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Research Article

Preparation of deuterated abscisic acid metabolites for use in mass spectrometry and feeding studies

L. Irina Zaharia, Marek M. Galka, Stephen J. Ambrose and Suzanne R. Abrams*
Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, Sask., Canada S7N 0W9

Summary

Four deuterium-labelled ABA catabolites of high isotopic purity were prepared for hormone profiling studies using HPLC-ESI-MS-MS. The catabolites, namely [7′,7′,7′-d_3]-PA, [7′,7′,7′-d_3]-DPA, [5,8′,8′,8′-d_4]-7′-hydroxyABA and [4,5,8′,8′,8′-d_5]-ABA glucose ester were prepared either by biotransformation or chemical synthesis starting with labelled ABA. The labelled compounds are employed as internal standards for plant hormone profiling by liquid chromatography–electrospray–tandem mass spectrometry. Copyright © 2005 John Wiley & Sons, Ltd.

Key Words: abscisic acid; ABA; deuterium-labelled ABA metabolite; phaseic acid; dihydrophaseic acid; 7′-hydroxyABA; abscisic acid glucose ester

Introduction

The biological significance of the plant hormone abscisic acid (ABA) and its multiple roles in various aspects of plant life cycle has been widely documented.[1] In plants, during normal development, as well as in response to environmental changes, the levels of ABA in plant tissues are regulated by biosynthesis, transport and catabolism processes. ABA can be metabolized by oxidation, reduction or conjugation (Figure 1).[2,3] The major metabolism pathway of natural ABA occurs through oxidation of 8′-methyl group resulting in 8′-hydroxyABA that rearranges to phaseic acid (PA). Its further enzymatic reduction leads to dihydrophaseic acid (DPA). Other metabolic steps include hydroxylation of 7′-methyl group of the ABA ring (7′-OH ABA),[2,4] hydroxylation of 9′-methyl group,[5] or conjugation as glucose esters

*Correspondence to: Suzanne R. Abrams, Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, Sask., Canada S7N 0W9. E-mail: sue.abrams@nrc-cnrc.gc.ca

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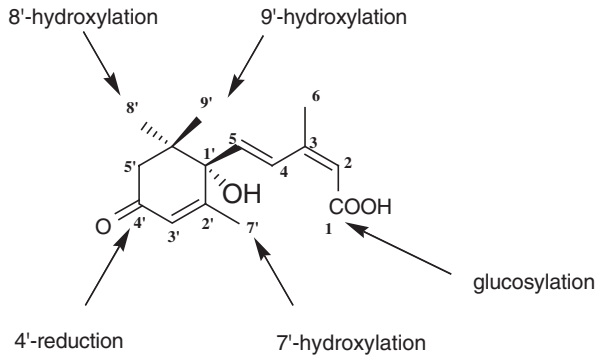


Figure 1. ABA metabolism sites

(ABAGE) or glucosides. While the activity of ABA is well documented, the activity of its metabolites has been less studied, due to the scarcity of the compounds. However, there are indications that the initial catabolites of ABA can have pronounced hormonal activity.[5–7]

To complement plant genomic studies on changes in gene expression and protein profiles, new analytical methods and internal standards are being developed for quantitation of ABA and catabolites involved in the regulation of important physiological processes in plants. While rigorous methods for measuring the ABA content in plant tissue have been developed, such methods for ABA catabolites quantitation are limited due to lack of internal standards. Early analytical methods employed to measure levels of ABA metabolites (PA, DPA, *epi*-DPA, ABAGE) consist of gas chromatography with electron capture detection (GC-ECD)[8,9] and GC coupled with mass spectrometry (GC-MS), methods which require partial purification and derivatization of the metabolites. The techniques used for quantitative determination of conjugated ABA are based on the analysis of ABA released by alkaline hydrolysis of the conjugate. However, Boyer and Zeevaart[10] developed a method in which ABAGE is measured by GC-ECD as its tetraacetate derivative. GC-MS with selected ion monitoring (GC-MS-SIM)[11] and multiple-ion monitoring (GC-MS-MIM) mode,[12] using isotopically labelled internal standards was frequently used for ABAGE quantification. Radioimmunoassays using monoclonal[13] and polyclonal[14,15] antibodies have also been used for quantitation of conjugated ABA.

