (+)-ABA in the lettuce seeds, and was almost equal to that of (+)-ABA in the rice seedlings. If the metabolism was suppressed, then the activity should be strengthened. Therefore, this result suggested that **5a** was metabolized in a manner similar to ABA.

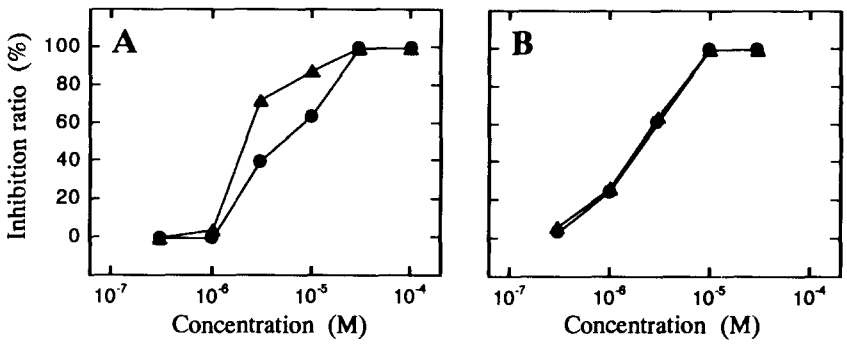


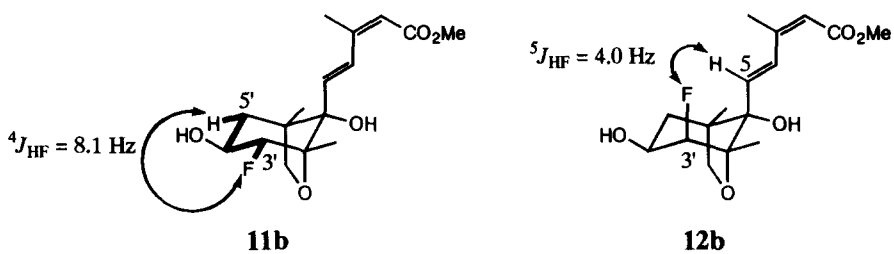
Fig. 3. Inhibitory activity of (+)-ABA (●) and **5a** (▲) in lettuce seed germination (A) and elongation of the second leaf sheath of rice seedling (B)

Metabolism in bean shoots

To understand how **5a** is metabolized, it was fed to bean shoots through the cut ends via transpiration stream for three days. The plant extract was partitioned under acidic conditions to give EtOAc and aqueous extracts.

Identification of 3'-fluoro-DPAs in the EtOAc extract. The EtOAc extract was treated with diazomethane to methylate the free metabolites. This methylated extract exhibited three major peaks by gas-liquid chromatography (GLC) using a XE-60 column and an electron-capture detector with high selectivity and sensitivity for ABA and its metabolites.[17] The retention time of the major peak agreed with that of **5b** in GLC analyses using not only XE-60 but also OV-17 and SE-30. Therefore, the compound corresponding to this peak was identified as **5b** derived from unmetabolized **5a**. Compounds giving the other two peaks were purified by column chromatography on silica gel followed by ODS. Finally, two compounds were isolated by HPLC. The more polar compound (**11b**) corresponded to the peak with the longer retention time in GLC with XE-60, whereas the less polar compound (**12b**) corresponded to the peak with the shorter retention time. Compounds **11b** and **12b** showed a molecular ion at *m/z* 314. In the ^{1}H NMR analysis, they showed similar signals to the methyl ester of DPA, except for those of the 3'-protons, which appeared at a field lower by about

2 ppm than those of the methyl ester of DPA[18] and which were split by large couplings over 50 Hz (Table 1). The presence of the fluorine at C-3' in each compound was confirmed by a ^{19}F signal which appeared as a double doublet split by couplings with the 3'- and 4'-protons (Table 2). These findings suggested that these compounds were the methyl ester of 3'-fluoro-DPAs which were epimeric at C-3'. The absolute configuration at C-3' was determined on the basis of the long-range ^{1}H-^{19}F couplings in ^{1}H NMR. Compound **11b** possessed the 5'*pro-R*-proton with large long-range ^{1}H-^{19}F coupling (8.1 Hz), which depends on the W-arrangement that can occur only when the 3'-fluorine is in the equatorial α-position (Fig. 4). These findings showed that **11b** was the methyl ester of 3'α-fluoro-DPA (**11a**). Compound **12b** had a 5-proton exhibiting the ^{1}H-^{19}F coupling (4.0 Hz) although it was separated by five σ-bonds, indicating that the 5-proton is spatially close to the 3'-fluorine. This type of relationship between the 5-proton and the 3'-fluorine is formed when the 3'-fluorine is in the axial β-position (Fig. 4), so **12b** was the methyl ester of 3'β-fluoro-DPA (**12a**) (Fig. 4). Considering that the configuration at C-1' in the precursor **5a** is the same as that of natural (+)-ABA, the absolute configuration of all the asymmetric carbons in **11b** was elucidated as (1'*S*,2'*S*,3'*R*,4'*S*,6'*R*), while that of **12b** as (1'*S*,2'*S*,3'*S*,4'*S*,6'*R*).



