

# Germination stimulant from root exudates of *Vigna unguiculata*

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**Abstract** Root exudate of *Vigna unguiculata* was extracted from a soil system consisting of charcoal and vermiculite. Germination stimulating activity for *Striga gesnerioides* was found in extracts of the soil system, and an active compound was isolated. The chemical structure of the active ingredient was determined to be (+)-4-*O*-acetylrobanchol, based on analysis of the spectral data of 1-D and 2-D NMR together with nuclear Overhauser effect (NOE) experiments. Application of the active compound to the seeds of *S. gesnerioides* at a concentration of  $0.35 \times 10^{-9}$  mol/disk led to 69% germination. The germination observed with application of GR-24, a positive control, at  $0.57 \times 10^{-10}$  mol/disk was 80%.

**Keywords** Strigolactones · Alectrol · Orobanchol · Germination releaser · *Striga gesnerioides*

## Introduction

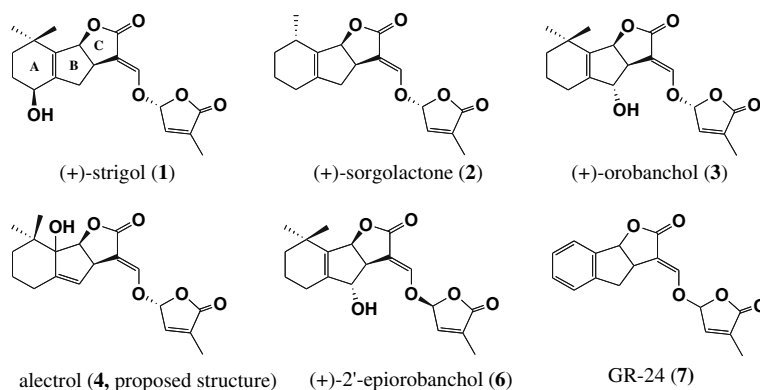
Strigolactones (1–4, Fig. 1) are indispensable signaling compounds in the life cycle of the root parasites *Striga* spp. and *Orobanche* spp. These compounds

serve as germination agents for seeds of these parasitic weeds, which may lie dormant in the soil for 15–20 years. Important food crops, such as maize, sorghum, millet, and rice, are host plants that receive immense damage by these parasitic weeds. The first naturally occurring germination stimulant discovered, (+)-strigol (1) (Cook et al. 1972), was isolated from root exudates of a false host plant, cotton (*Gossypium hisulium* L.), which is not attacked by the root parasites. Strigol is an extremely active ingredient, with concentrations of  $10^{-11}$  M being capable of releasing seed dormancy. Three other strigolactones (2–4) have been isolated from root exudates of host plants, namely (+)-sorgolactone (2) from sorghum (*Sorghum bicolor*) (Hauck et al. 1992), (+)-orobanchol (3) from red clover (*Trifolium pratense*) (Yokota et al. 1998), and alectrol (4) from cowpea (*Vigna unguiculata*) and red clover (Müller et al. 1992; Yokota et al. 1998). The deduced structures of 1–3 have been reconfirmed by synthetic studies (Sugimoto et al. 1998; Matsui et al. 1999; Hirayama and Mori 1999), although the structure of 4 proposed by Müller et al. (1992) was pointed out to be in error by the synthetic studies of Mori et al. (1998). The active compound which acts as a germination stimulant for the parasites, *Striga gesnerioides*, from cowpea has been isolated, but the determination of its chemical structure remains to be solved. In recent years, we reported on an anti-fungal compound isolated from root exudates of *Zea mays* (Park et al. 2004) and *Solanum abutiloides* (Yokose et al. 2004) using a soil

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**Fig. 1** Structures of the germination stimulants, (+)-strigol (1), (+)-sorgolactone (2), (+)-orobanchol (3), alectrol (4), (+)-2'-epiorobanchol (6), and GR-24 (7)



system, which consisted of charcoal and vermiculite. We applied this methodology to the investigation of germination stimuli for the seeds of *S. gesnerioides* produced by cowpea. In this report, we describe the isolation and structural elucidation of a novel strigolactone from cowpea root exudate.

