Research Article

Journal of Labelled Compounds and Radiopharmaceuticals

Received 29 January 2010, Revised 16 April 2010, Accepted 22 April 2010 Published online 5 August 2010 in Wiley Online Library

(wileyonlinelibrary.com) DOI: 10.1002/jlcr.1789

Preparation of multideuterated 5-deoxystrigol for use as an internal standard for quantitative LC/MS

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We prepared deuterium-labeled 5-deoxystrigol (5DS), a multifunctional plant second metabolite, using a simple set of procedures. The labeled compound contained mainly [3a,4,4,5,5,6′-D_6]-5DS and [D_5]-5DS and did not contain unlabeled 5DS. Therefore, multideuterated 5DS can be used as an internal standard in liquid chromatography/mass spectrometry (LC/MS) assays to quantitate the amounts of 5DS in plants and root exudates.

Keywords: strigolactone; multideuterated 5-deoxystrigol

Introduction

Strigolactones are multifunctional plant secondary metabolites that induce seed germination in the Orobanchaceae family of parasitic weeds,[1,2] trigger hyphal branching of arbuscular mycorrhizal (AM) fungi,[3] and inhibit shoot branching in a variety of plants.[4,5] The structural core of strigolactones consists of a carotenoid-derived tricyclic-lactone (the ABC-ring) and a butyrolactone group (the D-ring) that are connected via an enol ether bridge.[2,6] Many different kinds of strigolactones, chemically substituted at the A- and/or B-rings, have been isolated from root exudates of many different mono- and dicotyledonous plants.[6] The structurally simplest strigolactone is 5-deoxystrigol (**1**), which has geminal methyl groups at C-8 and no other A- and B-ring substituents (Figure 1). Therefore, **1** may be the precursor of other strigolactones with hydroxylated, acetylated, demethylated, and/or aromatized A- and/or B-rings.[1,6]

Strigolactones are released into the soil as a signal for symbiosis with AM fungi that help plants absorb essential minerals.[3] Strigolactone production and the relative amounts and types in a population differ among plant species.[6] Moreover, the strigolactone composition in root exudates is modulated greatly by the concentrations of soil nutrients, such as inorganic phosphate and nitrogen.[7] To understand how the relative amounts and types of various strigolactones are affected by the environment and to characterize their biosynthetic/catabolic pathways, an accurate, precise and reliable tool is needed that can identify and quantitate them in plant extracts. Liquid chromatography-tandem mass spectrometry (LC/MS-MS) with natural or synthetic standards has been used to identify and quantitate strigolactones found in the root exudates.[7] However, quantitative analysis of **1** is difficult without using an internal standard because the amount of **1** in plants is small and **1** is readily hydrolyzed to the enol of the tricyclic lactone and to the hydroxyl furanone in aqueous solution.[8] Umehara and colleagues quantitated the 2′-*epi*-**1** content in the root exudates and the roots of rice using [6′-D]-*epi*-**1** as a LC/MS-MS internal standard.[5] Although this monodeuterated **1** is easily and economically prepared, a tandem mass spectrometer is required to quantitate samples containing [6′-D]-*epi*-**1** because MS signals of the unlabeled and monodeuterated *epi*-**1** tend to overlap. Therefore, we developed, and describe herein, a simple method to synthesize multideuterated **1** that can be used as an internal standard for the accurate quantitative analysis of **1** using not only LC/MS-MS, but also gas chromatography and LC/MS.

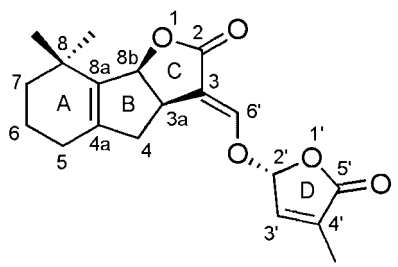

Figure 1. The structure of **1**.