11b **12b**

Fig. 4. Structures of the methyl esters **11b** and **12b**, and the long-range ^{1}H-^{19}F couplings in ^{1}H NMR

Identification and properties of 3'-fluoro-8'-HOABA and 3'-fluoro-PAs in the EtOAc extract. The presence of **11a** and **12a** suggested that 3'α- and 3'β-fluoro-PAs (**9a** and **10a**) which are the immediate precursors of **11a** and **12a**, respectively, must be in the EtOAc extract. These compounds seemed to be minor peaks in GLC with XE-60. In order to investigate the presence of the methyl esters **9b** and **10b** in the methylated EtOAc extract, **9b** and **10b** as the standard samples were prepared from **11b** and **12b**, respectively, by Jones oxidation.[19] These structures were ascertained by MS and ^{1}H and ^{19}F NMR data (Tables 1, 2). Immediately after purification, these compounds gave single peaks in HPLC and single spots in TLC. When **9b** was left for several hours at 25°C, however, half of it was converted into two compounds. The minor compound was identified as **10b** from the retention time in HPLC and its R*f* in TLC. The major one **8b** exhibited a peak with a retention time intermediate between **9b** and **10b** in HPLC and a spot with the

Table 1. ^{1}H NMR Data for **8b-12b** in $CDCl_3$

H	**8b**[a]	**9b**[a]	**10b**[a]	**11b**[b]	**12b**[b]
2	5.78 (br s)	5.85 (br s)	5.78 (br s)	5.79 (br s)	5.75 (br s)
4	7.90 (d, 16.0)	8.24 (d, 16.0)	8.09 (d, 15.8)	8.10 (d, 16.0)	8.00 (d, 16.0)
5	6.00 (d, 16.0)	6.20 (d, 16.0)	6.30 (dd, 15.8, $^5J_{HF}$ = 3.2)	6.13 (d, 16.0)	6.52 (dd, 16.0, $^5J_{HF}$ = 4.0)
6	2.02 (d, 1.3)	2.04 (d, 1.2)	1.99 (d, 1.3)	2.03 (d, 1.1)	2.04 (d, 1.2)
3'	—	4.77 (d, $^2J_{HF}$ = 46.8)	4.31 (dd, 1.1, $^2J_{HF}$ = 48.0)	4.33 (dd, 7.7, $^2J_{HF}$ = 50.0)	4.55 (ddd, 4.6, 1.2, $^2J_{HF}$ = 51.5)
4'	—	—	—	4.23 (m)	4.22 (m)
5'*pro-R*	2.45 (dd, 17.5, $^4J_{HF}$ = 1.5)	2.52 (dd, 17.4, $^4J_{HF}$ = 5.2)	2.53 (dd, 19.0, 1.1)	1.98 (ddd, 13.8, 4.7, $^4J_{HF}$ = 8.1)	1.95 (ddd, 14.1, 4.0, 1.2)
5'*pro-S*	2.54 (dd, 17.5, $^4J_{HF}$ = 3.9)	2.60 (dd, 17.4, 2.6)	2.66 (ddd, 19.0, 2.3, $^4J_{HF}$ = 0.6)	1.62 (ddd, 13.8, 11.0, 2.4)	1.70 (ddd, 14.1, 11.0, 1.9)
7'	1.91 (d, $^4J_{HF}$ = 3.6)	1.40 (d, $^4J_{HF}$ = 1.8)	1.37 (d, $^4J_{HF}$ = 2.6)	1.29 (d, $^4J_{HF}$ = 2.0)	1.34 (d, $^4J_{HF}$ = 1.0)
8'	3.62 (dd, 11.2, 5.0)	3.72 (d, 8.2, H*pro-S*)	3.71 (d, 7.7, H*pro-S*)	3.75 (d, 8.0, H*pro-S*)	3.68 (d, 8.7, H*pro-S*)
	3.99 (dd, 11.2, 5.0)	3.98 (dd, 8.2, 2.6, H*pro-R*)	4.06 (ddd, 7.7, 2.3, $^5J_{HF}$ = 1.9, H*pro-R*)	3.88 (dd, 8.0, 2.4, H*pro-R*)	3.86 (ddd, 8.7 1.9, $^5J_{HF}$ = 4.0, H*pro-R*)
9'	1.13 (s)	1.08 (s)	1.04 (s)	0.99 (s)	0.96 (s)
1'-OH	4.59 (s)	2.20 (s)	2.15 (s)	2.01 (s)	1.93 (s)
4'-OH	—	—	—	2.25 (d, 3.5)	1.83 (dd, 3.2, $^4J_{HF}$ = 10.1)
8'-OH	2.34 (dd, 5.0, 5.0)	—	—	—	—
OMe	3.71 (s)	3.74 (s)	3.73 (s)	3.73 (s)	3.72 (s)

a) 300 MHz. b) 500 MHz. Values for the chemical shifts are δ (ppm). Multiplicity of signals and coupling constants (Hz) are shown in parentheses. $^nJ_{HF}$ represents ^{1}H-^{19}F coupling constant separated by n bonds (n = 1,2,...).