## Material and methods

### General

Field desorption mass spectra (FD-MS) and electron ionization mass spectra (EI-MS) were obtained on JEOL JMS-SX102A and JMS-AX-300 mass spectrometers, respectively. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AMX-500 instrument ( $^1\text{H}$  at 500 MHz;  $^{13}\text{C}$  at 125 MHz). In the  $^1\text{H}$ -NMR spectra, chemical shifts are reported using TMS as an internal standard. In the  $^{13}\text{C}$ -NMR spectra, chemical shifts are reported as  $\delta$  (ppm) values relative to the carbon signals ( $\delta$  77.0) of  $\text{CDCl}_3$ . Silica gel 60 F<sub>254</sub> TLC plates (0.25 and 0.5 mm thickness; Merck, Darmstadt, Germany) were used for analytical and preparative TLC, respectively, and silica gel column chromatography was carried out using silica gel (Wako-gel C-200) provided by Wako Pure Chem. (Japan). GR-24 was purchased from Prof. Binne Zwanenburg, University of Nijmegen, The Netherlands. Charcoal (activated and granular type) and vermiculite (s size) were purchased from Wako Pure Chem. Co., Ltd. and Showa Vermiculite Co., Ltd., respectively.

### Source of seeds

Seeds of *S. gesnerioides* were collected in the northern part of Ghana in 2000 with the advice from

Dr. E. O. Owusu, University of Ghana, Legon. Seeds of cowpea were kindly gifted from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT).

### Plant material

Sixteen seeds of cowpea were sown in a planter filled with 11 L of a mixture of charcoal and vermiculite (1:2 v/v), and the total 64 plants across four planters were grown in a greenhouse for a period of three months from May. The plants were watered with tap water, and liquid HYPONeX (N-P-K: 6-10-5, HYPONeX Japan Co., Ltd.) was used as a nutrient source and given once every three days at a concentration of 1/1,000 from above the planters.

### Bioassay for seed germination of *S. gesnerioides*

A bioassay for evaluating the seed germination activity was performed according to the reported method (Visser 1975). Seeds of the parasites (*S. gesnerioides*) were preconditioned at 30°C in a water-saturated atmosphere for 10 days. A certain amount of test compound was dissolved with acetone (10  $\mu\text{l}$ ), and to this solution was added  $\text{H}_2\text{O}$  (990  $\mu\text{l}$ ). A portion (17  $\mu\text{l}$ ) of the solution was added to the disk having the seeds of *S. gesnerioides* with on it. Germination tests were determined after 24 hour. Each test was replicated by two times.

### Isolation of compound 5

The seed germination stimulatory activity of all extracts and fractions were evaluated using seeds of

the parasite, *S. gesnerioides*, as described above. After cultivation of the aerial parts of the plants, the mixture of charcoal and vermiculite was carefully separated from the roots in a plastic container filled with tap water. Stainless steel columns ( $\phi$  45 cm; 45 cm) were filled with the vermiculite/charcoal mixture, which was rinsed with water and eluted sequentially with EtOH (36 L  $\times$  2), EtOAc (36 L  $\times$  2), which gave fractions EtOAc-I and -II, and *n*-hexane (36 L  $\times$  2). Germination stimulant activity was observed in EtOAc-I, which was evaporated to dryness in vacuo to give a dark brown residue (255 mg). The residue was subjected to silica gel column chromatography (C-200, Wako gel, 45 g) using CHCl<sub>3</sub> (100 ml  $\times$  3), which gave Fr. 1–3, and 2% MeOH/CHCl<sub>3</sub> (100 ml  $\times$  3), which gave Fr. 4–6, and MeOH (300 ml). The mixed eluate of Fr. 5 and 6 was evaporated to dryness in vacuo to give a dark brown residue (45 mg). The residue was subjected to silica gel column chromatography (C-200, Wako gel, 4 g) using 10% EtOAc/CHCl<sub>3</sub> (50 ml) to give Fr. A–G. Fr. E was subjected to preparative TLC using *n*-hexane/EtOAc/AcOH (66:33:1, v/v/v) to give **5** (0.8 mg), and Fr. F was also subjected to preparative TLC using *n*-hexane/EtOAc/AcOH (66:33:1, v/v/v) to give **5** (0.4 mg).

