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Ent-2′-*epi*-Orobanchol and Its Acetate, As Germination Stimulants for *Striga gesnerioides* Seeds Isolated from Cowpea and Red Clover

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ABSTRACT: *Striga gesnerioides* is a root parasitic weed of economic significance to cowpea (*Vigna unguiculata*) crops in Western Africa. Seeds of the parasite germinate in response to cowpea root exudates. Germination stimulants for the seeds were isolated from the hydroponic culture filtrate of cowpea, and their structures were unambiguously determined as (−)-(3a*R*,4*R*,8b*R*,2′*R*)-*ent*-2′-*epi*-orobanchol and (+)-(3a*R*,4*R*,8b*R*,2′*R*)-*ent*-2′-*epi*-orobanchyl acetate, on the basis of mass, CD, and ^{1}H NMR spectra; optical rotatory power; and chromatographic behavior on HPLC. The alcohol was first isolated and identified from the cowpea root exudates, and the acetate may be the same compound that had been previously isolated from the exudates and designated as alectrol. Identity of the stimulants produced by cowpea to those produced by red clover (*Trifolium pratense*) was confirmed.

KEYWORDS: *Striga gesnerioides*, *Vigna unguiculata*, *ent*-2′-*epi*-orobanchol, *ent*-2′-*epi*-orobanchyl acetate, *Trifolium pratense*, strigolactone

INTRODUCTION

The root parasitic weeds *Striga*, *Orobanche*, and *Alectra* in the family Orobanchaceae only germinate when they are exposed to stimulants produced and released into the soil from the roots of host and some nonhost plants.[1,2] Most of the germination stimulants isolated thus far (Figure 1) possess the same basic skeleton and are collectively referred to as strigolactones, as first proposed by Butler.[3] The structural core of strigolactones is a tricyclic lactone (ABC ring) connected through an enol ether linkage to an α,β-unsatulated furanone moiety (D ring). Naturally occurring strigolactones include strigol (**1**),[4] sorgolactone (**2**),[5] alectrol,[6] orobanchol,[7] deoxystrigol (**3**),[8,9] fabacyl acetate (**4**),[10] and solanacol (**5**).[11,12] The original structure proposed for solanacol was incorrect.[11] The methyl groups in the A-ring are positioned ortho instead of para,[13] and the relative configuration at C2′ was the opposite.[12] Most of the naturally occurring strigolactones can be stereochemically divided into two groups: typical strigolactones and *ent*-2′-*epi*-strigolactones. The former, such as compounds **1**−**3**, have the (3a*R*,8b*S*)-configuration in the C ring and the (2′*R*)-configuration in the D ring (Figure 1), while the latter, such as compounds **4** and **5**, have the (3a*R*,8b*R*,2′*R*)-configuration in the CD part. Among stereoisomers of strigol,[14] sorgolactone,[15] and synthetic strigolactone GR24,[16] (2′*R*)-isomers are more active than their corresponding (2′*S*)-isomers in inducing *Striga hermonthica* seed germination.

Alectrol was originally isolated by Müller et al.[6] from root exudates of cowpea (*Vigna unguiculata*) as a germination stimulant for *Striga gesnerioides* and *Alectra vogelii*. The compound gave an ion at *m*/*z* 346 in EI-MS analysis and thus was recognized as an isomer of strigol. Alectrol was also identified in root exudates of red clover (*Trifolium pratense*) as a stimulant for *Orobanche minor*.[7] The structure **6** of alectol proposed by Müller et al.[6] was disproven by the synthetic studies of Mori et al.[17] Mori et al.[18] also showed that alectrol was not identical to orobanchol (**7**) because the reported ^{1}H NMR spectrum of alectrol did not match that of (±)-**7**. Wigchert et al.[19] then suggested an alternative structure for alectrol, although it has not been confirmed to date. Recently, alectrol was purified from root exudates of red clover and defined as (+)-orobanchyl acetate [(3a*S*,4*S*,8b*S*,2′*R*)-*O*-acetylorobanchol] (**8**).[20] The ESI-MS analysis of alectrol afforded the sodium adduct ion at *m*/*z* 411 [M + Na]$^+$.[20] Matsuura and co-workers[21] isolated a germination stimulant for *S. gesnerioides* seeds from cowpea root exudates and determined the stimulant as (+)-4-*O*-acetylorobanchol (**9**) that gave a protonated ion at *m*/*z* 389 in the FD-MS analysis, although its absolute configuration, in particular the stereochemistry at C4, remains to be elucidated.

