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SYNTHESIS, RESOLUTION AND BIOLOGICAL ACTIVITY OF 7′,7′-DIFLUOROABSCISIC ACID

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Key Word Index—*Bromus inermis*; Gramineae; bromegrass; *Lepidium sativum*; cress germination inhibition; freezing tolerance; abscisic acid analogues; abscisic acid; 7′,7′-difluoroabscisic acid.

Abstract—7′,7′-Difluoroabscisic acid was synthesized in seven steps from the known ketoaldehyde 4,4-ethylenedioxy-2-formal-6,6-dimethylcyclohex-2-en-1-one. Fluorination with diethylaminosulphur trifluoride afforded the key intermediate 2-difluoromethyl-4,4-ethylenedioxy-6,6-dimethylcyclohex-2-en-1-one which was further transformed to 7′,7′-difluoroabscisic acid. The racemic mixture and the individual optical isomers, obtained by resolution of the methyl esters by HPLC, displayed activity similar to the optical isomers of ABA in assays for germination in cress and freezing tolerance in bromegrass cell suspension culture.

INTRODUCTION

The plant hormone abscisic acid [(+)-ABA, **1**] is involved in the regulation of many diverse processes in growth and development in higher plants [1, 2]. Abscisic acid inhibits precocious seed germination [3] and is implicated in resistance to abiotic stresses [4]. Application of exogenous (+)-ABA, or more commonly, (±)-ABA, can lead to inhibition of seed germination in many species [5] and can also induce freezing tolerance in cell cultures of bromegrass [6], a useful model system for studying acclimation to cold. As part of our ongoing research on the roles of ABA and its metabolites in plant growth and development, we have undertaken to synthesize and investigate the biological activity of fluorinated analogues of the hormone.

Recently, fluorine-containing analogues of biologically active molecules have become important tools for studying receptors and active sites of enzymes [7]. Replacing a hydrogen with a fluorine only slightly changes the size or shape, but can greatly affect the electronic nature of the molecule, due to the strong electronegative properties of fluorine. Such substitutions can produce active analogues with increased or altered biological activity in comparison to the parent compound. The use of fluorinated analogues has been particularly prevalent in the field of prostacyclin and prostaglandin chemistry. One example is compound **2**, which was found to have an inhibitory action on rabbit platelet aggregation [8]. The fluorinated analogue **3** was found to have more persistent activity with its half-life greatly increased over the parent compound. The unique properties of fluorine have also been widely utilized in the design of mechanism-based enzyme inhibitors [9].

Our entry into fluorinated compounds arose from our recent synthesis of 7′-hydroxyabscisic acid (**4**), a catabolite of ABA, whose biological activity is unknown [10]. We chose as our synthetic target 7′,7′-difluoroabscisic acid (**5**), a molecule that could be made from an intermediate in the 7′-hydroxyabscisic acid synthesis. The fluorine-containing ABA analogue **5** was expected to have a similar size to ABA, and fit into some, but not necessarily all, putative ABA receptors. The electron withdrawing fluorine atoms would be expected to perturb the electronics of the enone system, with unknown consequences to the overall stability of the molecule in the plant system.

We report the synthesis of the target molecule, its resolution, and the activity of the racemic material in an assay for freezing tolerance and the activity of both antipodes in germination of cress.

RESULTS AND DISCUSSION

Synthesis

Our strategy for synthesizing 7′,7′-difluoroabscisic acid involved initially forming the difluoromethyl cyclohexenone ring system of ABA, followed by attachment of the side chain using methodology developed by Mayer [11] and commonly used in these laboratories [12]. For this, we required a suitably protected compound containing the features of the ABA ring system as well as having an aldehyde functionality at C-7′ (following the ABA numbering system) for introduction of the fluorine atoms. Ketoaldehyde **6**, which was an intermediate in our synthesis of 7′-hydroxyabscisic acid, served as a candidate for the introduction of two fluorine atoms at the 7′ position (Scheme 1).