Reversed-phase high performance liquid chromatography-tandem mass spectrometry (HPLC-MS-MS), suitable for polar compounds, is becoming widely used for the ABA metabolites analysis. Hogge *et al.*[16] reported the quantification of ABAGE using liquid chromatography–continuous flow secondary–ion mass spectrometry with reaction monitoring, while Schneider *et al.*[17] quantified ABAGE by liquid chromatography–electrospray ionization–

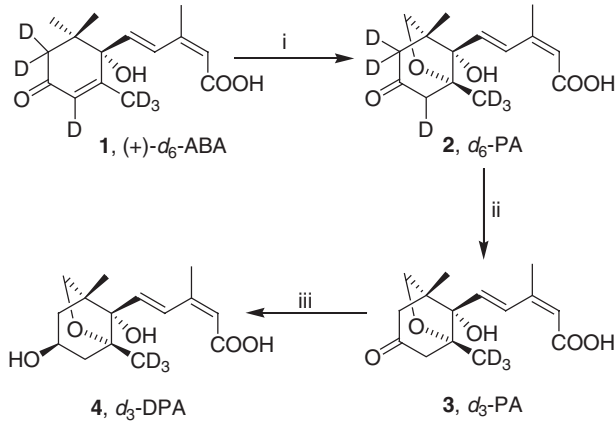
tandem mass spectrometry (LC-ESI-MS-MS). More recently, a method using LC-MS with multiple reaction monitoring (MRM) in negative ion ESI mode has been developed for plant hormone profiling including ABA metabolites.[5,18–20] This method requires deuterium-labelled internal standards of high isotopic purity, whose preparation is presented in this paper. The deuterium-labelled ABA metabolites have been prepared as biotransformation products of the labelled ABA[21] by maize cell suspension cultures.[22,23] The choice of deuterium-labelling at different positions of the molecule was designed such that these compounds, through MRM experiments, should exhibit specific diagnostic molecular ion-to-daughter ion transitions, distinct from those shown by their endogenous (non-labelled) counterparts found in the plant extracts. This was established upon extensive studies on fragmentation patterns for ABA metabolites deuterium-labelled at various positions, studies that will be presented elsewhere.

In this paper we report the preparation of specifically deuterated PA, DPA, 7′-OH ABA and ABAGE of high isotopic purity, to be used as internal standards for quantification of ABA metabolites by LC-ESI-MS-MS, to study the ABA metabolism in different plant systems.

Results and discussion

Deuterium-labelled PA and 7′-OH ABA were prepared through isolation of biotransformation products of the corresponding labelled ABA by maize cell suspension cultures, following a convenient procedure previously described.[22,24] High incorporation of label at key positions of the starting material was achieved through chemical synthesis,[21] suitable for large-scale preparation. The use of cell suspension culture had the advantage of providing a clean extract for PA or 7′-OH ABA isolation from the culture medium. Previous studies of ABA metabolism in maize cells showed that oxidation at the 8′ position was fast, while the enzymatic reduction at the 4′ position of PA to DPA was a slower process, which favoured the accumulation of PA.

Thus, the starting material for preparing [7′,7′,7′-d_3]-PA (**3**) was (+)-[3′,5′,5′,7′,7′,7′-d_6]-ABA (**1**) synthesized as previously described (Scheme 1).[25] Compound **1** was fed to the maize cells and incubated for 3 days. The main metabolism product [3′,5′,5′,7′,7′,7′-d_6]-PA (**2**) was isolated from the culture medium in 76% yield and further subjected to D-H exchange by treatment with an aqueous solution of NaOH. Preliminary MS results showed that after 2 h of treatment with NaOH, the isotopic distribution of the product was 84% d_3, 14% d_4 and 2% d_5, while after 6 h, the exchange of the more labile deuterium atoms at C-3′ and C-5′ was complete, as shown by ^{1}H-NMR and MS data. The isotopic distribution of the final product **3** showed 99% d_3 at C-7′ atom, as determined from the MS and ^{1}H-NMR. An alternate procedure,[26] reported only 86% deuterium incorporation for the same