Table 2. ^{19}F NMR (282 MHz) Data of **5a** in acetone-d_6 and **8b-12b** in $CDCl_3$

Compound	^{19}F chemical shift	Multiplicity	Coupling constants
5a	-135.4	s	
8b	-134.8	s	
9b	-204.5	d	$^2J_{FH}$ = 46.8 Hz
10b	-175.0	d	$^2J_{FH}$ = 48.0 Hz
11b	-133.1	dd	$^2J_{FH}$ = 50.0 Hz, $^3J_{FH}$ = 22.4 Hz
12b	-197.8	dd	$^2J_{FH}$ = 51.5 Hz, $^3J_{FH}$ = 25.8 Hz

Values for the chemical shifts are shown by ppm from CCl_3F as the internal standard.

lowest R*f* among three compounds in TLC. Isolated **8b** gave no signal corresponding to the 3'-proton of **9b**, and showed a signal for the 7'-protons at a field lower by 0.51 ppm than that of **9b** in ^{1}H NMR (Table 2). In ^{19}F NMR, a singlet signal was observed at -134.8 ppm (Table 3). MS revealed that the molecular weight was 312, which agreed with those of **9b** and **10b**. These data showed that **8b** was the methyl ester of 3'-fluoro-8'-HOABA (**8a**). Some of compound **8b** was spontaneously and easily converted to **9b** and **10b**, while **10b** was relatively stable and converted very slowly to **8b** and **9b**. Each compound was converted finally into an equilibrium mixture of **8b**, **9b** and **10b** at 25°C in the ratio of 7:6:1, as determined by HPLC (Fig. 5). This would be caused by the weakened electrophilicity of C-2' by the effect of fluorine as expected. Such mutual conversion cannot be observed between 8'-HOABA and PA.

The stability of **10b** compared with **8b** and **9b** suggests that the energy barrier against the conversion of **10b** to other isomers is higher than that of **8b** and **9b**. The small proportion of **10b** in equilibrium and the high energy barrier against the conversion indicated that the isomerization to **10b** is unfavorable both thermodynamically and kinetically. The thermodynamic instability of **10b** would be caused by 1,3-diaxial steric repulsion between the fluorine and the side chain. The **8b/10b** and **9b/10b** ratios in equilibrium suggest that **10b** is less stable by 1 kcal mol^{-1} than **8b** and **9b**. The transition state for enolization-ketonization between **10b** and the enol **13b** would be less stable than that between **9b** and **13b**. This is because there is less σ-π interaction between the σ-electron at the 3'-proton and the π-electron at the 4'-carbonyl when the entering or leaving proton has an equatorial orientation as opposed to an alternative axial orientation.[20] This would cause a high energy barrier against the isomerization of **10b**.

Compounds **8b**, **9b** and **10b** afforded extremely broad peaks in GLC with XE-60, probably because of the fast interconversion at high temperature and the inappropriate liquid phase or support. Detection of these compounds in the methylated EtOAc extract was thus difficult in GLC with XE-60. Apiezon Grease L® gave better separation than silicon. GLC of **8b**, **9b** and **10b** using Apiezon Grease L® gave the same chromatograms, which consisted of four peaks in the ratio of about 6:1:1:6, although they were still broad and overlapping. All four peaks gave the ion at *m/z* 312 in GC-MS analysis, suggesting that they were not degradation products but isomers converted by the high temperature. The two high peaks among the four in GLC will correspond to **8b** and **9b** although it is unknown which is which. One of the two small peaks will correspond to **10b**, and the other small one may correspond to the enol **13b**. The lack of the enol **13b** in HPLC using a mixture of MeOH and water as the solvent can be explained by its instability in polar solvents. Enols forming intramolecular hydrogen bonds are stabilized in apolar solvents especially in the gas phase, but they are labile in polar solvents such as MeOH and chloroform.[21] The enol **13b**, which can form intramolecular hydrogen bonding between the 4'-hydroxyl and 3'-fluoro groups, may have these features. Metabolites **8a-10a** would also be interconverted via **13a** *in vivo* (Fig. 5).

