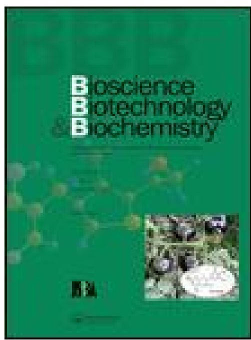

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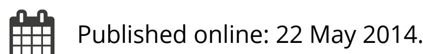

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7-Oxoorobanchyl Acetate and 7-Oxoorobanchol as Germination Stimulants for Root Parasitic Plants from Flax (*Linum usitatissimum*)

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Germination stimulants for root parasitic plants produced by flax (*Linum usitatissimum* L.) were purified and characterized. The root exudate of flax contained at least 8 active fractions, and liquid chromatography-tandem mass spectrometry (LC–MS/MS) and gas chromatography mass spectrometry (GC–MS) analyses suggested that there were 6 strigolactones. Two of them were identified as orobanchol and orobanchyl acetate by comparing NMR and GC–MS and LC–MS/MS data with those of synthetic standards. One of the two novel strigolactones was purified and determined as 7-oxoorobanchyl acetate [((3a*S*,4*S*,8b*S*,*E*)-8,8-dimethyl-3-(((*R*)-4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy)methylene)-2,7-dioxo-3,3a,4,5,6,7,8,8b-octahydro-2*H*-indeno[1,2-*b*]furan-4-yl acetate) by 1D and 2D NMR spectroscopic, and ESI– and EI–MS spectrometric analyses. The other one was also purified and identified as 7-oxoorobanchol. The remaining two compounds could not been characterized due to their scarcity.

Key words: flax; *Orobanche minor*; parasitic plants; strigolactone

Strigolactones were originally identified as germination stimulants for the root parasitic plants, *Striga* and *Orobanche* spp.,[1,2] and then as branching factors for arbuscular mycorrhizal (AM) fungi.[3] In addition to these functions as rhizosphere-signaling chemicals for symbiosis and parasitism, another role of strigolactones as a novel class of plant hormones inhibiting shoot branching has recently been unveiled.[4,5] It is therefore reasonable that even non-hosts of root parasitic plants and of AM fungi produce strigolactones,[6,7] because plants need strigolactones for their normal growth and development.

In general, plants produce strigolactones in extremely low quantities and they are unstable during the purification process. Although extensive studies have been conducted to isolate strigolactone-germination stimulants for root parasitic plants, only 10 of them have been characterized to date and at least several unknown strigolactones remain to be identified.[8]

From the late 1940s to the early 1950s, Brown *et al.* examined various plant species for the production of *O. minor* germination stimulants and found that a flax (linseed) root exudate was the most active among those from the plants examined.[9–11] They partially purified the active compounds and estimated that these compounds would contain lactone groups, because they were relatively stable in weekly acidic solutions and unstable in alkaline solutions. The UV spectrum of the purified compound(s) with λ_{max} at around 245 nm[11] suggested that the stimulant(s) were strigolactone(s). However, the germination stimulants produced by flax have not yet been characterized.

We report here the identification of strigolactones in a root exudate of flax (*Linum usitatissimum* L.) and the structural elucidation of two novel strigolactones, 7-oxoorobanchyl acetate and 7-oxoorobanchol (Fig. 1).

Results and Discussion

Flax plants were grown hydroponically and the root exudate was collected as described previously.[7] The root exudate was subjected to solvent partitioning to give a neutral EtOAc fraction. This was purified by a silica gel column chromatography eluted with *n*-hexane–EtOAc. The germination-stimulating activities were eluted in the 30% to 100% EtOAc fractions at a 100,000-fold dilution, indicating that the flax root exudate contained at least 8 active fractions (Fig. 2). LC–MS/MS and GC–MS analyses of these fractions suggested that there were 6 strigolactones as they showed typical fragmentation patterns in their mass spectra; a base peak at m/z 97 by GC–MS and neutral loss of 97 Da by LC–MS/MS (see later).