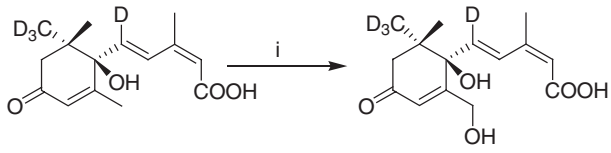
Scheme 1. (i) Maize (*Zea mays* L. cv Black Mexican Sweet) cell suspension culture; (ii) aq. NaOH, 6 h; (iii) $NaBH_4$, water–methanol, 5:1, v/v

compound d_3-PA (**3**). However, the procedure describes the introduction of deuterium atoms by treatment of d_0-PA with NaOD for 26 days, some of which will be exchanged back with the hydrogen atoms by treatment of the labelled PA with NaOH. The method reported here yields PA of higher isotopic purity, is more direct and due to the availability of labelled ABA used as starting material, it is suitable for large-scale preparation of deuterated PA.

Deuterium-labelled DPA was prepared using an improved method employed by Milborrow[27] for preparation of non-labelled epimeric methyl dihydrophaseates by reduction of methyl phaseate with $NaBH_4$, in a solvent mixture methanol–water, 1:2. A similar method[26] describing the reduction of the [7′,7′,7′-d_3]-PA methyl ester with $NaBH_4$ in methanol–water, 2:1, followed by basic hydrolysis to release the acid form reported a ratio of d_3-DPA (**4**) to d_3-*epi*-DPA of 1:2. We used the reduction of [7′,7′,7′-d_3]-PA (**3**) with $NaBH_4$ in a solvent mixture of methanol-water, 1:5 (v/v), affording [7′,7′,7′-d_3]-DPA (**4**) and [7′,7′,7′-d_3]-*epi*-DPA in a ratio of ca. 2:1. The deuterium incorporation in **4** was 100% d_3.

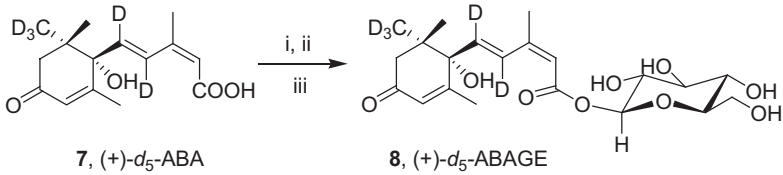
(-)-[5,8′,8′,8′-d_4]-7′-OH ABA (**6**) was prepared through the same method[22] by feeding (-)-[5,8′,8′,8′-d_4]-ABA (**5**)[21] to maize cell suspension culture (Scheme 2). The (-)-ABA enantiomer was preferred for feeding studies, since 7′-hydroxyABA is known to be the main metabolic product of the unnatural ABA in plants.[4] The natural hormone (+)-ABA is also metabolised to 7′-OH ABA, but as a minor product. The deuterium incorporation in **6** was 99% d_4.

Non-labelled and (+)-[4,5,8′,8′,8′-d_5]-ABAGE (**8**) were prepared using an improved synthesis to the previously described by Balsevich *et al.*,[28] as shown in Scheme 3. The labelled ABAGE is used not only as a standard, but also in



5, (-)-d_4-ABA **6**, d_4-7'OH ABA

Scheme 2. (i) Maize (*Zea mays* L. cv Black Mexican Sweet) cell suspension culture



7, (+)-d_5-ABA **8**, (+)-d_5-ABAGE

Scheme 3. (i) Cs_2CO_3; (ii) α-bromo-D-glucose tetraacetate, DMF; (iii) NH_3/MeOH

feeding studies, to determine if this ABA conjugate is a source of ABA *in planta*. Thus, the (+)-ABA glucose ester was prepared by reaction of the (+)-[4,5,8′,8′,8′-d_5]-ABA cesium salt with α-bromo-D-glucose tetraacetate,[29] followed by deacetylation of the intermediate with gaseous ammonia[30] instead of using a sunflower seeds enzyme preparation. The deuterium incorporation in **8** was 98% d_5.