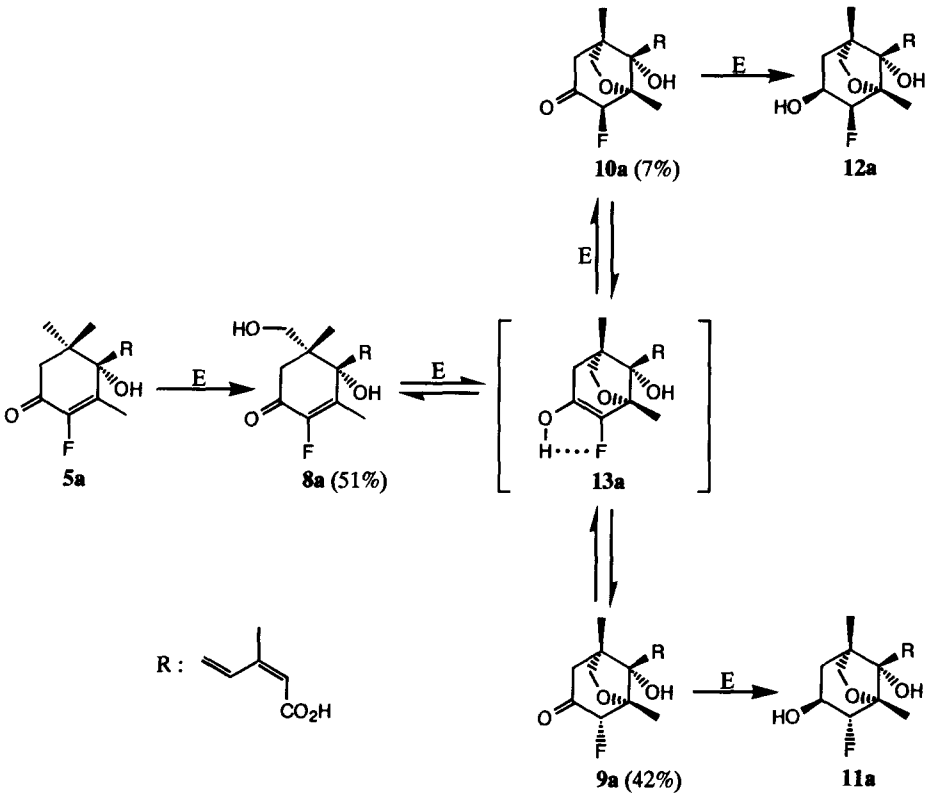


Fig. 5. Supposed oxidation pathway in the metabolism of **5a** in bean shoots
"E" means the step catalyzed by an enzyme. Percentages in the parentheses are the compositions of methyl esters **8b**, **9b** and **10b** in the final equilibrium in methanol.

GLC of the methylated EtOAc extract with Apiezon Grease L® confirmed the equilibrium mixture of **8b**, **9b** and **10b**. HPLC of the fractions purified from the extract revealed peaks with the same retention time as the standard samples of **8b** and **9b**. After isolation, these compounds were identified with **8b** and **9b** from the spectral data. A peak corresponding to **10b** was not found due to the small amount and masking by overlapping peaks, but it must also be in the extract. The ratio among unmetabolized **5a**, metabolites **8a**-**10a**, metabolite **11a**, and metabolite **12a** in the EtOAc extract was estimated to be 1:2:1:1 from the contents of their methyl esters determined by GLC analyses using XE-60 and Apiezon Grease L® (Table 3).

The slight increase of biological activity of **5a** in the lettuce seeds may be caused by the stability of **8a** which would maintain the activity, or else by the higher affinity of **5a** for the receptor involved in the inhibitory activity in lettuce seed germination than ABA. However, the stability of **8a** is not enough to give **5a** prominently high activity since **8a** could be metabolized to **9a** and **10a**, and then to **11a** and **12a**.

Table 3. The Recovery of **5a** and its Metabolites (**8a-12a**) from Bean Shoots

Compound	In the EtOAc extract	Released by hydrolysis of the conjugate fraction with alkali	Released by hydrolysis of the conjugate fraction with β-glucosidase
5a	0.6 mg	0.3 mg (3)	0.3 mg (3)
8a-10a	1.2 (13)	0.2 (2)	0.2 (2)
11a	0.6 (7)	trace	trace
12a	0.6 (7)	trace	trace

Compounds were analyzed as the methyl ester and their weights were determined in GLC with 1% XE-60 and 1% Apiezon Grease L® columns. The ratios of conversion of the applied **5a** (9 mg) into each metabolite were presented in percentage in the parentheses.

Involvement of enzymes in the cyclizing step. The ratio between metabolites **11a** and **12a**, 1:1, was inconsistent with the **9b/10b** ratio (6:1) in equilibrium. If the cyclization of **8a** occurs nonenzymatically, the ratio of **11a** to **12a** must be 6:1. This discrepancy supports the participation of the cyclization enzyme in this conversion[8]; metabolite **8a** would be enzymatically converted to **10a**, which then may be isomerized to **8a** and **9a** owing to equilibrium before being reduced to **12a**, or some of **8a** may be isomerized depending upon equilibrium to **9a** before being enzymatically cyclized (Fig. 5). The tendency of the 4'-carbonyl to accept nucleophiles would be enhanced more intensely by the 3'-axial, than by the equatorial fluorine, because the former can have a σ-π interaction with the π bond of the carbonyl more effectively than the latter. Therefore, the 4'-carbon in **10a** may be attacked by hydride ions more easily than that in **9a**, that is, **10a** may be metabolized more quickly than **9a**. Alternatively, **10a** may have higher affinity for the active site of a reductase than **9a**. These might contribute to the increase of **12a**.

Hydrolysis of the conjugates. The conjugate fraction from the aqueous extract was hydrolyzed with alkali or β-glucosidase before being extracted with EtOAc under acidic conditions and methylated for GLC analysis using XE-60 and Apiezon Grease L® columns. These extracts yielded similar results. Compounds **5b** and (**8b**, **9b** and **10b**) were found in the ratio of 3:2, and the **11b** and **12b** levels were low (Table 3). This suggested that most of the conjugated metabolites are C-1-glucosyl esters, not C-1'- or C-8'-glucosides. The total amount of the conjugated metabolites was less than that of the free metabolites, indicating that the oxidation pathway mostly concerns **5a** in bean shoots.

In conclusion, the introduction of fluorine at C-3' in ABA increased the electron density at C-2' so much that the methyl esters of 3'-fluoro-8'-HOABA and 3'-fluoro-PAs coexisted at equilibrium by the partial resistance of C-3' to attack by the 8'-hydroxyl group because of the effect the fluorine atom at C-3' has on C-2'.