In a previous communication,[22] we reported the importance of the stereochemistry of strigolactones for *S. gesnerioides* seed germination. A 2′-epimeric mixture of synthetic strigolactones, (±)-(3a*S**,4*S**,8b*S**)-4-hydroxy-GR24 (HO-GR24), induced negligible seed germination, whereas (−)-(3a*R*,4*R*,8b*R*,2′*R*)-HO-GR24 (**10**) having the same configuration as those of compounds **4** and **5** induced appreciable seed germination of *S. gesnerioides* (19.0% at 10 μM). The acetylated compound of **10** also induced seed germination (5.7%). In contrast, (+)-(3a*S*,4*S*,-8b*S*,2′*R*)-HO-GR24 (**11**) and its acetylated compound, whose configuration is the same as those of **1**−**3**, induced negligible seed germination (0.4 and 0.0%, respectively). Moreover, authentic compound **8** did not induce seed germination of *S. gesnerioides*.[22] These results provided the impetus to reinvestigate the absolute configuration of the natural germination stimulants produced by cowpea. In this study, reisolation of the germination stimulants for

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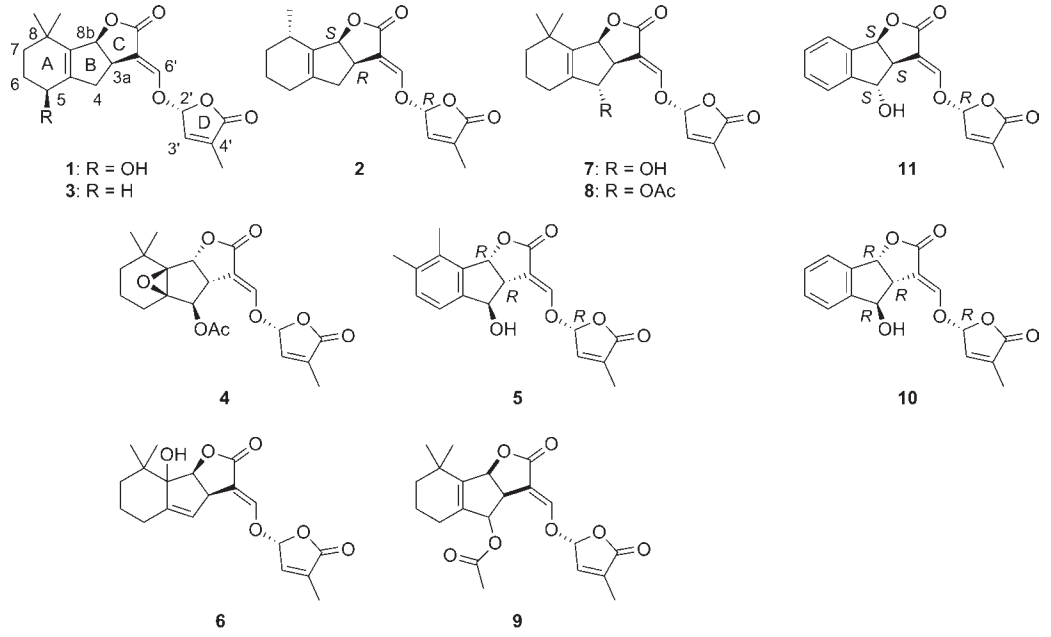

Figure 1. Structures of naturally occurring and synthetic strigolactones. The true structure of salanacol (**5**) has been reported by Chen et al.[12] The structures of alectrol (**6** and **9**) were proposed by Müller et al.[6] and Matsuura et al.,[21] respectively.

seeds of *S. gesnerioides* from cowpea root exudates and unambiguous structural determination of the stimulants were investigated.

MATERIALS AND METHODS

General. ^{1}H NMR spectra were recorded in $CDCl_3$ with a JNM-AL300 spectrometer (JEOL, Ltd., Tokyo, Japan) using tetramethylsilane as an internal standard, and chemical shifts are shown in δ (ppm). Circular dichroism (CD) spectra were recorded with a J-805 spectropolarimeter (JASCO Corp., Tokyo, Japan). Optical rotation was recorded with a DIP-1000 digital polarimeter (JASCO). LC-MS/MS analyses were performed using a system consisting of an Acquity Ultra Performance liquid chromatograph (UPLC) (Waters, Milford, MA), an Acquity quadruple tandem mass spectrometer (TQ Detector) (Waters), and data acquisition and analysis were performed using MassLynx 4.1 software (Waters). UPLC analytical conditions were column, 100 × 2.0 mm i.d., 2.5 μm, COSMOSIL Packed column 2.5C_{18}-MS-II, (Nacalai Tesque, Inc., Kyoto, Japan), 30 °C; solvent, 50−70% MeOH in H_2O (0−20 min, linear gradient); flow rate, 0.2 mL/min. The mass spectrometer was operated in positive ESI mode with the capillary voltage at 3 kV, the cone voltage at 30 V, the source temperature at 120 °C, and the desolvation gas temperature at 350 °C. The nebulizer and desolvation N_2 gas flows were 50 and 550 L/h, respectively. Fragmentation was performed by collision-induced dissociation with argon at 0.1 mL/min and a collision energy of 18 eV. Column chromatography was conducted on silica gel, Wakogel C-200 (Wako Pure Chemical Ind., Ltd., Osaka, Japan).