Compound **6** was selectively fluorinated at the aldehydic position using one equivalent of diethylaminosulphur trifluoride (DAST) [13]. The reaction was carried out at −78° to form, in 72% yield, compound **7**. The side chain was then introduced into the C-1′ position in 67% yield

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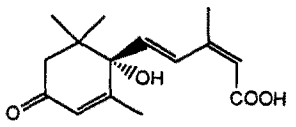

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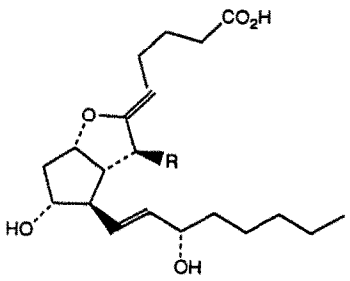
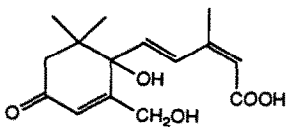


2: R=H
3: R=F



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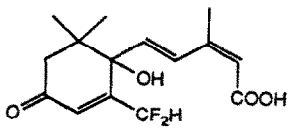

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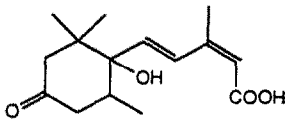

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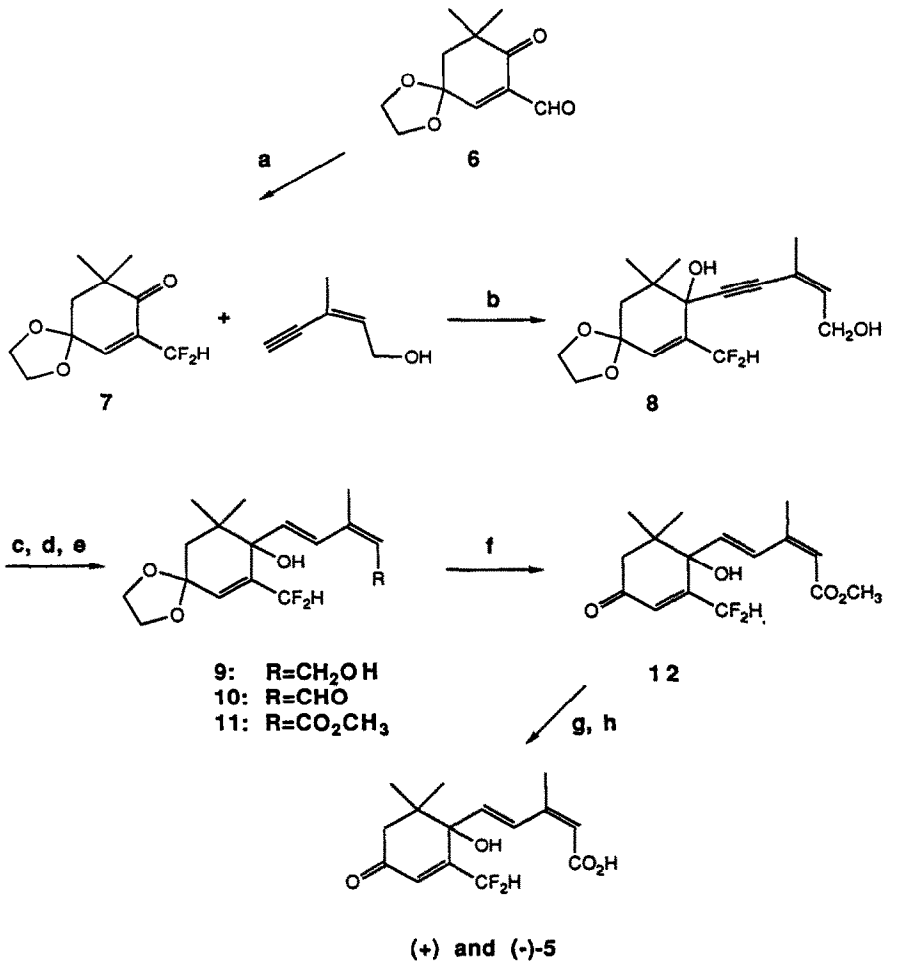
by nucleophilic attack of the carbonyl by the dianion of *cis*-3-methyl-2-penten-4-yn-ol. The alkylated product **8** contained all the carbons required for the ABA framework.