Compound **5**: colorless oil;  $[\alpha]_D^{26} + 20.0^\circ$  (c 0.23, CHCl<sub>3</sub>); FD-HR-MS  $m/z$ : 389.1605 [M + H]<sup>+</sup> (calcd. for C<sub>21</sub>H<sub>25</sub>O<sub>7</sub>, 389.1600); FD-MS: 389 [M + H]<sup>+</sup> (99), 292 (100), 97 (91); EI-MS (rel. int.): 346 (4), 328 (22), 249 (12), 232 (80), 231 (100), 203 (26), 97 (42); <sup>1</sup>H-NMR (Table 1); <sup>13</sup>C-NMR (Table 2).

## Results and discussion

Extraction of the components absorbed by the soil system was achieved by eluting compounds in the soil using ethanol, ethyl acetate, and *n*-hexane. Germination activity for *S. gesnerioides* was found in the ethyl acetate eluate. The ethyl acetate eluate derived from a total of 64 plants was concentrated and subjected to a series of silica gel column chromatography steps to give **5** (1.2 mg).

The FD-MS of **5** had a pseudomolecular ion peak at  $m/z$  389 [M + H]<sup>+</sup> and indicated cleavages from  $m/z$  389 (C<sub>21</sub>H<sub>25</sub>O<sub>7</sub>) to peaks at  $m/z$  292 (M<sup>+</sup>+H-C<sub>5</sub>H<sub>5</sub>O<sub>2</sub>) and 97 (C<sub>5</sub>H<sub>5</sub>O<sub>2</sub>). The ion at  $m/z$  97 is typical of strigolactones. The cleavage ion of  $m/z$  97 was also observed in EI-MS. The molecular formula of  $m/z$  389 [M + H]<sup>+</sup> was deduced to be C<sub>21</sub>H<sub>25</sub>O<sub>7</sub> by HR-FD-MS. The UV spectrum of **5** had a  $\lambda_{\max}$  at

**Table 1** <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 500 MHz) assignments of **5**, (+)-orobanchol (**3**), and (+)-2-epiorobanchol (**6**)

	Compound ( <b>5</b> )	Alectrol ( <b>4</b> ) <sup>a</sup>	(+)-orobanchol ( <b>3</b> ) <sup>b</sup>	2-epiorobanchol ( <b>6</b> ) <sup>b</sup>
H-9	7.46 (1H, d, <i>J</i> = 1.5 Hz)	7.44 (1H, d, <i>J</i> = 2.4 Hz)	7.46–7.48	7.50–7.52
H-3'	6.94 (1H, d, <i>J</i> = 1.5 Hz)	6.93 (1H, s)	6.94–6.96	6.95–6.98
H-2'	6.15 (1H, s)	6.14 (1H, s)	6.20–6.23	6.17–6.20
H-4	5.73 (1H, s)	5.72 (1H, s)	4.56–4.60	4.53–4.57
H-8b	5.61 (1H, d, <i>J</i> = 7.0 Hz)	5.59 (1H, d, <i>J</i> = 6.6 Hz)	5.59–5.63	5.58–5.63
H-3a	3.45 (1H, d, <i>J</i> = 7.0 Hz)	3.43 (1H, d, <i>J</i> = 6.6 Hz)	3.39–3.44	3.39–3.44
H-2''	2.05 (3H, s)	– <sup>c</sup>		
C4'-Me	2.03 (3H, s)	2.01 (3H, s)	2.01–2.04	2.01–2.03
H-5	1.87–1.99 (2H, m)	– <sup>c</sup>	2.09–2.22 (1H) 1.19–2.02 (1H)	1.91–2.00
H-6	1.64–1.76 (2H, m)	– <sup>c</sup>	1.66–1.74	1.63–1.73
H-7	1.36–1.52 (2H, m)	– <sup>c</sup>	1.37–1.52	1.35–1.51
C8-Me $\alpha$	1.15 (3H, s)	1.14 (3H, s)	1.14	1.13
C8-Me $\beta$	1.13 (3H, s)	1.11 (3H, s)	1.12	1.12