EXPERIMENTAL

General experimental procedures

The ^1H and ^{13}C NMR spectra were recorded with TMS as an internal standard at 300 or 500 MHz for ^1H and 125 MHz for ^{13}C using Bruker ARX500 and AC300 instruments. For clarity, the atoms of all the compounds with the carbon skeleton of ABA were numbered as in ABA in the assignment of peaks. ^{19}F NMR spectra were recorded at 282 MHz on a Bruker AC300. ^{19}F chemical shifts were reported in ppm from CCl_3F as an internal reference and the higher field resonance from the CCl_3F signal was assigned as negative. Mass spectra were recorded at 70 eV with a Jeol JMS-DX300/DA5000 mass spectrometer. CD spectra were recorded with a Jasco J-720w spectropolarimeter. Optical rotations were measured with a Jasco DIP-1000 digital polarimeter.

2'α,3'α-Dihydro-2'α,3'α-epoxyabscisic acid (**6a**)

To a stirred solution of (+)-ABA (200 mg, 0.76 mmol) in MeOH (20 ml) were added 30% H_2O_2 (0.2 ml) and 6 N NaOH (0.6 ml) at 0°C. The mixture was stirred for 8 hr at 0°C and made up to 40 ml with H_2O. After lowering the pH to 2 with 3 N HCl, the mixture was extracted with EtOAc (30 ml x 3). The organic layer was washed with H_2O, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by chromatography on silica gel (12 g) with hexane-EtOAc-AcOH (80:20:3) to afford **6a** (114 mg, 54% yield) as a colorless oil. ^1H NMR (500 MHz, $CDCl_3$): δ 0.94 (3H, s, H_3-9'), 0.99 (3H, s, H_3-8'), 1.39 (3H, s, H_3-7'), 1.83 (1H, d, J = 14.7 Hz, H-5'), 2.07 (3H, s, H_3-6), 2.67 (1H, s, OH), 2.84 (1H, d, J = 14.7 Hz, H-5'), 3.30 (1H, s, H-3'), 5.79 (1H, s, H-2), 6.11 (1H, d, J = 15.8 Hz, H-5), 8.03 (1H, d, J = 15.8 Hz, H-4); UV λ_{max}^{MeOH} nm (ε): 253 (15,700); $[\alpha]_D^{26}$ +6° (MeOH, *c* 0.41); EIMS *m/z* (rel. int.): 280 $[M]^+$ (6), 262 $[M - H_2O]^+$ (4), 251 (22), 206 (11), 196 (25), 178 (33), 151 (50), 135 (20), 121 (42), 111 (76), 94 (26), 85 (100); HREIMS: $[M]^+$ at *m/z* 280.1307 (calcd for $C_{15}H_{20}O_5$, *m/z* 280.1311).

Methyl 2'α,3'α-dihydro-2'α,3'α-epoxyabscisate (**6b**)

Method i). Ethereal CH_2N_2 was added to **6a** (100 mg, 0.36 mmol) in MeOH (3 ml) at room temperature until a yellow color persisted. The mixture was left for 1 hr at room temperature, and concentrated under reduced pressure to give **6b** (105 mg, quantitative yield) as a colorless oil.

Method ii). The methyl ester of (+)-ABA was prepared from (+)-ABA by the same method as method i). To a stirred mixture of the methyl ester of (+)-ABA (100 mg, 0.36 mmol) in dimethyl sulfoxide and 30% H_2O_2 (180 μl) was added dropwise, tetrabutylammonium fluoride (1.0 M tetrahydrofuran solution, 2.36 ml, 2.36 mmol) at room temperature under nitrogen. The mixture was stirred at the same temperature for 5 hr. H_2O (30 ml) was added and the product was extracted with EtOAc (20 ml x 3). The organic layer was washed with

H_2O, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by chromatography on silica gel (2 g) with hexane-EtOAc (8:2) to give **6b** (44 mg, 41% yield). ^1H NMR (500 MHz, $CDCl_3$): δ 0.93 (3H, s, H_3-9'), 0.99 (3H, s, H_3-8'), 1.39 (3H, s, H_3-7'), 1.83 (1H, dd, J = 14.6 and 0.7 Hz, H-5'), 2.03 (3H, d, J = 1.1 Hz, H_3-6), 2.83 (1H, d, J = 14.6 Hz, H-5'), 2.87 (1H, d, J = 0.7 Hz, OH), 3.29 (1H, br s, H-3'), 3.34 (3H, s, OMe), 5.77 (1H, br s, H-2), 6.06 (1H, d, J = 15.8 Hz, H-5), 8.07 (1H, d, J = 15.8 Hz, H-4); EIMS *m/z* (rel. int.): 294 $[M]^+$ (5), 276 $[M - H_2O]^+$ (2), 265 (15), 238 (12), 210 (17), 206 (17), 195 (27), 178 (41), 165 (17), 151 (56), 135 (23), 125 (100); HREIMS: $[M]^+$ at *m/z* 294.1464 (calcd for $C_{16}H_{22}O_5$, *m/z* 294.1467).