Results and discussion

Multideuterated **1** was prepared referring to published synthetic procedures,[9–11] as follows (Figure 2). Potassium hydroxide and 2,2-dimethylcyclohexanone were added to a solution of *ca* 50% (w/w) propargyl alcohol in H_2O, and the mixture was stirred overnight at 45°C[12] to give the diol (**2**) in 91% yield. Compound **2** was cyclized and alkylated to give **3**, which was converted to

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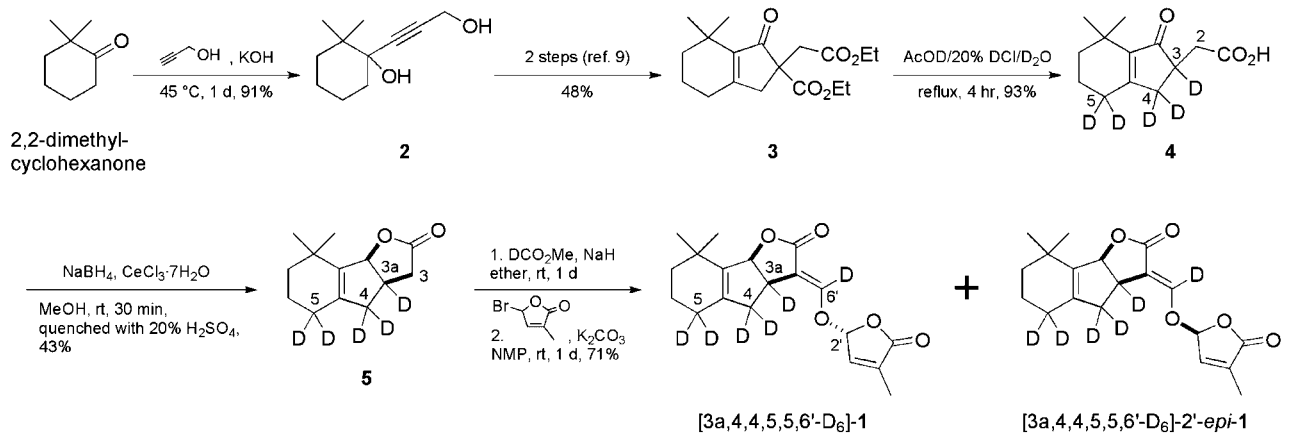

Figure 2. Synthesis of multideuterated **1**. Multideuterated **1** and **5** were found to be epimeric racemic mixtures.

the multideuterated oxo acid (**4**) by refluxing in AcOD (98 atom% D) and 20% DCl in D_2O (99+atom% D) for 4 hours. Compound **4** was then reduced to yield the multideuterated lactone (**5**).[10] Formylation of **5** with DCO_2Me (98+atom% D) and sodium hydride, and subsequent reaction with 5-bromo-3-methyl-2(5*H*)-furanone[9] gave racemic multideuterated **1** and its corresponding multideuterated 2′-epimer.[11] The epimeric mixtures could be separated by silica gel column chromatography.

The ^{1}H NMR spectra of multideuterated **1** and the 2′-epimer each exhibited a singlet signal at C-8b (5.51 ppm); the proton signal intensities of the C-6′ (7.46 ppm), C-3a (3.60 ppm), C-4 (2.70 and 2.31 ppm), and C-5 (1.90 ppm) were considerably reduced compared to unlabeled **1**.[13] In the positive-ion ESI LC/TOF-MS spectra of multideuterated **1** and its 2′-epimer, the ratio of the $[M+H]^+$ peak areas for the ions at *m/z* 337 (hexadeuterated), 336 (pentadeuterated), and 335 (tetradeuterated) was 100:68:18 and 100:70:23, respectively (Figure 3 and Table 1). On the other hand, the $[M+H]^+$ ions at *m/z* 331 of unlabeled compound were not detected in multideuterated **1** and *epi*-**1** (Table 1). Therefore, multideuterated **1** consists mainly of [3a,4,4,5,5,6′-D_6]-**1** and [D_5]-**1**. This result suggests that multideuterated **1** can be used as an internal MS standard.