The remaining transformations involved the reduction of the triple bond to the *trans* double bond, oxidation of the allylic alcohol to the acid level and removal of the ketal. This had to be carried out while keeping the *cis* configuration of the C-2/C-3 double bond intact, therefore requiring mild conditions. The reduction of the triple bond to the *cis–trans*-dienic system of **9** could be carried out in 70% yield using sodium bis(2-methoxyethoxy)aluminium hydride (Redal^{R}) at low temperature. The oxidation of the sensitive allylic alcohol was accomplished in two steps: first, oxidation to the aldehyde stage (**10**) using manganese dioxide; and secondly, a Corey oxidation to the ester (**11**) [14]. The ketal was then easily removed by reaction with a catalytic amount of *p*-toluenesulphonic acid in acetone, to afford methyl 7',7'-difluoroabscisic acid (**12**). At this stage, the racemic keto-ester was found to be resolvable on both analytical and preparative scales by HPLC on a chiral column.

The final step involved the basic hydrolysis of the racemic and two optically active esters forming (±)-**5**, (+)-**5** and (−)-**5** in high yields. The fluorinated compounds were compared to ABA in assays for freezing tolerance and germination inhibition.

Biological testing

Cell suspension cultures of *Bromus inermis* Leyss acclimate to cold when supplied with (±)-ABA [6]. Cells treated with 75 μM ABA and grown for seven days at 25° can survive slow cooling to greater than −40°, while untreated controls can tolerate only −10°. At equivalent concentrations, (±)-**5** confers similar levels of freezing tolerance as (±)-ABA (Table 1), and the difluorinated analogue is perceived by the bromegrass cells as equivalent to ABA. The fluorinated ABA analogue is unusual in this respect. We have screened ABA metabolites and a wide range of analogues in the bromegrass model system and found that the majority of compounds tested do not promote freezing tolerance (data not shown). One molecule that does substitute for ABA is 2',3'-dihydroabscisic



a. DAST, CH_2Cl_2; b. n-BuLi, THF, -78°; c. REDAL^R, THF, -15°; d. MnO_2, acetone; e. MnO_2, NaCN, AcOH, MeOH; f. p-TsOH, acetone; g. hplc resolution; h. 2M KOH, MeOH.

Scheme 1.

Table 1. The effect of (±)-abscisic acid and (±)-difluoroabscisic acid on the freezing tolerance of bromegrass cells

	LT_{50}* (°) conc. (μM)				
Sample	0	0.1	1	10	100
Control	−10.5 (0.4)				
0.1% DMSO	−10.5 (0.1)				
(±)-ABA		−9.2 (0.3)	−22.2 (2.9)	−30.0	< −40
(±)-5		−9.9 (0.5)	−13.4 (0.3)	−34.3	< −40

*LT_{50} (temperature for 50% survival) determined after seven days of treatment at 25° in the dark. Value in parentheses indicates standard error where available.

acid (13), a compound that cannot be metabolized in the same way as ABA. Our results suggest that the activity observed with (±)-5 may be due to a compatible fit with a receptor for ABA and not a metabolite, with the resulting signal triggering the cold hardening process.

Compounds (+)-5, (−)-5 and (±)-5 were compared to (±)-ABA in a germination assay using cress seed. The racemic difluorinated analogue (±)-5 is a potent germination inhibitor, nearly as active as (±)-ABA in inhibiting both root and shoot germination (see Fig. 1). The activit-