Chemicals. Racemic orobanchol and 2′-*epi*-orobanchol were prepared using a previously reported method.[23] (+)-Orobanchol, (+)-2′-*epi*-orobanchol, (+)-4-*epi*-orobanchol, and (+)-4,2′-bis*epi*-orobanchol were a generous gift from Emeritus Professor Kenji Mori (The University of Tokyo, Tokyo, Japan). Acetylation of orobanchol isomers was performed using the same method described previously.[22] Racemic 2′-*epi*-orobanchyl acetate was characterized as follows: ^{1}H NMR (300 MHz, $CDCl_3$), δ 1.13 and 1.16 (each 3H, s, Me_2-8), 1.38−1.52 (2H, m, H_2-7), 1.60−1.79 (2H, m, H_2-6), 1.86−2.02 (2H, m, H_2-5), 2.04 (3H, m, Me-4′), 2.05 (3H, s, AcO), 3.45 (1H, ddd, J = 7.3, 2.6, and 1.8 Hz, H-3a), 5.62 (1H, d, J = 7.3 Hz, H-8b), 5.74 (1H, s, H-4), 6.16 (1H, m, H-2′), 6.95 (1H, m, H-3′), 7.46 (1H, d, J = 2.6 Hz, H-6′); LC-ESI-MS/MS, daughter ions of m/z 389, m/z (base peak intensity %), 347 (17), 329 (27), 311 (3), 233 (100), 232 (21), 215 (15), 205 (20), 187 (14), 135 (6), 107 (2), 97 (93). (+)-Orobanchyl acetate (**8**) prepared from (+)-orobanchol[22] had CD (MeCN, c0.000049) λ_{ext} ($\Delta\varepsilon$) 264 (−2.1), 231 (+26.9) nm. (−)-2′-*Epi*-orobanchyl acetate prepared from (+)-2′-*epi*-orobanchol demonstrated $[\alpha]_D^{26}$ − 44.9 (c 0.011, $CHCl_3$) and CD (MeCN, c 0.000053) λ_{ext} ($\Delta\varepsilon$) 254 (+1.4), 219 (−12.8) nm.

Plant Material and Germination Bioassay. Seeds of *S. gesnerioides* (Willd.) Vatke were collected from mature plants parasitizing cowpea (*Vigna unguiculata* (L.) Walp.). Parasitic weed seeds were surface sterilized by immersion in 0.5% (w/v) NaOCl containing a few drops of Tween 20 and sonication for 3 min in an ultrasonic cleaner. After three rinses with distilled water and surface-drying in a laminar hood, *S. hermonthica* and *S. gesnerioides* seeds were pretreated (conditioned) for 10−12 days on 8-mm glass fiber filter paper disks (ca. 50 seeds each) placed on distilled water-saturated filter paper. Aliquots (20 μL) of dilution series of an aqueous solution of cowpea root exudates and strigolactones were assayed by applying them to the conditioned *Striga* seeds on 8-mm disks. The treated seeds were incubated at 30 °C and microscopically evaluated after 24 and 48 h for germination (radicle protrusion) of *S. hermonthica* and *S. gesnerioides*, respectively. *O. minor* seeds were conditioned at 23 °C for 6 days, treated with test solutions as described above, incubated for 5 days at 23 °C, and then examined for germination.

Hydroponic Culture and Collection of Cowpea and Red Clover Root Exudates. About 70 cowpea seedlings were grown hydroponically under phosphate-deficient conditions (40% Long Ashton nutrient solution).[24] The medium was continuously circulated with a pump, and cowpea root exudates of 1- to 8-week-old seedlings were collected by absorbing on activated charcoal (96 g) as described previously.[8] The cultivation and collection of root exudates were performed twice. The root exudates absorbed on charcoal were eluted with acetone for 2 weeks at 4 °C and then evaporated in vacuo to remove acetone. The residual aqueous solution (ca. 50 mL) was extracted with EtOAc (50 mL × 3), and the organic layer was dried over Na_2SO_4 and concentrated in vacuo.

About 400 red clover seeds were germinated at 23 °C for 2 days in the dark, and the germinated seedlings were grown hydroponically as described above. Root exudates of 6−21-day-old seedlings were collected and extracted with the same method.

