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Isolation and identification of alectrol as (+)-orobanchyl acetate, a germination stimulant for root parasitic plants

Xiaonan Xie [a,b], Kaori Yoneyama [a], Dai Kusumoto [a], Yoichi Yamada [c], Takao Yokota [d], Yasutomo Takeuchi [a], Koichi Yoneyama [a,*]

[a] *Weed Science Center, Utsunomiya University, 350 Mine-machi, Utsunomiya 321-8505, Japan*
[b] *United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan*
[c] *Faculty of Education, Utsunomiya University, 350 Mine-machi, Utsunomiya 321-8505, Japan*
[d] *Department of Biosciences, Teikyo University, 1-1 Toyosatodai, Utsunomiya 320-8551, Japan*

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Abstract

Alectrol, a germination stimulant for root parasitic plants, was purified from root exudates of red clover (*Trifolium pratense* L.) and identified as a strigolactone, (+)-orobanchyl acetate [(3a*S*,4*S*,8b*S*,*E*)-8,8-dimethyl-3-(((*R*)-4-methyl-5-oxo-2,5-dihydrofuran-2-yloxy)methylene)-2-oxo-3,3a,4,5,6,7,8,8b-octahydro-2*H*-indeno[1,2-*b*]furan-4-yl acetate], by 1D and 2D NMR spectroscopy and ESI- and EI-MS spectrometry. Orobanchyl acetate afforded an $[M–42]^+$ ion in EI-MS and thus had been recognized as an isomer of strigol. Orobanchyl acetate was detected in root exudates of soybean (*Glycine max* L.) and cowpea (*Vigina unguiculata* L.) along with orobanchol.

Keywords: *Trifolium pratense*; Leguminosae; *Orobanche minor*; Orobanchaceae; Alectrol; Orobanchyl acetate; Strigolactone

1. Introduction

Seed germination of root parasitic plants, *Striga*, *Orobanche,* and *Alectra* in the family Orobanchaceae, is induced by germination stimulants produced by and released from roots of host and some non-host plants (Parker and Riches, 1993; Joel et al., 1995). At least three different classes of plant secondary metabolites, dihydroquinones, sesquiterpene lactones, and strigolactones, have been shown to induce seed germination of root parasites (Bouwmeester et al., 2003). Among these germination stimulants, strigolactones appear to be widely distributed in the plant kingdom and thus play pivotal roles in the interactions between root parasites and host plants; these compounds are important host-recognition signals for arbuscular mycorrhizal fungi with which >80% of land plants form symbiotic relationships (Akiyama et al., 2005; Akiyama and Hayashi, 2006).

Alectrol was originally isolated as a germination stimulant of *Striga gesnerioides* and *Alectra volgelii* from root exudates of *Vigna unguiculata* (Müller et al., 1992), and then as a stimulant of *O. minor* from root exudates of *Trifolium pratense* (Yokota et al., 1998). Some other plant species including soybean (*Glycine max* L.) were found to produce alectrol as one of the major strigolactones (Yoneyama et al., 2006). The structure (**1**) originally proposed for alectrol was, however, proven to be incorrect by chemical synthesis (Mori et al., 1998). Wigchert et al. (1999) then suggested an alternative structure for it (**2**) which has not been confirmed to date.

In this paper we describe the isolation and identification of orobanchyl acetate (**3**), a novel germination stimulant for root parasitic plants from red clover (*Ttrifolium*

* Corresponding author. Tel.: +81 28 649 5152; fax: +81 28 649 5155.
E-mail address: yoneyama@cc.utsunomiya-u.ac.jp (K. Yoneyama).

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pratense) root exudates. This is the actual compound previously referred to as alectrol (**1**).

2. Results and discussion

Red clover was grown hydroponically and root exudates collected as described previously (Yoneyama et al., 2001, 2007). The root exudates were subjected to solvent partitioning to give a neutral EtOAc fraction. This was purified by a silica gel column chromatography eluted with *n*-hexane–EtOAc. Two major stimulant activities eluted in the 40% and 70% EtOAc fractions were found to contain two compounds, respectively, by LC/MS analysis; the 70% EtOAc fraction gave orobanchol as its purified product. The first compound, supposedly alectrol (**1** or **2**), eluted in the 40% EtOAc fraction was purified to homogeneity by HPLC with ODS and ODS-CN column chromatographies.