To confirm the effect that the reflux time had on deuterium incorporation, we refluxed **3** in AcOD/DCl/D_2O for 2, 4, or 6 hr and subsequently reduced **4** to give **5**. The deuterium content of **5** was measured by integrating the signals of its ^{1}H NMR spectrum. The C-3a and C-5 carbons were almost completely deuterated after 2 hr; whereas, an additional 2 hr was needed to extensively deuterate the C-4 carbon (Figure 4). Therefore, the deuteriums were readily introduced at C-3a, C-4, and C-5 carbons of compound **3**.

We next examined the stabilities of **4** and **5** in aqueous solution. The compounds were dissolved in water and left for 8 days in the shade. Little hydrogen-deuterium back-exchange occurred in water and 1 mM aqueous NaOH solution at room temperature (Table 2). This results show good stability of label at neutral and basic pH.

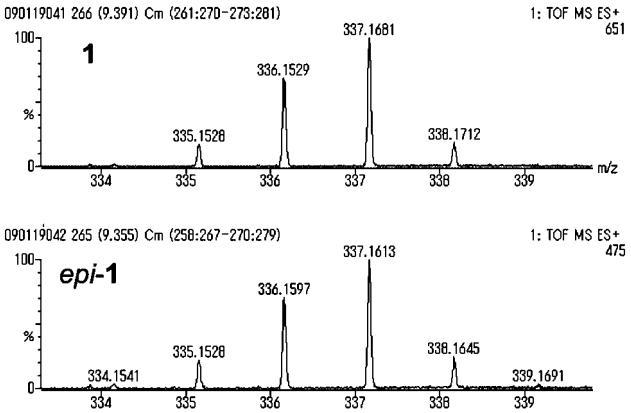

Figure 3. TOF-MS spectra of the multideuterated **1** and *epi*-**1**.

Table 1. TOF-MS absolute (and relative (%)) peak areas for the parent ions of the components of the multideuterated **1** and its corresponding 2′-epimer (*epi*-**1**).

Ion (*m/z*)	**1**	*epi*-**1**
331	not detected	not detected
334	3.3 (0.9%)	4.7 (1.7%)
335	29.3 (8.3%)	28.3 (10.4%)
336	112.4 (31.8%)	84.8 (31.2%)
337	166.4 (47.1%)	120.5 (44.3%)
338	41.8 (11.8%)	33.5 (12.3%)

Experimental

^{1}H NMR spectra were measured on a Jeol JNM-A500 (500 MHz) with tetramethylsilane as internal references. The chemicals, dehydrated solvents and reagents were purchased from Aldrich, Wako, and Kanto Chemical and Tokyo Chemical Industry. Reactions were monitored by TLC using silica gel plate (Silica gel 60 F_{254}, Merck) and hexane and EtOAc as mobile phase. Column chromatography was performed on silica gel (Wakogel C-200). LC/MS-MS analyses were carried out on a system of a quadruple/time-of-flight tandem mass spectrometer (Q-Tof premier, Waters) and an Acquity Ultra Performance liquid chromatograph (Waters) equipped with a reverse-phase column (Acquity UPLC BEH-C18, 2.1 × 50 mm, 1.7 μm; Waters).

1-(3-Hydroxyprop-1-ynyl)-2,2-dimethylcyclohexanol (2)

Potassium hydroxide (8.7 g, 0.16 mol) was added to a solution of 2-propyn-1-ol (3.5 g, 63 mmol) in H_2O (3.3 g) with stirring and warmed up to 45°C.[12] 2,2-dimethylcyclohexanone (5.1 g,