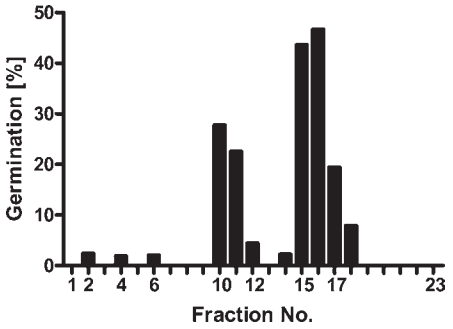

Figure 2. Distribution of the germination-inducing activity of cowpea root exudates toward *S. gesnerioides* after silica gel column separation. The eluates were diluted 30-fold and assayed.

Isolation of Germination Stimulants. The concentrated EtOAc extracts of cowpea root exudates were subjected to column chromatography on silica gel (25 g) with a stepwise gradient of *n*-hexane/EtOAc (100:0−0:100, 10% step, 30 mL × 2 in each step) and subsequent MeOH to give fractions 1−23. Distinct germination-inducing activity toward *S. gesnerioides* seeds was shown by fractions 10−12 (40−50% EtOAc) and 15−17 (70−80% EtOAc) (Figure 2). The active fractions were combined and concentrated in vacuo to give crude compounds **12** and **13**, eluted with 40−50% and 70−80% EtOAc, respectively. Crude compound **12** was subjected to chiral HPLC on a 250 × 10 mm i.d., 5 μm, CHIRALPAK IC (Daicel Chemical Ind., Ltd., Osaka, Japan) with 50% EtOH in hexane at 1 mL/min and eluted at 47 min. Crude compound **13** was subjected to 100% EtOH at 0.5 mL/min and eluted at 42 min. Eluents were monitored at 237 nm. These stimulants were passed through a silica gel column with 10−30% EtOAc in $CHCl_3$ to give compounds **12** (268 μg) and **13** (263 μg). The weights of these stimulants were estimated using an absorption coefficient for (−)-*ent*-2′-*epi*-5-deoxystrigol.[25]

Compound **12** was characterized as follows: colorless oil; $[\alpha]_D^{27}$ + 32.6 (*c* 0.025, $CHCl_3$), [$[\alpha]_D^{26}$ + 20.0 (*c* 0.23, $CHCl_3$)[21]]; CD (MeCN, *c* 0.000038) λ_{ext} ($\Delta\varepsilon$) 253 (−1.9), 218 (+14.4) nm; ^{1}H NMR (300 MHz, $CDCl_3$), δ 1.13 and 1.16 (each 3H, s, Me_2-8), 1.40−1.48 (2H, m, H_2-7), 1.60−1.72 (2H, m, H_2-6), 1.86−1.96 (2H, m, H_2-5), 2.04 (3H, m, Me-4′), 2.05 (3H, s, AcO), 3.45 (1H, ddd, *J* = 7.3, 2.7, and 1.7 Hz, H-3a), 5.62 (1H, d, *J* = 7.3 Hz, H-8b), 5.74 (1H, s, H-4), 6.16 (1H, m, H-2′), 6.95 (1H, m, H-3′), 7.46 (1H, d, *J* = 2.7 Hz, H-6′); LC-ESI-MS/MS, daughter ions of *m*/*z* 389, *m*/*z* (base peak intensity %), 347 (14), 329 (25), 311 (3), 265 (5), 233 (100), 232 (11), 215 (8), 205 (9), 203 (3), 187 (7), 135 (3), 107 (2), 97 (83); HR-ESI-MS *m*/*z* 411.1417 $[M + Na]^+$ ($C_{21}H_{24}NaO_7$ requires 411.1410). Compound **13** was characterized as follows: $[\alpha]_D^{28}$ − 57.9 (*c* 0.023, $CHCl_3$); CD (MeCN, *c* 0.000038) λ_{ext} ($\Delta\varepsilon$) 262 (−1.3), 221 (+9.5) nm; ^{1}H NMR (300 MHz, $CDCl_3$), δ 1.13 and 1.14 (each 3H, s, Me_2-8), 1.37−1.51 (2H, m, H_2-7), 1.66−1.74 (2H, m, H_2-6), 1.81−2.01 (1H, m, H-5), 2.04 (3H, m, Me-4′), 2.10−2.20 (1H, m, H-5), 3.42 (1H, ddd, *J* = 7.4, 2.7, and 1.7 Hz, H-3a), 4.56 (1H, brs, H-4), 5.62 (1H, d, *J* = 7.4 Hz, H-8b), 6.18 (1H, m, H-2′), 6.97 (1H, m, H-3′), 7.52 (1H, d, *J* = 1.7 Hz, H-6′); EI-MS *m*/*z* (rel. int), 346 $[M]^+$ (4), 328 $[M - H_2O]^+$ (2), 285 (8), 249 (16), 232 (41), 231 (54), 204 (39), 203 (34), 189 (21), 161 (17), 135 (10), 97 (100); HR-ESI-MS *m*/*z* 369.1310 $[M + Na]^+$ ($C_{19}H_{22}NaO_6$ requires 369.1314).