Experimental section

All chemicals were purchased from Sigma-Aldrich Canada Ltd., Oakville, Ont., Canada. Solvents were HPLC grade and were used as such. (+)-[3′,5′,5′,7′,7′,7′-d_6]-ABA (**1**), (-)-[5,8′,8′,8′-d_4]-ABA (**5**) and (+)-[4,5,8′,8′,8′-d_5]-ABA (**7**) were prepared as previously published.[21,25]

Suspension cell cultures of maize (*Zea mays* L. cv Black Mexican Sweet) were maintained as previously described.[22]

Supelco Amberlite XAD-2 resin, silica gel, Merck, grade 60, mesh size 230-400, 60 Å or Aldrich reversed-phase octadecyl-functionalized silica gel (RP C-18) were used for column chromatography (FCC). Preparative thin layer chromatography (prepTLC) was carried out on silica gel EM Science, Kieselgel 60 F_{254} plates (20 × 20 cm × 0.25 mm).

High-resolution mass measurements were performed by fast atom bombardment (FAB) ionization mass spectrometry on a VG 70-VSE magnetic

sector instrument using a nitrobenzyl alcohol-polyethylene glycol matrix, or on a Q-ToF Ultima™ Global mass spectrometer (Waters-Micromass, Manchester, UK) fitted with a nano-lockspray™ interface and nanoflow ESI source. Data was acquired in the negative ion mode and analysed using MassLynx™ 4.0 software (Waters-Micromass). Source conditions included a cone voltage of −120 V, capillary voltage of −2.4 kV and a source temperature of 80°C.

The isotopic distribution of the deuterium-labelled compounds was calculated from the analysis of the full scan continuum data acquired by HPLC-ESI-MS in the negative ion mode. The chromatographic system consisted of an Agilent (Palo Alto, CA) HP1100 series HPLC equipped with a binary pump, autosampler and a Genesis C-18 column (4 μm particle size silica, 2.1 mm i.d. × 100 mm). The LC was connected to a quadrupole tandem mass spectrometer (Quattro LC) fitted with a Z-spray™ electrospray ion source (Micromass, Manchester, UK). The mobile phase consisted of solvents A (acetonitrile–0.07% glacial acetic acid in water, 90:10) and B (0.07% glacial acetic acid in water). The HPLC gradient started at 15% A, increasing linearly to 33% A at 10 min, then to 100% A at 16.7 min, at a flow rate of 0.2 ml/min. The instrument was operated using Mass Lynx™ v3.5 software, and the data processed using the Quantify™ program. Continuum data was acquired over a range of 20 atomic mass units (amu) about the expected isotopic masses, using a scanning cycle time of 0.25 s/scan (80 amu/s), an ES cone voltage of 35 V and the source temperature of 150°C.

Fourier transform infrared (FTIR) spectra were obtained on a Perkin Elmer Paragon 1000 spectrometer using a diffuse reflectance cell.

NMR spectra were obtained on a Bruker Avance DRX 500 MHz spectrometer (Bruker Biospin Ltd., Milton, Ont., Canada), equipped with a cryoprobe. For ^{1}H (500 MHz) δ values were referenced to $CDCl_3$ ($CHCl_3$ at 7.25 ppm) and CD_3OD (CHD_2OD at 3.30 ppm) and for ^{13}C (125.8 MHz) referenced to $CDCl_3$ (77.23 ppm) and CD_3OD (49.15 ppm). For ^{1}H-NMR, spin coupling constants (*J* values) were reported to the nearest 0.5 Hz.