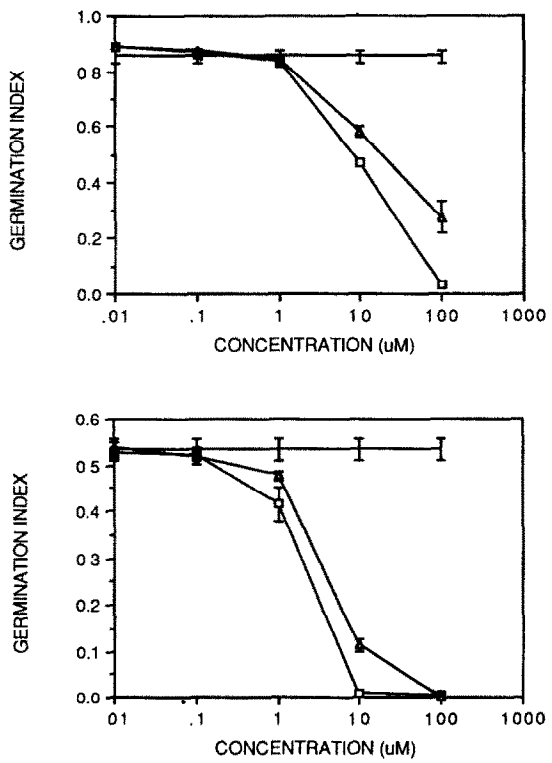


Fig. 1. Root germination in cress with (±)-ABA (□), (±)-5 (△), and 0.2% acetone (upper). Leaf germination in cress with (±)-ABA (□), (±)-5 (△), and 0.2% acetone (lower).

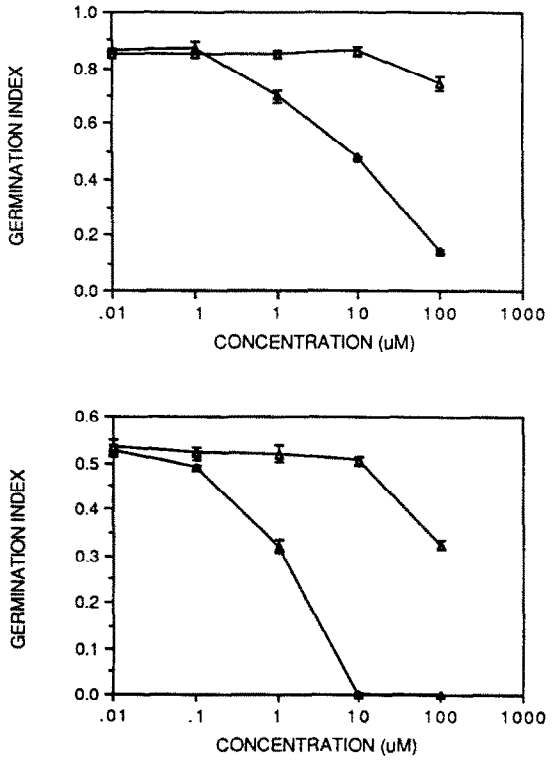


Fig. 2. Root germination in cress with (+)-5 (▲) and (−)-5 (△) (upper). Leaf germination in cress with (+)-5 (▲) and (−)-5 (△) (lower).

ies of the resolved difluoro analogues in cress seed germination tests are very different. The (+)-form is active, and the (−)-form shows only slight germination inhibition at 100 μM (see Fig. 2). The same pattern is found for the optically active forms of ABA (to be reported elsewhere).

The fluorinated ABA analogues (±)-5, (+)-5 and (−)-5 possess ABA-like activity in two assays and should prove to be valuable probes for determining the mode of action of ABA in other biological systems. We are investigating the metabolism and longevity of the fluorinated analogues and their application as enzyme inhibitors in plant cell cultures.

EXPERIMENTAL

General. Mp: uncorr.; ^{1}H NMR: 360 MHz, $CDCl_3$ with $CHCl_3$ as ref. For clarity, the conventional ABA numbering system is employed in assignments of peaks in the ^{1}H NMR spectra. Optical rotations: MeOH. Flash CC: Merck silica gel 60 (230–400 mesh); Analytical TLC: Merck silica gel 60 F254 plates (0.2 mm) with aluminium sheet backing; Prep. TLC: Chromatotron (Harrison Research) with circular glass plates precoated with silica gel F254 (1, 2, or 4 mm), where the radial flow of eluent and sample were centrifugally accelerated. GC-MS: DB-5 column (60 m) operated in either the EI mode or the CI mode. HR-EIMS: VG 70-250SEQ double-focusing hybrid spectrometer. Elemental analyses were carried out by the Microanalytical Laboratory of the University of Alberta, Edmonton, Canada.