Concentrated EtOAc extracts of red clover root exudates were subjected to purification and isolation procedures as described above. Isolated 2′-*epi*-orobanchol and its acetate had CD (MeCN, *c* 0.000015) λ_{ext} ($\Delta\varepsilon$) 262 (−1.7), 217 (+7.9) nm and CD (MeCN, *c* 0.0000077) λ_{ext} ($\Delta\varepsilon$) 261 (−2.7), 219 (+14.8) nm [CD (MeCN, *c* 0.00016) λ_{ext} ($\Delta\varepsilon$) 255 (−5.75), 217 (+52.70) nm[20]], respectively.

RESULTS AND DISCUSSION

Isolation of Germination Stimulants from Cowpea Root Exudates. Cowpea was grown hydroponically, and root exudates were collected as reported previously.[8] The root exudates were subjected to solvent partitioning to give an EtOAc fraction, which was purified by silica gel column chromatography with a mixture of *n*-hexane and EtOAc. Two major stimulants inducing *S. gesnerioides* seed germination were eluted in the 40−50% and 70−80% EtOAc fractions as shown in Figure 2. These stimulants were further purified by chiral column chromatography to obtain pure compounds **12** (eluted in the 40−50% EtOAc fractions) and **13** (eluted in the 70−80% EtOAc fractions).

Identification of Germination Stimulants in Cowpea Root Exudates. The ESI-MS analysis of compound **12** afforded the proton adduct ion at *m*/*z* 389 and the sodium adduct ion at *m*/*z* 411. The ESI-MS/MS analysis of the precursor ion at *m*/*z* 389 gave fragment ions at *m*/*z* 347 ($[M - Ac]^+$), 329 ($[M - OAc]^+$), 233, 205, and 97. These fragment ions were comparable to those for a cowpea stimulant reported previously.[21] However, compound **13** produced the proton adduct ion at *m*/*z* 347 and the sodium adduct ion at *m*/*z* 369 in ESI-MS analysis, suggesting that compound **13** is a strigol isomer.[26]

Table 1 shows ^{1}H NMR chemical shifts of compounds **12** and **13**, and alectrol reported previously.[6,20,21] The resonances of compound **12** were very similar to those previously reported for alectrol isolated from cowpea,[6,21] indicating that compound **12** is alectrol[6] and the same stimulant as that isolated by Matsuura et al.[21] (**9**). The resonance of H-4 of compound **12** was observed as a singlet, which suggests that the dihedral angle between H-4 and H-3a was ca. 90°, and hence the acetoxy group in compound **12** was in the α orientation. The resonance of H-6′ in compound **12** was δ 7.46, whereas the resonances of H-6′ in synthetic orobanchyl acetate[22] and 2′-*epi*-orobanchyl acetate were δ 7.58 and δ 7.46, respectively (Table 1). This result shows the structure of compound **12** as (±)-2′-*epi*-orobanchyl acetate. Moreover, the resonances of compound **12** were almost the same as those previously reported for alectrol isolated from red clover[20] (Table 1). The ^{1}H NMR spectrum of compound **13** was very similar to that of 2′-*epi*-orobanchol reported previously.[27] The resonance of H-4 of compound **13** was observed as a singlet, compared to those of 4-*epi*-orobanchol and 4,2′-bis*epi*-orobanchol observed as a doublet,[27] which suggests that the hydroxyl group in compound **13** was in the α orientation.

LC-ESI-MS/MS analysis using an ODS column with MeOH-H_2O as the eluting solvent, monitoring the transition of *m*/*z* 389 > 233 and *m*/*z* 411 > 254, further supported that compound **12** was 2′-*epi*-orobanchyl acetate. Compound **12** and authentic 2′-*epi*-orobanchyl acetate were eluted at 13.2 min, whereas orobanchyl acetate, 4-*epi*-orobanchyl acetate, and 4,2′-bis*epi*-orobanchyl acetate were eluted at 15.5, 13.4, and 12.2 min, respectively, in the LC conditions described in Material and Methods. Strigol was eluted at 9.0 min in the same LC condition, which is coincident with the information that alectrol was eluted slower than strigol in the reverse phase HPLC.[6] LC-ESI-MS/MS analysis monitoring the transition of *m*/*z* 347 > 205 and *m*/*z* 369 > 272 showed that the chromatographic behavior of compound **13** was identical to that of authentic 2′-*epi*-orobanchol (t_R 7.9 min) (Figure 3A,B). The retention time of compound **13** was inconsistent with those of orobanchol (**7**) (t_R 7.4 min), 4-*epi*-orobanchol (t_R 12.2 min), and 4,2′-bis*epi*-orobanchol (t_R 9.2 min) (Figure 3C−E), and with naturally occurring monohydroxylated strigolactones, strigol (t_R 9.0 min) and sorgomol (t_R 8.6 min). Therefore, we determined the relative configurations of compounds **12** and **13** as (±)-2′-*epi*-orobanchyl acetate