Preparation of (-)-[7′,7′,7′-d_3]-phaseic acid (**3**)

Two litres of medium containing maize cells (as described in Reference[22]) were incubated with 100 mg (+)-[3′,5′,5′,7′,7′,7′-d_6]-ABA (**1**), so that the final concentration in the flask was 185 μM. After 3 days of shaking in the dark, at room temperature, the media was filtered, and the cells were crushed in lq. N_2 followed by extraction with *iso*-PrOH (500 ml). The medium (2 l) was acidified with glacial acetic acid, and then passed through an Amberlite XAD-2 resin column previously equilibrated. The column was eluted successively with 1% aqueous acetic acid (500 ml) and *iso*-PrOH (1 l). The *iso*-PrOH wash was

mixed with the *iso*-PrOH cell extract and the solvent was evaporated to give a crude extract (820 mg). This extract was redissolved in water (100 ml) and extracted with hexane (2 × 200 ml). The remaining aqueous portion was dried and further fractionated by RP C-18 FCC, using a gradient elution with water–CH_3CN. Fractions eluted with 15–100% CH_3CN were further fractionated by prepTLC (EtOAc–$CHCl_3$–glacial acetic acid, 100:50:5, v/v/v). The pure (-)-[3′,5′,5′,7′,7′,7′-d_6]-PA recovered (**2**, 79.3 mg, 76% yield) was redissolved in a mixture MeOH–water (2 ml), then a solution of 1 M NaOH (50 µl) was added drop wise. The mixture was left stirring, in the dark, at room temperature. After 6 h, the solution was acidified with 3 N HCl and extracted with EtOAc (3 × 5 ml). The organic layers were dried over Na_2SO_4 and evaporated to dryness to give (-)-[7′,7′,7′-d_3]-PA (**3**, 72.1 mg, 91% yield).

^{1}H-NMR (CD_3OD): δ 8.09 (d, J = 16 Hz, 1 H,), 6.45 (d, J = 16 Hz, 1 H), 5.78 (s, 1 H), 3.93 (dd, J = 7.5, 3.0 Hz, 1 H), 3.66 (d, J = 7.5 Hz, 1 H), 2.79 (d, J = 18 Hz, 1 H), 2.70 (dd, J = 18, 2.5 Hz, 1 H), 2.46 (dd, J = 18, 2.5 Hz, 1 H), 2.38 (dd, J = 18, 2.5 Hz, 1 H), 2.06 (d, J = 1 Hz, 3 H), 1.00 (s, 3 H).

^{13}C-NMR (CD_3OD): δ 210.9 (s), 169.5 (s), 151.2 (s), 133.6 (d), 132.9 (d), 119.8 (d), 87.8 (s), 82.9 (s), 78.5 (t), 53.9 (t), 53.2 (t), 49.6 (s), 21.2 (q), 18.6 (m), 15.7 (q).

Optical rotation $[\alpha]_D^{25}$ –30 (*c* 0.4, MeOH).

HRMS-TOF-ES$^-$ m/z measured 282.1421 (282.1426 calculated for $C_{15}H_{16}D_3O_5$ $[M\text{-}H]^-$).

LC-ES-MS: 281 (1.3%), 282 ($[M\text{-}H]^-$, 82.7%), 283 (13.8%), 284 (1.9%); isotopic distribution: 99% d_3, 1% d_2.

LC-ESI-MS-MS: 282 ($[M\text{-}H]^-$), 208, 171, 142.

FT-IR ν_{max}: 3443, 2928, 1709, 1676, 1600, 1279, 1254 cm^{-1}.

Preparation of (-)-[7′,7′,7′-d_3]-dihydrophaseic acid (**4**)

$NaBH_4$ (22.7 mg, 0.6 mmol) was added to a stirred solution of [7′,7′,7′-d_3]-PA (**3**, 16.2 mg, 0.06 mmol) in H_2O–MeOH, 5:1 (v/v, 1 ml) kept in an ice bath. After stirring for 30 min at room temperature, the reaction mixture was acidified with 3 N HCl, and then extracted with EtOAc (3 × 5 ml). The organic phase was dried over Na_2SO_4 and evaporated to dryness to give 18.3 mg crude residue. Separation by prepTLC (CH_2Cl_2–CH_3OH–AcOH, 95:5:2) gave (-)-[7′,7′,7′-d_3]-DPA (**4**, 9.8 mg, 57%).