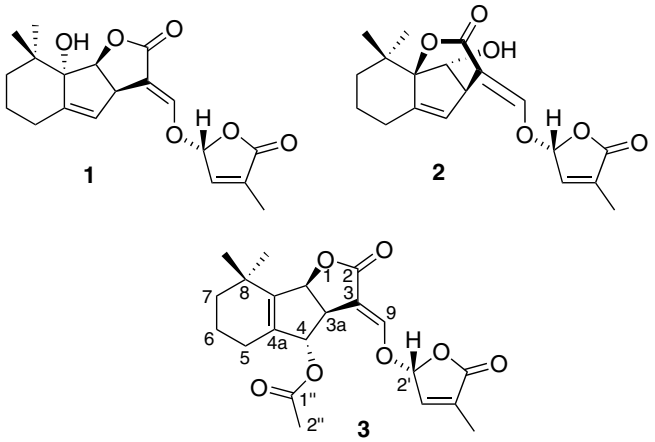

Fig. 1. Structures of alectrol proposed by Müller et al. (**1**) and Wigchert et al. (**2**), and orobanchyl acetate (**3**).

The ESI-MS analysis of "alectrol" afforded the sodium adduct ion at *m/z* 411 $[M+Na]^+$ along with the potassium adduct ion at *m/z* 427 $[M+K]^+$. This demonstrated that the molecular weight of "alectrol" was 388 rather than 346. The TOF/MS analysis also confirmed this (data not shown). In addition, the CID spectrum of "alectrol" indicated that the $[M+a]^+$ ion was converted with loss of AcOH to $[M+Na–AcOH]^+$ ion at *m/z* 351 and $[M+Na–AcOH–D\ ring]^+$ at *m/z* 254 (data not shown). Such evidence indicated that "alectrol" should be strigyl acetate previously identified from cotton (Sato et al., 2005) or its isomer. The R_t of "alectrol" (15 min) in ODS-HPLC (MeOH–H_2O, 6:4, v/v) was distinct from that of strigyl acetate (R_t, 12 min), suggesting that "alectrol" was isomeric to strigyl acetate.

The amount of the purified "alectrol" sample was enough to obtain the ^1H and ^{13}C NMR spectroscopic data for structural elucidation. Although it was not clear from the earlier studies (Müller et al., 1992; Yokota et al., 1998), acetyl methyl protons [δ_H 2.04 (3H, *s*, H_3-C2")] were clearly observed. The other signals in the ^1H NMR spectrum of "alectrol" were very similar to those of orobanchol except for the downfield shift of H-4 [δ_H 5.74 (1H, *s*)] by ca 1.0 ppm, clearly indicating that the 4-α-hydroxyl group of orobanchol is acetylated. The α-orientation of the acetyloxyl group was assigned since the H-4 appeared as a singlet and hence the dihedral angle between H-4 and H-3a was ca. 90°. The ^{13}C NMR, HMQC, HMBC and NOE analyses