2-Difluoromethyl-4,4-ethylenedioxy-6,6-dimethylcyclohex-2-en-1-one (7). To a soln of DAST (0.90 ml, 0.0068 mol) in CH_2Cl_2 (15 ml) cooled to −78° and under an atmosphere of Ar, was slowly added ketoaldehyde **6** (1.42 g, 0.0068 mol) in 5 ml CH_2Cl_2. The mixture was allowed to gradually warm to room temp. and was then quenched with H_2O (25 ml). The layers were sepd and the aq. layer extracted with 3 × 25 ml of CH_2Cl_2. The combined organic layers were washed with satd NaCl soln, dried over Na_2SO_4 and concd, to produce 1.13 g (72%) of a pure oil HR-EIMS: $[M]^+$ at *m/z* 232.0921 ($C_{11}H_{14}O_3F_2$ requires 232.0911); IR ν_{max} cm^{-1}: 1685 (C=O); ^{1}H NMR· δ6.78 (*m*, 1H, H-3'), 6.45 (*dt*, 1H, J = 1 Hz, J' = 54.7 Hz, H-7'), 4.01 (*m*, 4H, OCH_2), 2.08 (*s*, 2H, H-5'), 1.19 (*s*, 6H, C-7', C-8', Me). (Found: C, 56.98; H, 5.86. $C_{11}H_{14}O_3F_2$ requires: C, 56.90; H, 6.08).

(2E)-5-(2-Difluoromethyl-4,4-ethylenedioxy-6,6-dimethylcyclohex-2-enyl)-3-methylpent-2-en-4-yn-1-ol (8). To a soln of *cis* 3-methylpent-2-en-4-yn-1-ol (1.2 eq, 0.54 g) in THF (5 ml) under an Ar atmosphere at −78° was slowly added *n*-BuLi (2.4 eq, 1.6 M soln in hexanes, 7.0 ml) The reaction mixture was stirred for 30 min upon which time ketone **7** (1.08 g, 0.0047 mol) dissolved in 10 ml THF was added. The reaction was allowed to warm to room temp. where it was stirred for 1.75 hr. After quenching with H_2O (10 ml), the mixture was extracted into Et_2O (3 × 30 ml) and the combined Et_2O extracts washed with satd NaCl soln, dried over Na_2SO_4 and concd. Flash chromatography (40% EtOAc in hexane) gave 1.02 g (67%) of the alkyne. HR-EIMS: $[M]^+$ at *m/z* 328.1513 ($C_{17}H_{22}O_4F_2$ requires 328.1486); IR ν_{max} cm^{-1}: 3600 (O–H); ^{1}H NMR· δ6.40 (1H, *t*, J = 55.4 Hz, H-7'), 5.96 (1H, *s*, H-3'), 5.88 (1H, *m*, H-2), 4.24 (2H, *d*, J = 7 Hz, H-1), 3.95 (4H, *s*, OCH_2), 2.68 (1H, *br s*, OH), 2.00 (1H, *d*, J = 14.3 Hz, H-5'), 1.92 (1H, *d*, J = 14.3 Hz, H-5'), 1.85 (3H, *s*, C-6, Me), 1.13 (3H, *s*, C-8', Me), 1.10 (3H, *s*, C-9', Me). (Found: C, 61.95; H, 6.92. $C_{17}H_{22}O_4F_2$ requires: C, 62.19; H, 6.75).