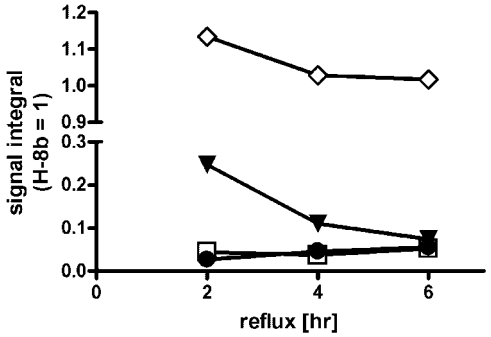

Figure 4. The relative levels of deuterium incorporation at the C-3a, C-4, and C-5 positions of **5** as a function of reflux time. Compound **3** was refluxed in AcOD/DCl/D_2O for 2, 4 or 6 hr to give **4**, which was subsequently reduced to give **5**. The plot displays the relative hydrogen content (obtained by integrating the peak area and normalizing it to that of the C-8b hydrogen) at the C-3a carbon, open squares; the C-4 carbon (2.6 ppm), filled triangles; the C-5 carbon, filled circles; and the C-3 carbon (2.3 ppm), open diamond of **5** versus reflux time. The C-3 carbon was not deuterated.

Table 2. Integrated ^{1}H NMR signal intensities for the spectra of **5** and **4** just after the samples were dissolved in water and eight days later.

Position in **5**	before[a,b]	after[a,b]	
3a (1H)	0.0573	0.0678	
4 (δ 2.6, 1H)	0.0722	0.0496	
4 (δ 2.1, 1H)	0.0896	0.2339[c]	
5 (2H)	0.1441	0.1668	
Position in **4**	Before[d,e]	After[d,e]	NaOH[d,f]
3 (1H)	0.0563	0.0525	0.0536
4 (δ 2.7, 1H)	0.0560	0.0455	0.0535
4 (δ 2.2, 1H)	0.0685	0.0704	0.0628
5 (2H)	0.1145	0.1068	0.1135

[a]The compounds (*ca* 5 mg each) were first dissolved in small amounts of MeOH, and then H_2O (50 ml) was added. The ^{1}H-NMR spectrum of the sample labeled 'before' was taken immediately after the sample was dissolved in water; whereas, the ^{1}H-NMR spectrum of the sample labeled 'after' was taken eight days later.
[b]The values were normalized to a signal intensity of 1 for H-8b.
[c]The increased signal intensity may be attributable to the appearance of a new compound with a ^{1}H signal at 2.1 ppm.
[d]The samples were treated as described above.
[e]The values were normalized to a signal intensity of 1 for H-2b.
[f]After dissolving compound **4** in water (50 ml), 1 M NaOH (50 μl) was added.

40 mmol) was added to the mixture and stirred overnight at 40°C. H_2O (70 ml) was added to the resulting mixture and the solution was extracted with EtOAc (15 ml × 5). The organic layer was washed with saturated NH_4Cl, brine and H_2O, dried over Na_2SO_4 and concentrated. The residual oil was purified by column chromatography with 10–40% EtOAc in hexane to give **2** (6.6 g, 36 mmol, 91% yield) as a white solid. The ^{1}H NMR spectrum of **2** was consistent with the previous report.[9]

Ethyl 2-(2-ethoxy-2-oxoethyl)-7,7-dimethyl-1-oxo-2,3,4,5,6,7-hexahydro-1H-indene-2-carboxylate (3)

The diester **3** (0.45 g, 1.4 mmol, 48% yield) was prepared from **2** (0.48 g, 2.9 mmol) by the literature procedure.[9]

2-(2,3,3,4,4-Pentadeutero-7,7-dimethyl-1-oxo-2,3,4,5,6,7-hexahydro-1H-inden-2-yl)acetic acid (4)

The diester **3** (0.45 g, 1.4 mmol) was dissolved in AcOD (98 atom % D, 1 ml, purchased from ACROS), and 20% DCl in D_2O (99+ atom % D, 1 ml, ACROS) was added to the solution. The mixture was refluxed for 4 hr and diluted with H_2O (50 ml), which was extracted with EtOAc (12 ml × 5). The organic layer was washed with brine and H_2O, dried over Na_2SO_4 and concentrated. The residual oil was purified by column chromatography with 30% EtOAc in hexane to give **4** (0.29 g, 1.3 mmol, 93% yield) as a yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.16 and 1.17 (each 3H, s, Me_2-7), 1.48 (2H, m H_2-6), 1.70 (2H, m, H_2-5), 2.39 and 2.83 (each 1H, d, J = 17.0 Hz, CH_2CO_2H).