^{1}H-NMR (CD_3OD): δ 7.86 (d, J = 16.0 Hz, 1 H), 6.40 (d, J = 16 Hz, 1 H), 5.79 (s, 1 H), 4.09 (m, 1 H), 3.79 (dd, J = 2, 7.5 Hz, 1 H), 3.69 (d, J = 7.5 Hz, 1 H), 2.02 (s, 3 H), 2.01 (m, 1 H), 1.83 (ddd, J = 13.5, 7, 1.5 Hz, 1 H), 1.72 (dd, J = 10.5, 13.5 Hz, 1 H), 1.65 (ddd, J = 13.5, 11, 2 Hz, 1 H), 0.92 (s, 3 H).

^{13}C-NMR (CD_3OD): δ 172.0 (s), 147.2 (s), 133.2 (d), 132.3 (d), 122.9 (d), 87.7 (s), 83.2 (s), 77.3 (t), 66.1 (d), 49.5 (s), 45.9 (t), 44.5 (t), 20.9 (q), 18.9 (m), 16.4 (q).

Optical rotation $[\alpha]_D^{25}$– 12 (*c* 0.9, MeOH).

HRMS-TOF-ES$^-$ *m/z* measured 284.1575 (284.1582 calculated for $C_{15}H_{18}D_3O_5$ [M-H]$^-$).

LC-ES-MS: 284 ([M-H]$^-$, 83.4%), 285 (14.4%), 286 (2.2%); isotopic distribution: 100% d_3.

LC-ESI-MS-MS: 284 ([M-H]$^-$), 240, 204, 192, 174, 126.

FT-IR ν_{max}: 3390, 2928,1686, 1560, 1406, 1244, 1182, 994 cm^{-1}.

Preparation of (-)-[5,8′,8′,8′-d_4]-7′-hydroxy abscisic acid (**6**)

(-)-[5,8′,8′,8′-d_4]-7′OH ABA (**6**) was obtained from the biotransformation of (-)-[5,8′,8′,8′-d_4]-ABA (**5**) by maize cell suspension cultures, following a similar procedure (feeding and purification) as described above for **3**. Thus, from feeding studies using **5** (49.3 mg), 7.9 mg of (-)-[5,8′,8′,8′-d_4]-7′OH ABA (**6,** 23% yield) were isolated, with the recovery of 15 mg starting material **5**.

^{1}H-NMR ($CDCl_3$): δ 7.62 (s, 1 H), 6.19 (s, 1 H), 5.72 (s, 1 H), 4.44 (d, J = 16.5 Hz, 1 H), 4.24 (d, J = 16.5 Hz, 1 H), 2.41 (d, J = 17 Hz, 1 H), 2.25 (d, J = 17 Hz, 1 H), 1.99 (s, 3 H), 1.01 (s, 3 H).

^{13}C-NMR ($CDCl_3$): δ 199.2 (s), 170.4 (s), 163.3 (s), 150.8 (s), 135.9 (m), 128.7 (d), 124.6 (d), 118.8 (d), 79.6 (s), 62.3 (t), 49.4 (t), 41.8 (s), 23.7 (q), 22.0 (m), 21.3 (q).

Optical rotation $[\alpha]_D^{25}$ –326 (*c* 0.7, MeOH).

HRMS-TOF-ES$^-$ *m/z* measured 283.1483 (283.1489 calculated for $C_{15}H_{15}D_4O_5$ [M-H]$^-$).

LC-ES-MS: 282 (0.8%), 283 ([M-H]$^-$, 83.3%), 284 (13.8%), 285 (1.9%); isotopic distribution: 99% d_4, 1% d_3.

LC-ESI-MS-MS: 283 ([M-H]$^-$), 221, 209, 203, 171, 154.