(2Z,4E)-5-(2-Difluoromethyl-4,4-ethylenedioxy-6,6-dimethylcyclo-hex-2-enyl)-3-methylpenta-2,4-dien-1-ol (9). Alkyne **8** (506 mg, 1.54 mmol) in 10 ml THF was cooled to −15° in an

ice/salt bath under an Ar atmosphere. Red-Al (3 eq, 3.4 M in toluene, 1.4 ml) was slowly added. The soln was warmed to room temp. and stirred for 4 hr. H_2O was added to quench the reaction and the mixture extracted into Et_2O (3 × 30 ml). The combined organic extracts were washed with satd NaCl soln and dried over Na_2SO_4. Flash chromatography (40% EtOAc in hexane) produced 354 mg (70% yield) of diene **9**. CIMS *m/z* (rel. int.): 348 $[M+NH_4]^+$ (33), 313 $[M+H-H_2O]^+$ (100); IR ν_{max} cm^{-1}: 3410 (O–H); ^1H NMR: δ6.63 (1H, *d*, *J* = 15.8 Hz, H-5), 6.06 (1H, *t*, *J* = 55.1 Hz, H-7′, overlapping *s*, 1H, H-3′), 5.70 (1H, *d*, *J* = 15.8 Hz, H-4), 5.59 (1H, *t*, *J* = 7 Hz, H-2), 4.25–4.32 (2H, *m*, H-1), 3.92–4.03 (4H, *m*, OCH_2), 1.97 (1H, *d*, *J* = 14.3 Hz, H-5′), 1.83 (3H, *s*, C-6, Me), 1.77 (1H, *d*, *J* = 14.3 Hz, H-5′), 1.06 (3H, *s*, C-8′, Me), 0.91 (3H, *s*, C-9′, Me). (Found: C, 61.71; H, 7.40. $C_{17}H_{24}O_4F_2$ requires: C, 61.81; H, 7.32).

(2Z, 4E)-5-(2-*Difluoromethyl*-4,4-*ethylenedioxy*-6,6-*dimethyl*-*cyclohex*-2-*enyl*)-3-*methylpenta*-2,4-*dien*-1-*al* (**10**). A mixture of MnO_2 (20 eq, 360 mg) and diene **9** (68 mg, 0.21 mmol) was stirred in 5 ml Me_2CO at room temp. for 1 hr in the dark. The suspension was filtered and the cake of MnO_2 washed with 100 ml CH_2Cl_2. After concn, 52 mg (76% yield) of the aldehyde was recovered and required no further purification for the subsequent step. CIMS *m/z* (rel. int.): 346 $[M+NH_4]^+$ (27), 311 $[M+H-H_2O]^+$ (100%); IR ν_{max} cm^{-1}: 3600 (O–H), 1665 (C=O); ^1H NMR: δ10.17 (1H, *d*, *J* = 8 Hz, H-1), 7.32 (1H, *d*, *J* = 15.6 Hz, H-5), 6.11 (1H, *d*, *J* = 15.6 Hz, H-4), 6.10 (1H, *t*, *J* = 55 Hz, H-7′), 6.08 (1H, *s*, H-3′), 5.88 (1H, *d*, *J* = 8 Hz, H-2), 3.94–4.04 (4H, *m*, OCH_2), 2.06 (3H, *s*, C-6, Me), 1.93 (1H, *d*, *J* = 15 Hz, H-5′), 1.83 (1H, *d*, *J* = 15 Hz, H-5′), 1.09 (3H, *s*, C-8′, Me), 0.94 (3H, *s*, C-9′, Me). (Found: C, 62.26; H, 6.56. $C_{17}H_{22}O_4F_2$ requires: C, 62.19; H, 6.75).