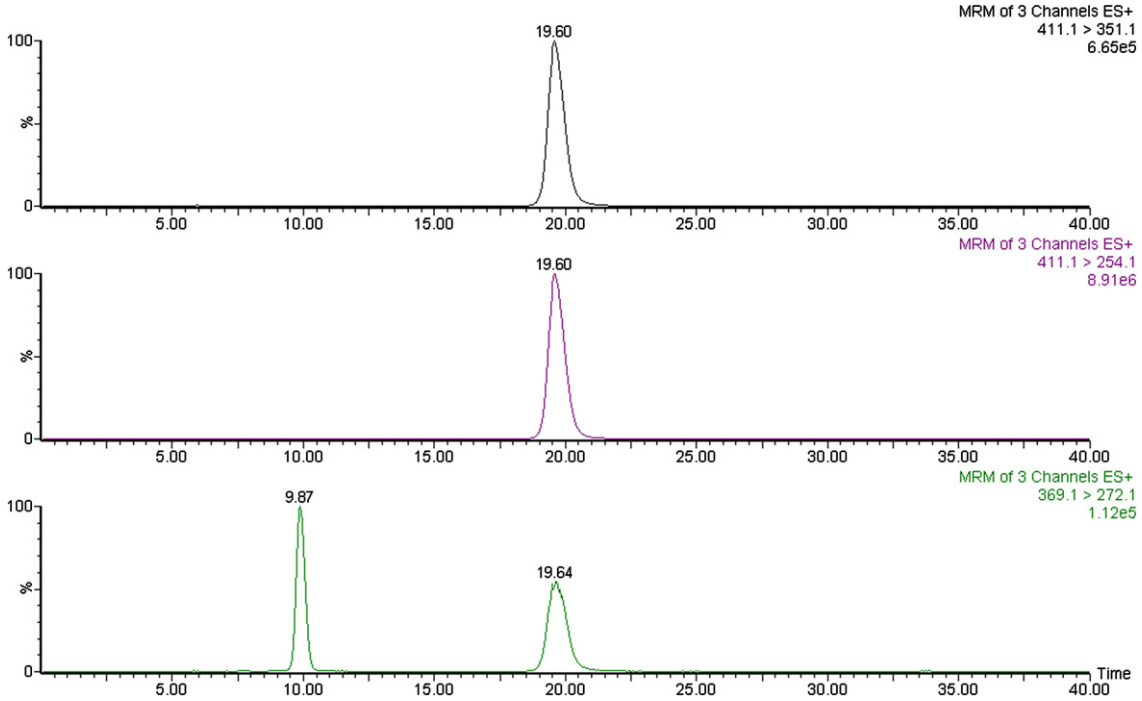

Fig. 2. Three-channel MRM chromatogram of red clover root exudates.

also supported the structure of "alectrol" as orobanchyl acetate (**3**). This was further confirmed by direct spectroscopic comparison with (+)-orobanchyl acetate (**3**) prepared from (+)-orobanchol (Hirayama and Mori, 1999). Accordingly, "alectrol" is orobanchyl acetate (**3**) (see Fig. 1).

Fig. 2 shows the three-channel MRM profiles obtained by LC–ESI-MS/MS of crude EtOAc extracts of red clover root exudates. HPLC separation was performed with an ODS-Phenyl column (Inertsil Ph, 2.1 × 250 mm, 5 μm; GL Science Inc. Tokyo, Japan) with MeOH–H_2O (6:4, v/v) as eluting solvent. The upper and mid channels show the transitions of *m/z* 411 > 351 and *m/z* 411 > 254, respectively at 19.6 min, elucidating the presence of orobanchyl acetate (**3**). The lower channel is for monitoring the transition of *m/z* 369 > 272 for the detection of strigol isomers, where orobanchol appeared as a peak at 9.9 min. It should be noted that orobanchyl acetate (**3**) (R_t, 19.6 min) gave a distinct peak in the MRM channel for strigol isomers while the peak intensity was ca. 1/10 and 1/100 that of the other two MRM channels. It is likely that acetates (and probably other derivatives) of strigolactones do not afford pseudo-molecular ions by EI-MS analysis. For example, in the EI-MS spectrum of strigyl acetate, only an $[M–42]^+$ ion was observed at *m/z* 346 as in the case of orobanchyl acetate (**3**). Levels of orobanchyl acetate (**3**) in the growth media recovered on day 7 and later during the incubation could directly be detected by LC/MS/MS, indicating that the acetate (**3**) is not an artifact produced from orobanchol during the EtOAc extraction and concentration procedure.

