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) 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 compounds with 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 λmax at around 245 nm 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, orobanchol 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...
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]+ (calcld. for C21H23O8Na, 425.1212) corresponding to a molecular formula of C21H23O8. 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 1H- and 13C-NMR spectroscopic data (Table 1) revealed similarities between compound 3 and orobanchyl acetate (1).12,13 The chemical shift of H-4 [δH 5.82 (1H, bs)] in compound 3 indicated that the acetylxy1 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 13C chemical shift of this carbonyl carbon in compound 3 appeared at δc 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 δc 212.4. Thus, compound 3 was determined to be 7-oxoorobanchyl acetate, whose structure is well supported by the HMBC, HMQC, 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 ((3aS,4S,8bS,E)-8,8-dimethyl-3-(((R)-4-methyl-5-oxo-2,5-dihydorufuran-2-yl)oxy)methylene)-2,7-dioxo-3,4a,5,6,7,8,8b-octahydro-2H-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 1H-NMR spectrum was obtained due to the scarcity of 4, the structure was confirmed by direct spectroscopic comparison of its acetate with 7-oxoorobanchyl acetate (3). The acetate prepared from compound 4 gave 1H-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 tobacco18 and tomato19 are formed via these oxidized orobanchol derivatives.20

**Experimental**

*Instruments.* 1H- and 13C-NMR spectra were recorded in CDCl3 (δH 7.26, δC 77.0) by a JEOL Lambda 400 spectrometer. The standard...
obtained with a JEOL JMS-Q1000GC/K9 instrument, using a DB-5 and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase cycling were used for the HMQC, HMBC, and NOE spectral analyses. CD spectra were recorded with a JASCO pulse sequence and phase c

Table 1. NMR Spectral Data for Compound 3

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<tr>
<th>No.</th>
<th>δ^1H (mult., J/Hz)</th>
<th>δ^13C</th>
<th>DEPT and HMQC</th>
<th>H-8b 1H–1H COSY</th>
<th>NOESY</th>
<th>HMBC</th>
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<td>3a</td>
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<td>H-8b</td>
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<td>2.03 (s)</td>
<td>20.9</td>
<td>CH₃</td>
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*Carbonyl carbon of an acetyl group.
**Methyl carbon of an acetyl group.

![Fig. 3. Germination-Stimulating Activities of Strigolactones Found in the Linum usitatissimum Root Exudate toward O. minor and O. ramosa Seeds.](image)

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).

Germination (%) = \frac{\text{germinated seeds}}{\text{total seeds}} \times 100

Carrier gas (3 ml min⁻¹). The operating conditions were the same as those reported earlier. 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. 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. 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. 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 liters of tap water and 1 mol CaCl₂. The root exudate released into culture medium was adsorbed to activated charcoal, using circulation pumps. 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 K₂HPO₄ (pH 8.3), dried with Na₂SO₄, and rotary evaporated to dryness. The residue was dissolved in 50 ml of acetone, passed through an activated charcoal column, and evaporated again.
over MgSO₄ and concentrated in vacuo. This crude extract 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 16.6 kg 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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References