Two known strigolactones, orobanchyl acetate (**1**) and orobanchol (**2**), were found in the 40% and 70% EtOAc fractions, respectively, by the LC–MS/MS analysis, and the identities of these strigolactones were further confirmed by a GC–MS analysis after their purification by HPLC (data not shown). The 50% and 60% EtOAc fractions, which were found to contain a novel strigolactone (**3**) by the LC–MS/MS analysis, were combined

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Abbreviations: GC–MS, gas chromatography-mass spectrometry; HR–ESI–TOF–MS, high resolution-electrospray ionization-time of flight-mass spectrometry; LC–MS/MS, liquid chromatography-tandem mass spectrometry

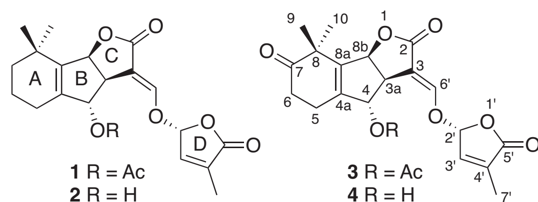

Fig. 1. Structures of the Strigolactones Identified in the Flax Root Exudate.

Orobanchyl acetate (**1**), orobanchol (**2**), 7-oxoorobanchyl acetate (**3**), and 7-oxoorobanchol (**4**).

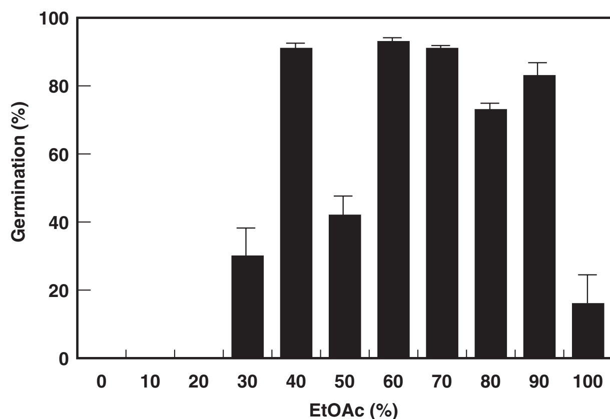

Fig. 2. Distribution of the Germination-Stimulating Activity of the *Linum usitatissimum* Root Exudate toward *Orobanche minor* Seeds after Silica Gel Column Chromatography.

The germination-stimulating activity of each sample was examined at a 100,000-fold dilution. Each value is presented as the mean ± SEM (n = 3).

and successively subjected to silica gel column chromatography and reversed-phase HPLC to yield pure compound **3**. Another novel strigolactone (**4**) was isolated from the 90% EtOAc fraction after its purification by silica gel column chromatography and by reversed-phase HPLC with ODS and ODS-CN columns. In addition to orobanchol, the 70% EtOAc fraction was found to contain small amounts of two novel strigolactones. Unfortunately, the characterization of these compounds was not possible due to their scarcity.

The HR–ESI–TOF–MS analysis of compound **3** afforded the sodium adduct ion at m/z 425.1241 $[M + Na]^+$ (calcd. for $C_{21}H_{22}O_8Na$, 425.1212) corresponding to a molecular formula of $C_{21}H_{22}O_8$. The CID spectrum of **3** in the ESI–MS/MS analysis, where the $[M + Na]^+$ ion at m/z 425 was converted with loss of AcOH to the $[M + Na–AcOH]^+$ ion at m/z 365 and $[M + Na–AcOH–D\ ring]^+$ at m/z 268 (data not shown), suggested that compound **3** would be either oxo-orobanchyl acetate or hydroxy-didehydro-orobanchyl acetate, or its isomer.

The ^{1}H- and ^{13}C-NMR spectroscopic data (Table 1) revealed similarities between compound **3** and orobanchyl acetate (**1**).[12,13] The chemical shift of H-4 [δ_H 5.82 (1H, *bs*)] in compound **3** indicated that the acetyloxyl group was attached to C-4. The lack of signals of two methylene protons and the large downfield shifts (*ca.* 0.4–1.0 ppm) of the remaining four methylene protons in compound **3**, as compared to those in orobanchyl acetate (**1**), clearly indicated that one of the methylene moieties, C-5, C-6 or C-7, in the A ring had been replaced with a carbonyl group. The ^{13}C chemical shift of this carbonyl carbon in compound **3** appeared at δ_C 212.4. These results suggested that compound **3** was 5-, 6-, or 7-oxoorobanchyl acetate.