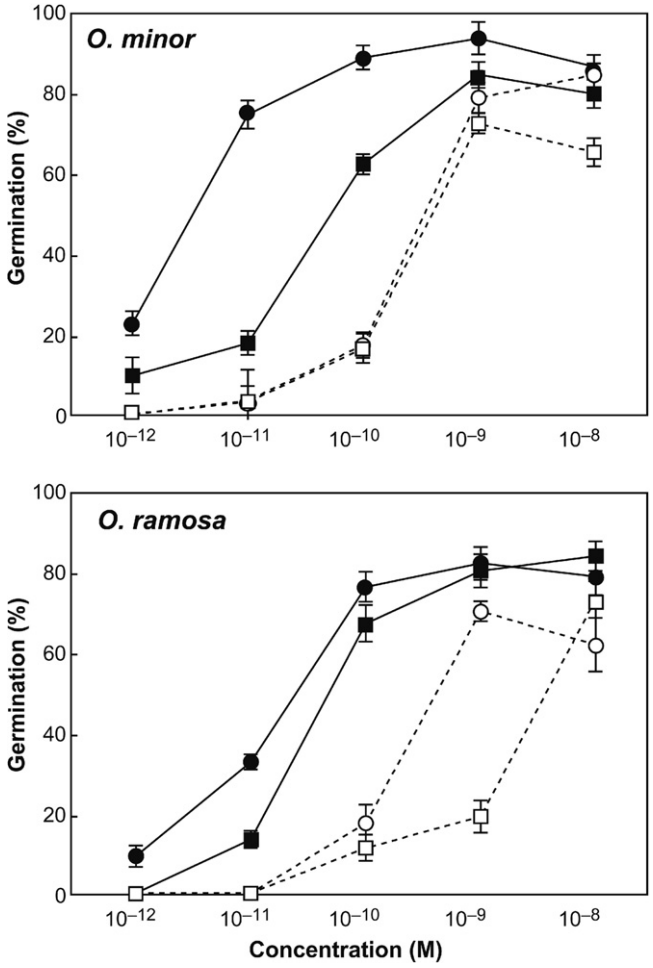

Fig. 3. Germination stimulation activity of (+)-orobanchol (closed circle), (+)-orobanchyl acetate (**3**) (open circle), (±)-strigol (closed square) and (±)-strigyl acetate (open square) on *O. minor* and *O. ramosa* seeds.

Germination stimulation activities of (+)-orobanchyl acetate (**3**), (+)-orobanchol, (±)-strigol and (±)-strigyl acetate on *O. ramosa* and *O. minor* seeds are shown in Fig. 3. In the case of orobanchol, the acetylation of the hydroxyl group in (**3**) resulted in about 100-fold and 10-fold reduction in germination stimulation activity on *O. minor* and *O. ramosa*, respectively. Similar reduction of activity associated with the acetylation of the hydroxyl group was observed with strigol but the reduction of activity was more significant against *O. ramosa* (100-fold) than *O. minor* (10-fold). These results suggest that acetates of hydroxy-strigolactones are less active as germination stimulants. However, we found that they are more stable through purification procedures than the corresponding hydroxy-strigolactones, suggesting that they may be more stable in subterranean environments and thus may play more significant roles in both germination stimulation of root parasites and induction of hyphal branching in AM fungi.

3. Concluding remarks

"Alectrol" was first isolated from cowpea root exudates (Müller et al., 1992). We detected "alectrol" in the root exudates of red clover (Yokota et al., 1998), soybean, and other leguminous plants (Yoneyama et al., 2006). All of these plants exude orobanchol. Ohashi et al. recently also isolated orobanchyl acetate (**3**) from cowpea root exudate (Ohashi, 2006). Further study is needed to clarify the roles of these acetate analogs in the host recognition by root parasitic plants and by AM symbionts.

4. Experimental

4.1. General procedures

^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ (δ_H 7.26, δ_C 77.0) on a JEOL Lambda 400 spectrometer. The standard pulse sequence and phase cycling were used for HMQC, HMBC, and NOE spectra. CD spectra were recorded with a JASCO J-720W spectropolarimeter in MeCN. EI-GC/MS spectra were obtained with a JEOL JMX-500 and a JOEL JMS-Q1000GC/K9 using a DB-5 (J&W Scientific, Agilent) capillary column (4 or 5 m × 0.25 mm) with He carrier gas (3 ml min^{-1}). The operating conditions were the same as reported earlier (Yokota et al., 1998). ESI-LC/MS analyses were performed using a Quattro LC tandem MS instrument from Micromass

(Manchester, UK). LC/MS analytical conditions were essentially the same as in (Sato et al., 2003, 2005). Column chromatography was conducted on silica gel (Wakogel C-300, Wako Pure Chemical Industries, Japan).

(+)-Orobanchol was a generous gift of Emeritus Professor Kenji Mori (The University of Tokyo, Japan). (±)-Strigyl acetate was prepared according to the literature (Frischmuth et al., 1991). (+)-Orobanchyl acetate (**3**) was prepared in a similar manner, with the crude product purified by silica gel CC (*n*-hexane–EtOAc) and preparative ODS-HPLC (MeCN–H_2O, 6:4, v/v). The other chemicals of analytical grade and HPLC solvents were obtained from Kanto Chemical Co. Ltd. and Wako Pure Chemical Industries Ltd.

4.2. 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* L. seeds were kindly provided by Prof. A. G. T. Babiker (ARC, Sudan), whereas seeds of red clover (*Trifolium pratense* cv. Makimidori) were obtained from a local supplier.