Methyl 3'-fluoroabscisate (**5b**)

A stirred solution of **6b** (21 mg, 0.071 mmol) in *N*-ethyldiisopropylamine (0.5 ml) and *N*-ethyldiisopropylamine tris(hydrofluoride)[14] (40 μl) was heated at 145°C for 4 hr. The solution was poured into 3N HCl (20 ml) and extracted with EtOAc (20 ml x 3). The organic layer was washed with saturated aqueous $NaHCO_3$ and H_2O, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by chromatography on silica gel (2 g) to give **5b** (6 mg, 28% yield) as a colorless oil. ^1H NMR (500 MHz, $CDCl_3$): δ 1.04 (3H, s, H_3-9'), 1.14 (3H, s, H_3-8'), 1.87 (3H, d, $^4J_{HF}$ = 3.5 Hz, H_3-7'), 2.02 (3H, d, J = 1.1 Hz, H_3-6), 2.11 (1H, s, OH), 2.43 (1H, dd, J = 16.9 and $^4J_{HF}$ = 4.4 Hz, H-5'*pro-R*), 2.49 (1H, d, J = 16.9 Hz, H-5'*pro-S*), 3.71 (3H, s, OMe), 5.78 (1H, br s, H-2), 6.09 (1H, d, J = 16.1 Hz, H-5), 7.88 (1H, d, J = 16.1 Hz, H-4); UV λ_{max}^{MeOH} nm (ε): 262 (23,100); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3500, 2950, 1690, 1650, 1630, 1600; EIMS *m/z* (rel. int.): 296 $[M]^+$ (4), 278 $[M - H_2O]^+$ (5), 264 (6), 246 (4), 223 (6), 208 (70), 183 (46), 180 (50), 165 (15), 152 (55), 125 (100); HREIMS: $[M]^+$ at *m/z* 296.1440 (calcd for $C_{16}H_{21}O_4F$, *m/z* 296.1424).

(+)-3'-Fluoroabscisic acid (**5a**)

To a solution of **5b** (42 mg, 0.14 mmol) in MeOH (1 ml) was added 1N NaOH (0.8 ml). The mixture was stirred at room temperature for 3 hr, then H_2O (30 ml) was added. After lowering the pH to 2 with 3N HCl, the mixture was extracted with EtOAc (20 ml x 3). The organic layer was washed with H_2O, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was chromatographed on silica gel (16 g) under pressure to afford **5a** (38 mg, 95% yield) as a white amorphous solid. ^1H NMR (500 MHz, acetone-d_6): δ 1.07 (3H, s, H_3-9'), 1.11 (3H, s, H_3-8'), 1.85 (3H, d, $^4J_{HF}$ = 3.5 Hz, H_3-7'), 2.07 (3H, d, J = 1.0 Hz, H_3-6), 2.33 (1H, br d, J = 16.8, H-5'), 2.63 (1H, d, J = 16.8 Hz, H-5'), 5.78 (1H, br s, H-2), 6.39 (1H, d, J = 16.0 Hz, H-5), 7.93 (1H, d, J = 16.0 Hz, H-4); ^{13}C NMR (125 MHz, acetone-d_6): δ 10.4 (d, $^3J_{CF}$ = 4.7 Hz, C-7'), 21.0 (C-6), 23.2 (C-8'), 24.3 (C-9'), 42.1 (C-6'), 49.3 (d, $^3J_{CF}$ = 4.0 Hz, C-5'), 79.5 (d, $^3J_{CF}$ = 5.1 Hz, C-1'), 118.7 (C-2), 128.8 (C-4), 137.4 (d, $^4J_{CF}$ = 2.5 Hz, C-5), 138.3 (d, $^2J_{CF}$ = 6.4 Hz, C-2'), 150.7 (C-3), 151.0 (d, $^1J_{CF}$ = 258.4 Hz, C-3'), 167.0 (C-1), 189.0 (d,

$^2J_{CF}$ = 20.5 Hz, C-4'), : $[\alpha]_D^{17}$ +264° (MeOH, *c* 0.32); CD λ_{ext}^{MeOH} nm ($\Delta\varepsilon$): 327 (-1.5), 260 (+27.2), 231 (-21.6); UV λ_{max}^{MeOH} nm (ε): 247 (24,300); EIMS *m/z* (rel. int.): 282 $[M]^+$ (6), 264 $[M - H_2O]^+$ (22), 249 (15), 239 (15), 223 (13), 208 (100), 183 (85), 180 (68), 165 (22), 152 (66), 124 (17), 111 (48); HREIMS: $[M]^+$ at *m/z* 282.1263 (calcd for $C_{15}H_{19}O_4F$, *m/z* 282.1267). The ^1H, ^{13}C and ^{19}F NMR data are listed in Tables 1 and 3.

(±)-3'-Fluoroabscisic acid [(±)-**5a**] *and HPLC analysis with a chiral column*

In the same manner as **5a**, (±)-**5a** was synthesized from (±)-ABA. The racemate (±)-**5a** gave two separate peaks with retention times of 8.2 and 9.4 min in HPLC with a Chiralcel OD column (4.6 x 250 mm, Daicel; solvent, 11% *i*-PrOH in hexane containing 0.1% TFA; flow rate, 1.0 ml min^{-1}; detection, 254 nm). Optically active **5a** afforded only one peak at a retention time of 8.2 min under the same conditions.

Bioassays

Details have been reported.[9] The germinated lettuce (*Lactuca sativa* L. cv. Grand Rapids) seeds were counted after incubation with the test solution at 25°C for 48 hr. To assay elongation, the length of the second leaf sheath of rice (*Oryza sativa* L. cv. Nihonbare) seedlings was measured after incubation with the test solution in continuous light at 30°C for 7 days.