3a,4,4,5,5-Pentadeutero-8,8-dimethyl-3,3a,4,5,6,7,8,8b-octahydro-2H-indeno[1,2-b]furan-2-one (5)

$CeCl_3 \cdot 7H_2O$ (0.98 g, 2.6 mmol) was added to a solution of **4** (0.28 g, 1.2 mmol) in MeOH (20 ml) and dissolved with stirring. $NaBH_4$ (0.30 g, 7.9 mmol) was carefully added to the mixture at 0°C, and stirred at room temperature. After 1 hr, $NaBH_4$ (*ca* 0.3 g) was added to the mixture to reduce the unreacted **4**, which was stirred for 30 min. The resulting mixture was acidified with 20% H_2SO_4 (4 ml) and diluted with H_2O (150 ml). The solution was extracted with EtOAc (18 ml × 6). The organic layer was washed with saturated $NaHCO_3$ (to remove the unreacted **4**) and H_2O, dried over Na_2SO_4 and concentrated. The residual oil was purified by column chromatography with 15% EtOAc in hexane to give the lactone **5** (0.11 g, 0.54 mmol, 43% yield) as a pale yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.08 and 1.10 (each 3H, s, Me_2-8), 1.37 and 1.47 (each 1H, m, H_2-7), 1.66 (2H, m, H_2-6), 2.32 and 2.79 (each 1H, d, J = 18.3 Hz, H_2-3), 5.47 (1H, s, H-8b).

(3a*R**,8b*S**,2′*R**)-3a,4,4,5,5.6′-hexadeutero-5-deoxystrigol and its 2′-epimer

DCO_2Me (98+atom % D, 0.3 ml, ACROS) was added to a solution of **5** (0.11 g, 0.50 mmol) in dry ether (2.5 ml), and sodium hydride (40 mg) was then added to the mixture at room temperature. The mixture was stirred at 15 hr at same temperature, and the reaction was quenched with 1 M HCl (3 ml). The solution was diluted to 30 ml with H_2O and extracted with EtOAc (10 ml × 5). The organic layer was washed with H_2O, dried over Na_2SO_4 and concentrated. The residual yellow oil was dissolved in *N*-methyl-2-pyrrolidinone (1 ml). Potassium carbonate (0.14 g, 1.0 mmol) and then 5-bromo-3-methyl-2(5*H*)-furanone[9] (0.20 g, 1.1 mmol) were added to the solution, which was stirred for 1 d at room temperature. The resulting mixture was poured into 1 M HCl (10 ml), diluted with H_2O (20 ml) and extracted with EtOAc (6 ml × 5). The organic layer was washed with H_2O, dried over Na_2SO_4 and concentrated. The residual oil was purified by column chromatography with 25–30% EtOAc in hexane to give multideuterated **1** (71 mg, 0.21 mmol, 42% yield), its 2′-epimer (48 mg, 0.14 mmol, 29% yield), and mixture of these diastereomers (45 mg). Multideuterated **1**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.10 and 1.12 (each 3H, s, Me_2-8), 1.34-1.39 and 1.45–1.50 (each 1H, m, H_2-7), 1.62–1.68 (2H, m, H_2-6), 2.03 (3H, m, Me-4′), 5.51 (1H, s, H-8b), 6.15 (1H, m, H-2′), 6.93 (1H, m, H-3′). Epimer of multideuterated **1**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.09 and 1.11 (each 3H, s, Me_2-8), 1.33–1.38 and 1.45–1.50 (each 1H, m, H_2-7), 1.60–1.70 (2H, m, H_2-6), 2.03 (3H, m, Me-4′), 5.52 (1H, s, H-8b), 6.15 (1H, m, H-2′), 6.94 (1H, m, H-3′).

Hydrogen-deuterium exchange timecourse experiment

Compound **3** (45.8 mg) was dissolved in AcOD (98 atom % D, 0.5 ml), and 20% DCl in D_2O (99+atom % D, 0.5 ml) was added to the solution. The mixture was refluxed at 135°C and *ca* 0.3 ml of the resulting mixture was taken every 2 hr; the reflux was finally performed for 6 hr. The mixture was then worked-up and the product was reduced with $NaBH_4$ as described above to give compound **5**.