The clear evidence for the position of the carbonyl group in compound **3** was obtained by an HMBC analysis, where a strong correlation was observed between H-9 (H-10) and the carbonyl carbon at δ_C 212.4. Thus, compound **3** was determined to be 7-oxoorobanchyl acetate, whose structure is well supported by the HMQC, HMBC, and NOE data (Table 1). The C-2′ stereochemistry of 7-oxoorobanchyl acetate (**3**) was assigned as being *R* based on the negative sign of its CD spectrum (Supplemental Fig. 1; see *Biosci. Biotechnol. Biochem.* Web site) at around 270 nm.[14] Consequently, the chemical structure of 7-oxoorobanchyl acetate (**3**) was determined as ((3a*S*,4*S*,8b*S*,*E*)-8,8-dimethyl-3-(((*R*)-4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy)methylene)-2,7-dioxo-3,3a,4,5,6,7,8,8b-octahydro-2*H*-indeno[1,2-*b*] furan-4-yl acetate.

The corresponding free alcohol, 7-oxoorobanchol (**4**), was isolated from the 90% EtOAc fraction after purification by silica gel column chromatography and by reversed phase HPLC with ODS and ODS-CN columns. Although only the ^{1}H-NMR spectrum was obtained due to the scarcity of **4**, the structure was confirmed by a direct spectroscopic comparison of its acetate with 7-oxoorobanchyl acetate (**3**). The acetate prepared from compound **4** gave ^{1}H-NMR and CD spectra identical to those of compound **3**, and the identity was confirmed by LC–MS and GC–MS analyses. Therefore, the C-2′ stereochemistry of 7-oxoorobanchol (**4**) was also assigned as being *R* which was supported by the negative sign of its CD spectrum at around 270 nm (Supplemental Fig. 1; see *Biosci. Biotechnol. Biochem.* Web site).

The germination-stimulating activities of orobanchyl acetate (**1**), orobanchol (**2**), 7-oxoorobanchyl acetate (**3**) and 7-oxoorobanchol (**4**) toward *O. minor* and *O. ramosa* (*Phelipanche ramosa*) seeds are shown in Fig. 3. In the case of orobanchol, like strigol, acetylation of the hydroxyl group resulted in a significant reduction of germination-stimulating activity.[15,16] By contrast, 7-oxoorobanchyl acetate (**3**) was more active than 7-oxoorobanchol (**4**) toward both *O. minor* and *O. ramosa* seeds. The relatively weak activity of 7-oxoorobanchol (**4**) may be attributable to its instability or low lipophilicity which made it difficult to reach its receptor site(s).

According to the proposed biosynthetic pathway for strigolactones, 5-deoxystrigol is the key intermediate.[17] Allylic hydroxylation of 5-deoxystrigol affords strigol and orobanchol. Oxidation also occurs at the homoallylic positions, leading to sorgomol and 7-oxo- and 7-hydroxy-strigolactones. It is likely that the didehydro-orobanchol (or -strigol) isomers and solanacol detected in the root exudates of tobacco[18] and tomato[19] are formed *via* these oxidized orobanchol derivatives.[20]

Experimental

Instruments. ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ (δ_H 7.26, δ_C 77.0) by a JEOL Lambda 400 spectrometer. The standard

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Table 1. NMR Spectral Data for Compound **3**