Table 1. ^{1}H NMR Chemical Shifts of Compounds 12 and 13, Alectrol, and Authentic Standards[a]

	12	13	alectrol[6]	9[21]	alectrol from red clover[20]	orobanchyl acetate[22]	2′-*epi*-orobanchyl acetate
H-6′	7.46	7.52	7.45	7.46	7.46	7.58	7.46
H-3′	6.95	6.97	6.9	6.94	6.94	6.97	6.95
H-2′	6.16	6.18	6.1	6.15	6.15	6.13	6.16
H-4	5.74	4.56	5.75	5.73	5.77	5.80	5.74
H-8b	5.62	5.62	5.6	5.61	5.61	5.62	5.62
H-3a	3.45	3.42	3.45	3.45	3.45	3.44	3.45
H-2″	2.05	*b*	NL[c]	2.05	2.04	2.06	2.05
Me-C4	2.04	2.04	2.0	2.03	2.03	2.01	2.04
H_2-5	1.86−1.96	2.10−2.20, 1.81−2.01	NL	1.87−1.99	1.96	1.88−1.98	1.86−2.02
H_2-6	1.60−1.72	1.66−1.74	NL	1.64−1.76	1.70	1.61−1.76	1.60−1.79
H_2-7	1.40−1.48	1.37−1.51	NL	1.36−1.52	1.38−1.52	1.41−1.52	1.38−1.52
Me_2-C8	1.16, 1.13	1.14, 1.13	1.16, 1.14	1.15, 1.13	1.16, 1.14	1.16, 1.14	1.16, 1.13

[a] The multiplicity and coupling constants of **12**, **13**, and 2′-*epi*-orobanchyl acetate were described in Materials and Methods. [b] Compound **13** had no acetyl groups. [c] Data are not listed.

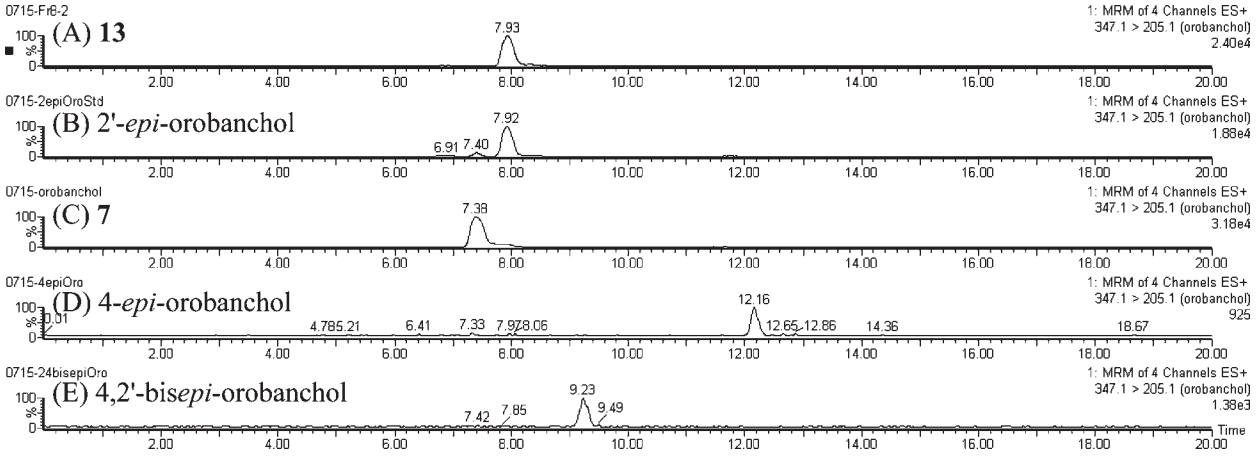

Figure 3. LC-MS/MS analysis of compound **13** (A), 2′-*epi*-orobanchol (B), orobanchol (**7**) (C), 4-*epi*-orobanchol (D), and 4,2′-bis*epi*-orobanchol (E). These MRM chromatograms are displayed with the transition of *m*/*z* 347 > 205.

and (±)-2′-*epi*-orobanchol, respectively. Compound **13** was first isolated from cowpea root exudates, although Müller et al.[6] had indicated the presence of the second stimulant in the exudates.