FT-IR ν_{max}: 3380, 2963, 1660, 1248, 1063 cm^{-1}.

*Preparation of (+)-[4,5,8′,8′,8′-d_5]- ABA-1-O-β-*D*-glucopyranoside* (**8**)

An aqueous solution (5 ml) of Cs_2CO_3 (60.5 mg, 0.2 mmol) was added to a solution of (+)-[4,5,8′,8′,8′-d_5]-ABA (**7**, 50 mg, 0.2 mmol) in MeOH (5 ml). After stirring at room temperature for 2 h, the reaction mixture was evaporated to dryness. To the solution of the cesium salt in DMF (5 ml), α-bromo-D-glucose tetraacetate (114 mg, 0.3 mmol) was added. After refluxing for 1 h at 60°C, the mixture was diluted with water (5 ml) and extracted with EtOAc (3 × 10 ml). The combined organic extracts were washed successively with 0.1 M NaOH (2 × 10 ml), water (2 × 10 ml) and brine (2 × 10 ml), and then were dried over Na_2SO_4 and concentrated under reduced pressure. The resulting extract was fractionated by prepTLC (EtOAc–hexanes, 1:1, v/v) to give (+)-[4,5,8′,8′,8′-d_5]-ABA β-D-glucosyl tetraacetate ester (63 mg, 57%). Through a solution of (+)-[4,5,8′,8′,8′-d_5]-ABA β-D-glucosyl tetraacetate ester

(50 mg, 0.1 mmol) in MeOH (5 ml), kept in an ice bath, gaseous ammonia was bubbled. After 1 h, the solvent was evaporated and the mixture was fractionated by prepTLC (CH_2Cl_2–MeOH–AcOH, 90:10:5, v/v/v) to give (+)-[4,5,8′,8′,8′-d_5]-ABA β-D-glucose ester (**8**, 10.5 mg, 29%).

^{1}H-NMR (CD_3OD): δ 5.92 (s, 1 H), 5.81 (s, 1 H) 5.49 (d, $J = 8$ Hz, 1 H), 3.84 (dd, $J = 8$, 3 Hz, 1 H), 3.66 (dd, $J = 17$, 5 Hz, 1 H), 3.35 (m, 4 H), 2.52 (d, $J = 17$ Hz, 1 H), 2.18 (d, $J = 17$ Hz, 1 H), 2.06 (s, 3 H), 1.92 (s, 3 H), 1.02 (s, 3 H).

^{13}C-NMR (CD_3OD): δ 200.9 (s), 166.3 (s), 165.8 (s), 153.5 (d), 127.7 (d), 118.1 (s), 95.4 (d), 94.0 (s), 80.5 (s), 78.8 (d), 78.0 (d), 73.9 (d), 71.1 (d), 62.3 (d), 50.6 (t), 42.6 (s), 24.6 (q), 21.3 (q), 19.6 (q).

Optical rotation $[\alpha]_D^{25} + 200$ (*c* 0.2, MeOH).

HRMS-FAB$^+$ *m/z* measured 432.2283 (432.2282 calculated for $C_{21}H_{25}D_5O_9$ $[M+H]^+$).

LC-ES-MS: 426 (0.2%), 429 (1.3%), 430 ($[M-H]^-$, 97.9%), 433 (0.5%); isotopic distribution: 98% d_5, 1% d_4.

LC-ESI-MS-MS: 430 ($[M-H]^-$), 310, 268, 156.

FT-IR ν_{max}: 3364, 1718, 1654, 1071 cm^{-1}.

Conclusions

Thus, we report the preparation of deuterium-labelled ABA metabolites of high purity and high isotopic incorporation at specific positions, suitable to be used as internal standards in the hormone metabolic profiling by LC-ES-MS-MS. The standards are being employed in on going research on ABA metabolism in seed development and germination, namely lettuce seeds,[18] western white pine seeds[19] and *Brassica napus* siliques,[5] as well as on the role of ABA in controlling developmental switching in *Marsilea quadrifolia*.[31–33]

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