No.	δ^1H (mult., J Hz)	δ^{13}C	DEPT and HMQC	^{1}H–^{1}H COSY	NOESY	HMBC
2		170.1	C			
3		109.6	C			
3a	3.56 (*ddd*, 7.3, 2.4, 1.9)	46.2	CH	H-8b	H-8b	C-3, C-4
4	5.82 (*bs*)	81.7	CH			C-3, C-8a
4a		139.6	C			
5	2.29–2.54 (*m*)	22.9	CH_2	H-6		C-4a, C-7
6	2.42–2.77 (*m*)	34.2	CH_2	H-5		C-7, C-9
7		212.4	C			
8		45.4	C			
8a		145.8	C			
8b	5.60 (*d*, 7.3)	84.9	CH	H-3a	H-3a	C-4, C-4a
9	1.34 (*s*)	26.5	CH_3			C-7, C-8, C-8a, C-10
10	1.31 (*s*)	21.7	CH_3		H-8b	C-7, C-8, C-8a, C-9
2′	6.17 (*t*, 1.5)	99.9	CH	H-3′	H-6′	
3′	6.95 (*t*, 1.5)	140.6	CH	H-2′	H-7′	
4′		136.3	C			
5′		169.9	C			
6′	7.56 (*d*, 2.4)	151.3	CH		H-2′	C-3, C-3a, C-2′
7′	2.04 (*t*, 1.5)	10.7	CH_3		H-3′	
1″*		169.4	C			
2″**	2.03 (*s*)	20.9	CH_3			

*Carbonyl carbon of an acetyl group.
**Methyl carbon of an acetyl group.

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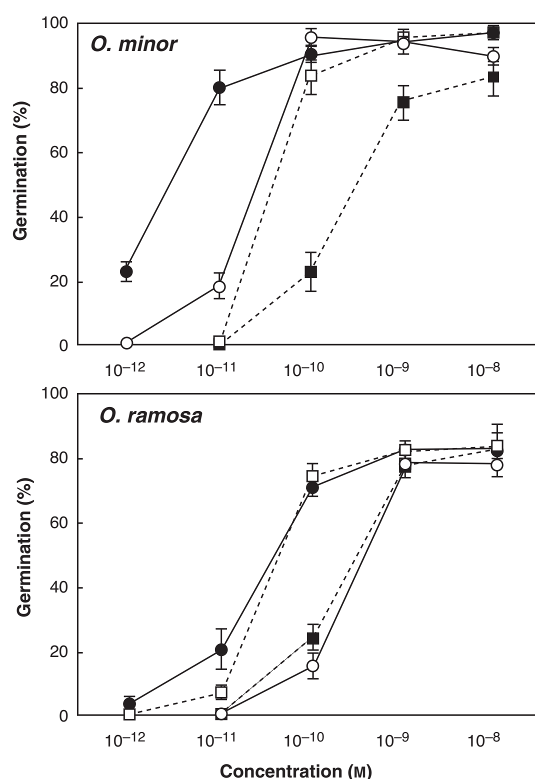

Fig. 3. Germination-Stimulating Activities of Strigolactones Found in the *Linum usitatissimum* Root Exudate toward *O. minor* and *O. ramosa* Seeds.

The germination-stimulating activities of **1** (unfilled circle), **2** (filled circle), **3** (unfilled square) and **4** (filled square) toward *O. minor* and *O. ramosa* seeds were determined as explained in the Experimental section. Each value is presented as the mean ± SEM (n = 3).

pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO J-720W spectropolarimeter in MeCN. EI–GC–MS spectra were obtained with a JEOL JMS-Q1000GC/K9 instrument, using a DB-5 (J&W Scientific, Agilent) capillary column (0.25 mm × 5 m) with He carrier gas (3 ml min^{-1}). The operating conditions were the same as those reported earlier.[21] ESI–LC–MS analyses were performed with a Quattro LC tandem MS instrument from Micromass (Manchester, UK). The LC–MS analytical conditions were essentially the same as those described previously.[15,22] Column chromatography was conducted on silica gel (Wakogel C-300, Wako Pure Chemical Industries, Japan).

Chemicals. (+)-Orobanchol was generously provided by Emeritus Professor Kenji Mori (The University of Tokyo, Japan). (+)-Orobanchyl acetate was prepared as reported previously.[16] The other chemicals of analytical grade and HPLC solvents were obtained from Kanto Chemical Co., Ltd., and Wako Pure Chemical Industries Ltd.