Germination Stimulants in Red Clover Root Exudates. As described above, the ^{1}H NMR spectrum of alectrol isolated from red clover[20] was almost the same as those for compound **12**, 2′-*epi*-orobanchyl acetate. To confirm the relative configuration of the germination stimulants from red clover, we analyzed root exudates of red clover collected by charcoal, using LC-MS/MS with a precursor ion scanning of *m*/*z* 97 (D ring). The analysis indicated the presence of two strigolactone-like compounds with the retention times at 7.9 and 13.2 min, which were consistent with those of authentic 2′-*epi*-orobanchol and 2′-*epi*-orobanchyl acetate, respectively. These compounds were detected with high sensitivity by multireaction monitoring (MRM), the transitions of *m*/*z* 347 > 205, *m*/*z* 347 > 233, and *m*/*z* 369 > 272 for orobanchol, and *m*/*z* 389 > 233 and *m*/*z* 411 > 254 for orobanchyl acetate. Other strigolactones such as **1**−**3**, **7**, **8**, and sorgomol were not detected by the precursor ion scan. These results indicate that the major strigolactones in root exudates of red clover are 2′-*epi*-orobanchol and its acetylated compound. It should be noted that neither (±)-**7** nor (±)-**8** was detected.

Absolute Configuration of Germination Stimulants for *Striga generioides* Seeds. The absolute configurations of cowpea stimulants were established by comparison of their CD spectra and optical rotations with those of authentic compounds. Compounds **12** and **13** had positive Cotton effects around 220 nm, whereas synthetic (+)-2′-*epi*-orobanchol and its acetate had negative Cotton effects (Figure 4). The result indicates that compounds **12** and **13** were the enantiomers of the authentic compounds. The CD spectrum of compound **12** is consistent with that of alectrol that showed a positive Cotton effect at 220 nm.[6] Compounds **12** and **13** had negative Cotton effects around 260 nm, which suggests the *R*-configuration at C-2′ of cowpea stimulants.[28,29] Although (+)-(2′*R*)-orobanchol (**7**) and its acetate (**8**) also had positive Cotton effects below 250 nm, the maximum absorption and its wavelength differed from those of cowpea stimulants (Figure 4). The optical rotation of compound **12** showed an $[\alpha]_D$ value of +33° (*c* 0.025, $CHCl_3$), whereas the $[\alpha]_D$ value of acetylated (+)-2′-*epi*-orobanchol was −45° (*c* 0.011, $CHCl_3$). Matsuura et al.[21] reported the optical rotation of the cowpea stimulant as +20° (*c* 0.30, $CHCl_3$). The $[\alpha]_D$ value of acetylated (+)-orobanchol was +148° (*c* 0.054, $CHCl_3$).[22] The optical rotation of compound **13** showed an $[\alpha]_D$ value of −58° (*c* 0.023, $CHCl_3$), whereas the $[\alpha]_D$ value of (+)-2′-*epi*-orobanchol

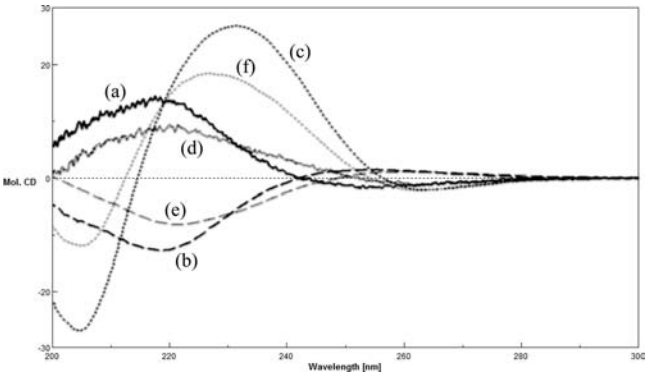

Figure 4. CD spectra of (a) compound **12**, (b) (−)-2′-*epi*-orobanchyl acetate, (c) (+)-**8**, (d) compound **13**, (e) (+)-2′-*epi*-orobanchol, and (f) (+)-**7**. Scan rates for cowpea stimulants and authentic compounds were 50 and 200 nm/min, respectively.