Plant material and application of ***5a***[22]

Bean plants (*Phaseolus vulgaris* L. cv. Kentucky Wonder) were grown in a greenhouse for 20 days until the primary leaves were mature and the first trifoliate leaf half expanded. The temperature in the greenhouse was maintained below 23°C during the day, and above 15°C during the night. To introduce compounds via the transpiration stream, the stems were cut at 0.5 cm above root apex under degassed water. For application to bean shoots, an acetone (0.6 ml) solution of **5a** (9 mg) was mixed with about 50 µl of Tween 20 and filled up to 150 ml with H_2O. Thirty shoots were placed in the solution for three days under continuous light (5000 lux) at 25°C. The vessel was covered with a sheet of black polyethylene to prevent photo-isomerization. HPLC analysis of the solutions after the application showed that 8.5 mg of **5a** was incorporated into the shoots.

Extraction from bean shoots

Plant samples (66 g, fresh weight) that incorporated **5a** were frozen in liquid nitrogen, pulverized and extracted with MeOH (400 ml) containing 10 µg ml^{-1} 2,6-di-*tert*-butyl-4-methylphenol for three days. After filtration, the tissue residue was washed several times with methanol. The combined filtrates were concentrated to a small volume and were brought to 250 ml with H_2O. After lowering the pH to 2 with 3 N HCl, the solution was extracted with EtOAc. The organic layer was washed with H_2O and concentrated under

reduced pressure to give the EtOAc extract (334 mg). The aqueous layer, after adjusting the pH to 5, was concentrated under reduced pressure to give the aqueous extract (38 mg).

Isolation of metabolites from the EtOAc extract

To a solution of the EtOAc extract (310 mg) in EtOAc (2 ml) was added ethereal CH_2N_2 until a yellow color persisted. The mixture was left for 0.5 hr at room temperature, then the solvent was removed. The residue was applied to a column packed with silica gel (15 g) and eluted with 10, 20, 30, 40, 60 and 80% in toluene. The 40 and 60% EtOAc fractions were combined and concentrated under reduced pressure. The residue was purified further by chromatography using ODS (AM120-S50, YMC, 16g) with 60% MeOH in H_2O to give 2 mg of a crude oil. The crude oil (2 mg) was injected into an HPLC column (μBondasphere 5μ C18-100Å, 19 x 150 mm, Waters; solvent, 55% MeOH; flow rate, 10 ml min^{-1}; detection, 254 nm). The materials with retention times of 8.2 and 13.9 min were collected to give **11b** (0.3 mg) and **12b** (0.3 mg) as colorless oils, respectively. The eluate containing other materials was injected into the HPLC column again. The material with a retention time of 9.8 min was collected to give **8b** (0.1 mg) as a colorless oil. The 30% EtOAc fraction was concentrated, suspended in MeOH-H_2O (9:1), then chromatographed in portions over Sep-Pak® C18 (original type, Millipore) using 30, 60 and 80% MeOH. The 60% MeOH fraction was injected into a HPLC column (AQ 311, 6 x 100 mm, YMC; solvent, 55% MeOH; flow rate, 2 ml min^{-1}; detection, 254 nm). The material with a retention time of 2.7 min was collected to give **9b** (0.1 mg) as a colorless oil. **11b**: $[\alpha]_D^{21}$ -80° (MeOH, *c* 0.014); UV λ_{max}^{MeOH} nm (ε): 264 (22,000); EIMS *m/z* (rel. int.): 314 $[M]^+$ (12), 296 $[M - H_2O]^+$ (16), 282 (5), 264 (6), 237 (15), 221 (6), 195 (7), 177 (14), 163 (21), 154 (34), 135 (30), 122 (100); HREIMS: $[M]^+$ at *m/z* 314.1522 (calcd for $C_{16}H_{23}O_5F$, *m/z* 314.1529). **12b**: $[\alpha]_D^{21}$: -197° (MeOH, *c* 0.0158); UV λ_{max}^{MeOH} nm (ε): 267 (22,000); EIMS *m/z* (rel. int.): 314 $[M]^+$ (8), 296 $[M - H_2O]^+$ (17), 282 (6), 264 (6), 237 (8), 219 (5), 195 (10), 177 (14), 163 (24), 154 (29), 135 (31), 125 (100); HREIMS: $[M]^+$ at *m/z* 314.1502 (calcd for $C_{16}H_{23}O_5F$, *m/z* 314.1529). **8b**: UV λ_{max}^{MeOH} nm (ε): 263 (24,000); EIMS *m/z* (rel. int.): 312 $[M]^+$ (6), 294 $[M - H_2O]^+$ (13), 280 (14), 264 (24), 249 (10), 239 (15), 221 (10), 208 (30), 199 (18), 179 (23), 163 (14), 152 (29), 125 (100); HREIMS: $[M]^+$ at *m/z* 312.1376 (calcd for $C_{16}H_{21}O_5F$, *m/z* 312.1373). **9b**: UV λ_{max}^{MeOH} nm (ε): 263 (18,500); EIMS *m/z* (rel. int.): 312 $[M]^+$ (34), 294 $[M - H_2O]^+$ (5), 292 (3), 280 (14), 264 (8), 249 (5), 239 (10), 221 (10), 208 (14), 177 (24), 163 (31), 154 (32), 135 (38), 125 (100); HREIMS: $[M]^+$ at *m/z* 312.1366 (calcd for $C_{16}H_{21}O_5F$, *m/z* 312.1373). The ^1H and ^{19}F NMR spectral data are listed in Tables 1 and 2. The value of the optical rotation of **8b** and **9b** was too small to be measured.