Plant material. O. minor Sm. seeds were collected from mature plants that were parasites of red clover grown in the Watarase basin of Tochigi Prefecture, Japan. *O. ramosa* seeds were kindly provided by Prof. A. G. T. Babiker (ARC, Sudan). Seeds of flax (*Linum usitatissimum* cv. Norlin) were generously supplied by Ms. Kiyomi Hashirikawa (AMA Supporters, Sapporo, Japan) and Amakousya (Sapporo, Japan).

Orobanche seed germination assay. Germination assays on *O. minor* and *O. ramosa* seeds were conducted as reported previously.[23,24] The temperatures for conditioning and germination were 23 °C and 18 °C for *O. minor* and *O. ramosa*, respectively. Each test solution, unless otherwise mentioned, contained 0.1% (v/v) acetone.

Hydroponic culture of flax and collection of the root exudate. Flax seeds were surface-sterilized in 70% EtOH for 2 min and then in 1% NaClO for 2 min. Approximately 500 seedlings were transferred to a strainer (28 × 23 × 9 cm, W × L × H) lined with a sheet of gauze moistened by placing it in a slightly larger container (28.5 × 23.5 × 11 cm, W × L × H) containing 1 liter of sterilized tap water as the culture medium in a growth room maintained at 17–22 °C under natural daylight conditions. In total, ten strainers were used to collect the root exudate. The plants were grown for a week, and then the two strainers were transferred to a larger container (53.5 × 33.5 × 14 cm, W × L × H) containing 10 liter of tap water and 1 mM $CaCl_2$. The root exudate released into culture medium was adsorbed to activated charcoal, using circulation pumps.[3] The tap water medium and activated charcoal were exchanged every 3–4 d. The root exudate adsorbed to the charcoal was eluted with acetone. After the acetone had been evaporated *in vacuo*, the residue was dissolved in 50 ml of water and then extracted 3 times with 50 ml of EtOAc. The EtOAc extracts were combined, washed with 0.2 M K_2HPO_4 (pH 8.3), dried

over $MgSO_4$, and concentrated *in vacuo*. This crude extracts was stored in sealed glass vials at 4 °C until needed.

Identification of orobanchyl acetate and orobanchol. The crude extract (352 mg) collected from a total of *ca.* 5000 seedlings of flax grown hydroponically was fractionated by silica gel CC (250 × 20 mm), using a gradient of *n*-hexane–EtOAc (100:0–0:100, 10% steps) to give fractions 1–11. One-thousandth of each fraction was dissolved in 100 µl of 60% MeOH and filtered, and 10 µl was injected into the HPLC instrument connected to the tandem mass spectrometer operated in the ESI positive mode. Orobanchol and orobanchyl acetate were detected by monitoring the transitions m/z 369 > 272 and 411 > 254, respectively. Fractions 5 and 8 were found to contain orobanchyl acetate and orobanchol, respectively. One-hundredth of each fraction was then successively purified by silica gel CC and reversed phase HPLC monitored at 240 nm. The peaks detected were collected and analyzed by GC–MS. The identities of orobanchol and orobanchyl acetate were confirmed by comparing their retention times in the LC–MS/MS and GC–MS data, and their fragmentation patterns in the GC–MS data with those of synthetic standards.[13)]

Isolation of 7-oxoorobanchyl acetate and 7-oxoorobanchol. Fractions 6 and 7 of the first silica gel CC were combined (64.3 mg) and subjected to silica gel CC, using *n*-hexane–EtOAc (60:40) as the eluting solvent system. The combined active fractions (34.4 mg) were purified by isocratic (40% $MeCN/H_2O$) HPLC in an ODS column (Mightysil RP-18, 10 × 250 mm, 10 µm; Kanto Chemicals, Japan) at a flow rate of 3.0 ml min^{-1} to give pure 7-oxoorobanchyl acetate (**3**, 2.35 mg, t_R 30.1 min, detection at 238 nm).