Methyl (2Z,4E)-5-(2-*difluoromethyl*-4,4-*ethylenedioxy*-6,6-*dimethylcyclohex*-2-*enyl*)-3-*methylpenta*-2,4-*dienoate* (**11**). To aldehyde **10** (119 mg, 0.36 mmol) in MeOH (5 ml) was added sequentially MnO_2 (16 eq, 500 mg), NaCN (2.4 eq, 42 mg) and HOAc (1 eq, 20 μl). The reaction was stirred for 4 hr at room temp. The suspension was filtered and washed with MeOH. After concn, the residue was partitioned between Et_2O and H_2O and the organic layer washed with satd NaCl soln, dried over Na_2SO_4 and concd. The crude product was purified on the Chromatotron using 25% EtOAc in hexane producing 98 mg (76% yield) of the desired ester. HR-EIMS: $[M]^+$ at *m/z* 358.1553 ($C_{18}H_{24}O_5F_2$ requires 358.1591); IR ν_{max} cm^{-1}: 3610 (O–H), 1700 (C=O); ^1H NMR: δ7.77 (1H, *d*, *J* = 16.1 Hz, H-5), 6.11 (1H, *t*, *J* = 55 Hz, H-7′), 6.06 (1H, *d*, *J* = 16.1 Hz, H-4, overlapping *s*, 1H, H-3′), 5.70 (*s*, 1H, H-2), 3.93–4.04 (4H, *m*, OCH_2), 3.69 (3H, *s*, CO_2Me), 1.97 (3H, *s*, C-6, Me), 1.93 (1H, *d*, *J* = 14.5 Hz, H-5′), 1.82 (1H, *dd*, *J* = 16.1, *J′* = 1 Hz, H-5′), 1.08 (3H, *s*, C-8′, Me), 0.92 (3H, *s*, C-9′, Me). (Found: C, 60.54; H, 6.91. $C_{18}H_{24}O_5F_2$ requires: C, 60.33; H, 6.75).

Methyl 7′,7′-*difluoroabscisate* (**12**). To a soln of ketal **11** (78 mg, 0.22 mmol) dissolved in 5 ml Me_2CO was added pTsOH (0.1 eq, 4 mg). After stirring at room temp. for 6 hr, the Me_2CO was evapd and the residue taken up in satd aq. $NaHCO_3$ and extracted into Et_2O (3 × 30 ml). The combined Et_2O extracts were washed with satd NaCl soln, dried over Na_2SO_4 and concd. The crude product was purified on the Chromatotron using 25% EtOAc in hexane giving 63 mg (91% yield). Crystals, mp 105.0–105.5° (hexane–Et_2O); HR-EIMS: $[M]^+$ at *m/z* 314.1348 ($C_{16}H_{20}O_4F_2$ requires 314.1330); IR ν_{max} cm^{-1}: 3610 (O–H), 1700 (C=O); ^1H NMR: δ7.84 (1H, *d*, *J* = 16.1 Hz, H-5), 6.31 (1H, *t*, *J* = 54.3 Hz, H-7′), 6.34 (1H, *s*, H-3′), 6.11 (1H, *d*, *J* = 16.1 Hz, H-4), 5.75 (1H, *s*, H-2), 3.66 (3H, *s*, CO_2Me), 2.54 (1H, *s*, OH), 2.47 (1H, *d*, *J* = 7.1 Hz, H-5′), 2.38 (1H, *d*, *J* = 7.1 Hz, H-5′), 1.98 (3H, *d*, *J* = 1 Hz, C-6, Me), 1.10 (3H, *s*, C-8′, Me), 1.02 (3H, *s*, C-9′, Me).

The racemic mixture could be separated into its respective enantiomers on the Chiracel OD chiral HPLC column using 10% isopropanol and 90% hexane as eluent. The (+) enantiomer displayed a R_t of 9.77 min while the (−) enantiomer showed a longer R_t of 17.08 min at a flow rate of 3 ml min^{-1}. (+)-Methyl 7′,7′-difluoroabscisate showed the following properties: $[\alpha]_D^{20}$ (+) 286.3; mp 91–93°. (−)-Methyl 7′,7′-difluoroabscisate showed the following properties: $[\alpha]_D^{20}$ (−) 306.76; mp 90–91°.