Hydrolysis of the conjugate fraction with alkali and β-glucosidase

The aqueous extract (38 mg) was roughly purified by chromatography on ODS (16 g). The first fraction that eluted with H_2O was discarded, and the second MeOH fraction was concentrated to give the conjugate fraction (34 mg). To the conjugate fraction (15 mg) was added 1 N NaOH (45 ml), and the mixture was left at room temperature for 6 hr. The conjugate fraction (15 mg) was made up in 0.05 M citrate-sodium citrate buffer (pH 5.0, 30 ml), and β-glucosidase (EC 3.2.1.21, Sigma G0395, 235 units) was added. The mixture was incubated for 6 hr at 37°C. The residual conjugate fraction (4 mg) was not hydrolyzed. After lowering the pH to 2 with 3 N HCl, these solutions were extracted with EtOAc, respectively. The organic layers were washed with saturated aqueous NaCl, dried over Na_2SO_4 and concentrated to give 13, 12 and 3 mg of crude oils, respectively, which were treated with ethereal CH_2N_2 before analysis by GLC. Free compounds (**5a** and **8a**-**12a**) were not detected in the organic layer of non-hydrolyzed-conjugate fraction.

Preparation of methyl 3'α-fluorophaseate (**9b**) *and methyl 3'β-fluorophaseate* (**10b**)

To a stirred solution of **11b** (0.1 mg) in acetone (0.5 ml) was added Jones reagent[19] (25 μl) at 5°C. The mixture was stirred for 0.5 hr at 5°C. H_2O (5 ml) was added to the mixture, and the solution was extracted with EtOAc. The organic layer was washed with H_2O, dried over Na_2SO_4 and concentrated under reduced pressure. The residual oil was purified by chromatography on silica gel (0.8 g) with hexane-EtOAc (7:3) to afford 0.1 mg of **9b** quantitatively as a colorless oil. In the same manner, **12b** (0.1 mg) gave **10b** (0.1 mg) as a colorless oil. **10b**: UV λ_{max}^{MeOH} nm (ε): 264 (18,000); EIMS *m/z* (rel. int.): 312 $[M]^+$ (28), 294 $[M - H_2O]^+$ (4), 292 (4), 280 (15), 264 (5), 251 (5), 233 (12), 208 (10), 191 (12), 177 (24), 163 (29), 154 (31), 135 (34), 125 (100); HREIMS: $[M]^+$ at *m/z* 312.1367 (calcd for $C_{16}H_{21}O_5F$, *m/z* 312.1373). The ^1H and ^{19}F NMR spectral data are listed in Tables 1 and 2. The other data for **9b** are described above. The values of the optical rotation of **10b** were too small to be measured.

Gas-liquid chromatography (GLC) and TLC

GLC proceeded using 1% XE-60 (support, Chromosorb W) column (3.2 mm x 2 m) at 210°C or 1% Apiezon Grease L® (support, Shimalite Q®) column (3.2 mm x 0.5 m) at 190°C at a nitrogen flow of 60 ml min^{-1}, with an electron-capture detector. The retention times of **5b**, **11b** and **12b** in XE-60 were 5.8, 8.1, and 4.6 min, respectively and those in Apiezon Grease L® were 6.0, 9.4, and 8.0 min, respectively. The retention times of the four peaks of **8b**, **9b** and **10b** in Apiezon Grease L®, respectively, were 6.0, 7.8, 10.2 and 12.1 min. Contents of the metabolites in extracts were determined on the basis of each peak area considering the difference in the sensitivity of the electron-capture detector which was calculated from the analysis of standard samples (see Table 3). Metabolites were analyzed by silica gel TLC using Kieselgel 60

F_{254} (thickness, 0.2 mm, Merck) in a solution of hexane-EtOAc (1:1). The R*f* values of **8b**, **9b**, **10b**, **11b** and **12b** were 0.50, 0.61, 0.73, 0.22 and 0.28, respectively.

GC-MS

GC-MS was conducted with 1% Apiezon Grease L® column (2.6 mm x 0.1 m) and a helium flow of 150 ml min^{-1}. The temperature was maintained at 200°C for initial 10 min, then raised from 200 to 220°C at a rate of 2°C min^{-1}. The ions observed in all the four peaks (retention time 8.2, 11.6, 14.4, and 16.4 min) of a mixture of **8b**, **9b** and **10b** at equilibrium, *m/z* (rel. int. of peaks in the ratio of 6:1:1:6, respectively): 312 $[M]^+$ (8, 8, 10, 10), 294 (3, 8, 5, 6), 280 (9, 8, 7, 7), 264 (9, 17, 17, 12), 249 (6, 8, 14, 10), 233 (12, 12, 14, 16), 221 (10, 22, 20, 16), 208 (10, 20, 14, 10), 191 (12, 25, 20, 18), 177 (17, 21, 22, 20), 163 (30, 21, 33, 22), 154 (20, 25, 33, 17), 135 (32, 32, 32, 29), 125 (100, 100, 100, 100).

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