Fraction 10 (10.3 mg) was subjected to silica gel CC, using *n*-hexane–EtOAc (60:40) as the eluting solvent system. The combined active fractions (7.4 mg) were separated by preparative ODS-HPLC (Mightysil RP-18, 10 × 250 mm, 10 µm; Kanto Chemicals, Japan) with an $MeCN/H_2O$ gradient system (30:70 to 0:100 over 40 min) as the eluent at a flow rate of 3.0 ml min^{-1}. The active fraction (0.92 mg) eluted as a single peak at 11.4 min (detection at 238 nm) was collected. This fraction was purified by isocratic (25% $MeCN/H_2O$) HPLC on a Develosil ODS-CN column (4.5 × 250 mm, 5 µm; Nomura Chemicals, Japan) at a flow rate of 1.0 ml min^{-1} to give 7-oxoorobanchol (**4**, 0.33 mg, t_R 22.8 min).

*(+) 7-Oxoorobanchyl acetate (**3**).* $[\alpha]_D^{24.1}$ 22.98 (*c* 0.15, MeCN). UV λ_{max}^{MeCN} nm: 239. CD (MeCN; *c* 0.0016) λ_{max} ($\Delta\varepsilon$) nm: 220 (52.71), 252 (−8.23). GC–EIMS, 70 eV, m/z (rel. int.): 402 $[M]^+$ (1), 360 (4), 342 (11), 314 (3), 263 (9), 245 (40), 228 (15), 218 (13), 97 (100). See Table 1 for the ^1H- and ^{13}C-NMR spectra.

*7-Oxoorobanchol (**4**).* UV λ_{max}^{MeCN} nm: 239. CD (MeCN; *c* 0.00074) λ_{max} ($\Delta\varepsilon$) nm: 221 (9.89), 259 (−1.01). GC–EIMS, 70 eV, m/z (rel. int.): 360 $[M]^+$ (4), 345 (2), 263 (17), 245 (23), 218 (14), 97 (100). HR–ESI–TOF–MS m/z: 361.1302 $[M + H]^+$ (calcd. for $C_{19}H_{20}O_7$, m/z 361.1287). ^1H-NMR (400 MHz, $CDCl_3$) δ: 1.32 (3H, *s*, H-9), 1.35 (3H, *s*, H-10), 2.04 (3H, *t*, $J = 1.5$ Hz, H-7′), 2.31–2.51 (2H, *m*, 5-H), 2.42–2.78 (2H, *m*, 6-H), 3.58 (1H, *ddd*, $J = 7.3$, 2.4 and 1.9 Hz, 3a-H), 4.68 (1H, *bs*, 4-H), 5.60 (1H, *d*, $J = 7.3$ Hz, 8b-H), 6.17 (1H, *t*, $J = 1.5$ Hz, 2′-H), 6.95 (1H, *t*, $J = 1.5$ Hz, 3′-H), 7.54 (1H, *d*, $J = 2.4$ Hz, 6′-H).

*Acetylation of compound **4**.* Compound **4** (150 µg) was treated with acetic anhydride (100 µl) and dry pyridine (100 µl) at 4 °C. After completing the reaction, the mixture was diluted with EtOAc, and the EtOAc solution was washed with brine, dried, and concentrated *in vacuo*. The crude product was purified by silica gel CC (*n*-hexane–EtOAc, 60:40) and by HPLC, affording *ca.* 160 µg of the pure acetate. The ^1H-NMR and CD spectra of the acetate were identical to those of compound **3**, and they showed the same retention times and spectra in the LC–MS/MS and GC–MS analytical data.

Acknowledgments

We are grateful to Emeritus Professor Kenji Mori (The University of Tokyo) for generously providing (+)-orobanchol. We thank Ms. Kiyomi Hashirikawa (AMA Supporters) and Amakousya for the supply of flax seeds. We also thank Dr. Y. Jikumaru (RIKEN) for HR–ESI–TOF–MS measurements. This work was supported by a Grant-in-Aid for Scientific Research (1820810) from Japan Society for the Promotion of Science (JSPS).

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