Deuterium stability experiments

The compounds (*ca* 4–5 mg) were dissolved in small amount of MeOH, and then H_2O (50 ml) was added. The solution was left for 8 days at room temperature in the shade. The compounds were extracted with EtOAc, and hydrogen-deuterium exchange was measured by integrating the signals of the ^1H NMR spectra.

Conclusion

We developed a straightforward procedure for synthesis multi-deuterated **1** that primarily contains [3a,4,4,5,5,6′-D_6]-**1** and [D_5]-**1**. Although the MS spectrum of the labeled compound shows the isotope cluster, multideuterated **1** does not contain unlabeled **1**. This finding shows that multideuterated **1** can be used as an internal standard for LC/MS analyses of **1**. We plan to use the labeled **1** as an internal MS standard for the identification and quantitation of **1** found in plants and root exudates.

Acknowledgements

This work was supported by the Program for Promotion of Basic Research Activities for Innovative Bioscience (PROBRAIN) of Bio-oriented Technology Research Advancement Institution, Tokyo, Japan.

References

[1] J. A. López-Ráez, R. Matusova, C. Cardoso, M. Jamil, T. Charnikhova, W. Kohlen, C. Ruyter-Spira, F. Verstappen, H. Bouwmeester, *Pest Manag. Sci.* **2009**, *65*, 471.
[2] B. Zwanenburg, A. S. Mwakaboko, A. Reizelman, G. Anilkumar, D. Sethumadhavan, *Pest Manag. Sci.* **2009**, *65*, 478.
[3] K. Akiyama, H. Hayashi, *Ann. Bot*. **2006**, *7*, 925.
[4] V. Gomez-Roldan, S. Fermas, P. B. Brewer, V. Puech-Pagès, E. A. Dun, J. P. Pillot, F. Letisse, R. Matusova, S. Danoun, J. C. Portais, H. Bouwmeester, G. Bécard, C. A. Beveridge, C. Rameau, S. F. Rochange, *Nature* **2008**, *455*, 189.
[5] M. Umehara, A. Hanada, S. Yoshida, K. Akiyama, T. Arite, N. Takeda-Kamiya, H. Magome, Y. Kamiya, K. Shirasu, K. Yoneyama, J. Kyozuka, S. Yamaguchi, *Nature* **2008**, *455*, 195.
[6] K. Yoneyama, X. Xie, K. Yoneyama, Y. Takeuchi, *Pest Manag. Sci.* **2009**, *65*, 467.
[7] K. Yoneyama, X. Xie, H. Sekimoto, Y. Takeuchi, S. Ogasawara, K. Akiyama, H. Hayashi, K. Yoneyama, *New Phytol.* **2008**, *179*, 484.
[8] K. Akiyama, S. Ito, H. Hayashi, *Reg. Plant Growth & Dev*. **2008**, *43*(Suppl.), 23.
[9] G. A. MacAlpine, R. A. Raphaerl, A. Shaw, A. W. Taylor, H.-J. Wild, *J. Chem. Soc. Perkin 1*, **1976**, 410.
[10] J. W. J. F. Thuring, N. W. J. T. Heinsman, R. W. A. W. M. Jacobs, G. H. L. Nefkens, B. Zwanenburg, *J. Agric. Food Chem.* **1997**, *45*, 507.
[11] J. Matsui, M. Bando, M. Kido, Y. Takeuchi, K. Mori, *Eur. J. Org. Chem*. **1999**, 2183.
[12] T. Hiyama, M. Shinoda, H. Saimoto, H. Nozaki, *Bull. Chem. Soc. Jpn*. **1981**, *54*, 2747.
[13] K. Frischmuth, E. Samson,A. Kranz, P. Welzel, H. Meuer, W. S. Shelderick, *Tetrahedron* **1991**, *47*, 9793.