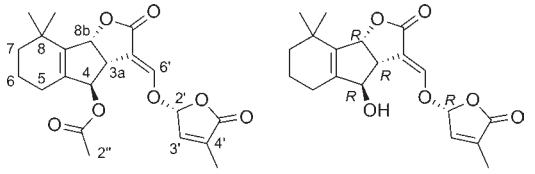

Figure 5. Structures of compounds **12** and **13**.

reported previously was +71° (*c* 0.30, $CHCl_3$).[27] Therefore, we concluded that compounds **12** and **13** are (+)-(3a*R*,4*R*,8b*R*,2′*R*)-*ent*-2′-*epi*-orobanchyl acetate and (−)-(3a*R*,4*R*,8b*R*,2′*R*)-*ent*-2′-*epi*-orobanchol, respectively (Figure 5).

The CD spectra of germination stimulants isolated from root exudates of red clover also showed negative Cotton effects around 260 nm and positive Cotton effects around 220 nm. The CD of the acetate was comparable to that of alectrol isolated by Xie et al.[20] and was consistent with the CD curve of compound **12**. The CD spectrum of the alcohol was identical to that of compound **13**. These results demonstrate that germination stimulants in root exudates of red clover are *ent*-2′-*epi*-orobanchyl acetate (**12**) and *ent*-2′-*epi*-orobanchol (**13**).

Germination-Inducing Activity. The germination-inducing activities of compounds **12** and **13** toward *S. gesnerioides*, *S. hermonthica*, and *O. minor* seeds are shown in Figure 6. *S. gesnerioides* seeds germinated appreciably in response to 100 pM compound **12** and 1 nM compound **13** but negligibly to their respective enantiomers at the same concentration (data not shown). (+)-2′-*Epi*-orobanchol and its acetate slightly induced germination at as high as 10 μM concentration. This configuration preference is the same as that observed using stereoisomers of HO-GR24; that is, (−)-(3a*R*,4*R*,8b*R*,2′*R*)-HO-GR24 (**10**) having the same configuration as that of **13** induced appreciable seed germination of *S. gesnerioides* seeds (19.0% at 10 μM), whereas its enantiomer, (+)-(3a*S*,4*S*,8b*S*,2′*S*)-HO-GR24, induced negligible seed germination (0.7%).[22] *O. minor* seeds germinated appreciably in response to 1 pM compound **12** and 100 pM compound **13**. In contrast, *S. hermonthica* germinated in response to 1 μM compounds **12** and **13**. Higher activities of compounds **12** and **13** toward *S. gesnerioides* seeds are consistent with the previous report that alectrol was a highly potent germination stimulant for *A. vogelii* and *S. gesnerioides*.[6]

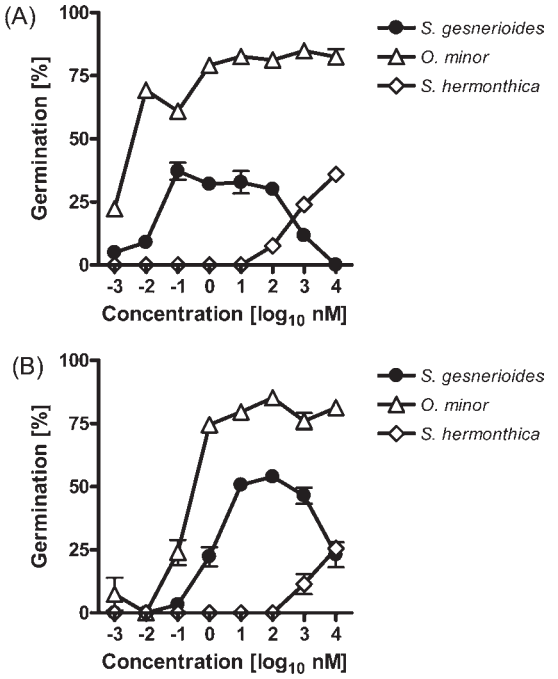

Figure 6. Germination-inducing activities of compounds **12** (A) and **13** (B) toward *S. gesnerioides* (●), *S. hermonthica* (◇), and *O. minor* (△). Data are shown as the mean ± SEM from more than 3 replicate tests.

The present work demonstrated that two major germination stimulants produced by cowpea are identical to the red clover products. The correct structure of alectrol was established as *ent*-2′-*epi*-orobanchyl acetate. Moreover, another strigolactone was found to be *ent*-2′-*epi*-orobanchol. On this occasion of unambiguous structural determination of major germination stimulants produced by cowpea and red clover, assignment of new names for the strigolactones is necessary to avoid confusion after this report. We would like to designate compounds **13** and **12** as gesnerol and gesneryl acetate, respectively, for their germination-inducing activity toward *S. gesnerioides* seeds. The configuration of the C ring and C2′ of strigolactones identified to date is the same as either strigol or gesnerol, suggesting that their deoxy derivatives are key intermediates for the generation of a variety of strigolactones. Further studies will clarify relationships between the plant metabolism of strigolactones and structural requirements for host recognition by parasitic weeds and AM symbionts.

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