4.3. Orobanche seed germination assay

Germination assay on *O. minor* and *O. ramosa* seeds were conducted as reported previously (Zhou et al., 2004; Chae et al., 2004). Temperature for conditioning and germination was 23 °C and 18 °C for *O. minor* and *O. ramosa*, respectively. Each test solution, unless otherwise mentioned, contained 0.1% (v/v) acetone.

4.4. Hydroponic culture of red clover and collection of root exudates

Approximately, 5000 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 l of sterilized tap H_2O as the culture medium. These strainers in the containers were transferred to a growth room at 25 °C under continuous fluorescent illumination (*ca.* 60 μmol m^{-2} s^{-1}). The tap H_2O medium was replaced with 1 l of fresh sterilized tap H_2O every 3–4 days. The collected root exudate samples plus washings (*ca.* 1.5 l) were extracted with EtOAc (3 × 1.5 l). The EtOAc extracts were combined, washed with 0.2 M K_2HPO_4 (pH 8.3), dried ($MgSO_4$), and concentrated *in vacuo*.

4.5. Isolation of alectrol

The crude EtOAc extracts collected during a 4-week incubation were combined (127 mg) and subjected to silica gel CC using a gradient of *n*-hexane–EtOAc (100:0–0:100) as eluting solvent system to give fractions 1 through 11 (Fr. 1–11). Two major germination stimulant activities were eluted in Frs. 5 (*n*-hexane–EtOAc, 60:40) and 8 (*n*-hexane–EtOAc, 30:70), which were found to contain "alectrol" and orobanchol, respectively, by ESI-LC/MS analysis (Sato et al., 2005). Fr. 5 (16.0 mg) was further separated by preparative ODS-HPLC (Mightysil RP-18, 10 × 250 mm, 10 μm; Kanto Chemicals, Japan) with an MeCN/H_2O gradient system (60:40 to 0:100 over 40 min) as eluent at a flow rate of 3.0 ml min^{-1}. Detection was carried out at 236 nm. The active fraction (1.32 mg) eluted as a single peak at 14.4 min was collected. This fraction was purified by isocratic (MeCN–H_2O, 4:6, v/v) HPLC on an ODS-CN column (Develosil CN-UG-5, 4.6 × 250 mm, 5 μm; Nomura Chemical Co. Ltd., Seto, Japan) at a flow rate of 1.0 ml min^{-1} to give pure "alectrol" (0.58 mg, R_t 32.8 min) which was identified as orobanchyl acetate (**3**).

4.6. (+)-Orobanchyl acetate (*3*)

^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.14 (3H, *s*, 8-Me), 1.16 (3H, *s*, 8-Me), 1.38–1.52 (2H, *m*, 7-CH_2), 1.70 (2H, *m*, 6-CH_2), 1.96 (2H, *m*, 5-CH_2), 2.03 (3H, *t*, J = 1.5 Hz, 4'-Me), 2.04 (3H, *s*, 2''-H), 3.45 (1H, *ddd*, J = 7.3, 2.9 and 2.0 Hz, 3a-H), 5.61 (1H, *d*, J = 7.8 Hz, 8b-H), 5.74 (1H, *s*, 4-H), 6.15 (1H, *t*, J = 1.5 Hz, 2'-H), 6.94 (1H, *t*, J = 1.5 Hz, 3'-H), 7.46 (1H, d, J = 2.9 Hz, 9-H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 10.7 (C4'-Me), 18.8 (C6), 21.0 (C2''), 23.7 (C5), 27.4 (C8-Me), 27.9 (C8-Me), 28.0 (C8), 38.7 (C7), 45.3 (C3a), 82.9 (C4), 85.5 (C8b), 99.7 (C2'), 110.6 (C3), 136.2 (C8a), 136.2 (C4'), 140.9 (C4a), 150.4 (C9), 170.1 (C2), 170.7 (C5'), 171.8 (C1''). GC-EIMS, 70 eV, *m/z* (rel. int): 346 (10), 328 (25), 249 (15), 232 (80), 231 (100), 217 (28), 203 (30), 97 (95). CD (MeCN; *c* 0.00016) λ_{max} ($\Delta\varepsilon$) 255 (–5.75), 217 (+52.70) nm.

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Appendix A. Supplementary data

The CD spectrum of orobanchyl acetate, and the three-channel MRM chromatograms of red clover root exudate (recovered growth medium was analyzed directly without solvent extraction) and of the $CHCl_3$ extract of cowpea root exudates were supplied as PDF files. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2007.07.017.

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