(+) *and* (−)-7′,7′-*Difluoroabscisate* (**5**). To a soln of (+)-**12** (18.4 mg) in 1 ml MeOH was added 2 ml 2 M KOH. The mixture was stirred at room temp. for 2 hr, at which time it was diluted with 10 ml H_2O and washed with CH_2Cl_2 (3 × 20 ml). The organic layer was discarded and the aq. layer acidified with 1M HCl and then extracted into CH_2Cl_2 (3 × 20 ml). The combined organic extracts were washed with satd NaCl soln and dried over Na_2SO_4 to yield, after concn, 16.5 mg (95% yield) of (+)-7′,7′-difluoroabscisic acid. Crystals, mp 155–156° (hexane–Et_2O); $[\alpha]_D^{20}$ (+)-283.25; CIMS: *m/z* (rel. int.): 318 $[M+NH_4]^+$ (100), 300 $[M]^+$ (23); IR ν_{max} cm^{-1}: 3610 (O–H), 1680 (C=O); ^1H NMR: δ7.79 (1H, *d*, *J* = 16.2 Hz, H-5), 6.32 (1H, *t*, *J* = 55.6 Hz, H-7′), 6.37 (1H, *s*, H-3′), 6.14 (1H, *d*, *J* = 16.3 Hz, H-4), 5.79 (1H, *s*, H-2), 2.53 (1H, *d*, *J* = 17.2 Hz, H-5′), 2.41 (1H, *d*, *J* = 17.2 Hz, H-5′), 2.06 (1H, *d*, *J* = 1 Hz, C-6, Me), 1.14 (3H, *s*, C-8′, Me), 1.06 (3H, *s*, C-9′, Me).

(−)-7′,7′-Difluoroabscisic acid showed identical ^1H NMR, IR and MS as the (+)-isomer as well as the following properties: $[\alpha]_D^{20}$ (−)-296.19; mp 156–158°.

Seed germination assay and index. The effect of compound **5** on seed germination was determined using cress seeds (*Lepidium sativum* L.) imbibed at 25° in the dark. All experiments were replicated a minimum of three times, with 100 seeds per 9 cm Petri dish. The seeds were placed on two layers of Whatman #1 filter paper to which was added either 4 or 5 ml of soln. The effect of the compounds on germination was determined over a range of 0.01 to 100 μM and the time for both radicle and shoot emergence was noted every 3 or 6 hr or as otherwise noted. Root emergence is defined as radicles equal or greater than seed length exhibiting positive geotropism. Shoot emergence is defined as being when the cotyledonary leaves expand, sloughing the testa. The compounds were initially dissolved in Me_2CO and made up to vol. with a final Me_2CO concn of 0.2% for the 100 μM concn. In the serial dilutions the Me_2CO concn was kept constant at 0.2%.

A weighted germination index (GI) was calculated as per the method of ref. [15] which gives maximum weight to seeds that germinate first and less weight to those that germinate subsequently:

$$GI = \frac{(3 \times n_1 + 2.5 \times n_2 + \cdots t \times n_i)}{(\text{total days} \times \text{total seeds})},$$

where $n_1, n_2, \ldots n_i$ are the number of seeds that germinated on the first, second and subsequent time points, respectively; 3, 2.5, . . . *t* are the weights given to the number germinated on the first, second and subsequent time points, respectively (in this case, in days and fractions of days). The maximum GI is 1.

Freeze tolerance assay. Bromegrass (*Bromus inermis* Leyss.) cell suspension cultures were grown in 50 ml of 0.5 Ericksson's (ER) media (containing B_5 micronutrient and vitamin solutions) as previously described [6]. The fluorinated analogue and ABA were each dissolved in DMSO and made up to vol. with final DMSO concns of 0.2% for the 100 μM concn. In the serial dilutions the DMSO concn was kept constant at 0.2%. The cells were treated with varying concs (0.1–100 μM) of either compound **1** or **5**. Freezing resistance was measured as described in ref. [6] and the LT_{50} (50% killing temperature) values were

determined by the TTC (2,3,5-triphenyl tetrazolium chloride) reduction method [16]. Cell cultures were routinely screened for bacterial and fungal contamination by microscopy and plating on 2% YPD medium (2% glucose, 25% bactopeptone, and 1% yeast extract).

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