<sup>a</sup> Resonances were referred from the reported data of Yokota et al. (1998)

<sup>b</sup> Resonances were referred from the reported data of Hirayama et al. (1999)

<sup>c</sup> Data are not listed (Yokota et al. 1998)

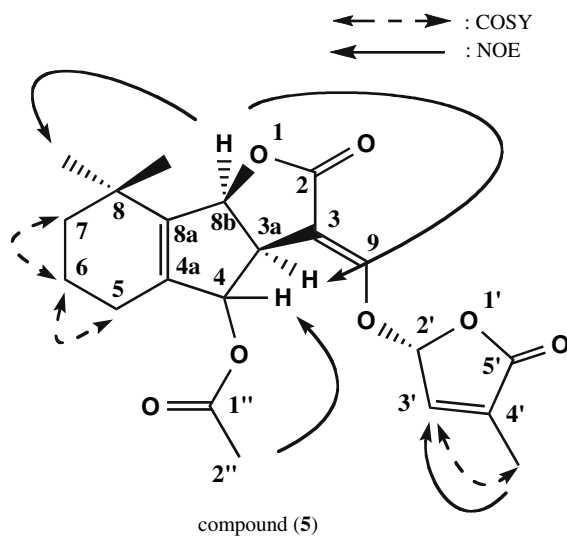
**Table 2**  $^{13}\text{C}$ -NMR ( $\text{CDCl}_3$ , 125 MHz) assignments of **5**, (+)-orobanchol (**3**), and (+)-2-epiorobanchol (**6**)

	Compound ( <b>5</b> )	(+)-orobanchol ( <b>3</b> ) <sup>a</sup>	2-epiorobanchol ( <b>6</b> ) <sup>a</sup>
C-2	170.5 <sup>b</sup>	171.0 <sup>b</sup>	171.0 <sup>b</sup>
C-3	110.5	111.3	111.2
C-3a	45.2	48.3	48.2
C-4	82.8	83.0	83.0
C-4a	140.1	143.0	143.0
C-5	23.7	23.6	23.5
C-6	18.8	18.9	18.9
C-7	38.7	38.7	38.8
C-8	32.3	32.1	32.1
C-8a	146.5	144.1	144.1
C-8b	85.5	85.9	85.9
C-9	150.4	150.5	151.1
C-2'	99.7	100.3	100.6
C-3'	140.9	140.9	141.0
C-4'	136.5	136.3	136.0
C-5'	170.2 <sup>b</sup>	170.1 <sup>b</sup>	170.2 <sup>b</sup>
C-1''	169.7		
C-2''	21.0		

<sup>a</sup> Resonances were refer from the reported data of Hirayama et al. (1999)

<sup>b</sup> Values are interchangeable within each column

236 nm, which was observed by a photodiode array detector (data not shown), the same as the spectrum of **1**. The  $^{13}\text{C}$ - and  $^1\text{H}$ -NMR spectra (Tables 1 and 2) indicated the presence of four methyl and three carbonyl carbons and three pairs of olefin carbons. Further confirmation by  $^1\text{H}$ - $^1\text{H}$  correlated spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond correlation (HMBC), and NOE differential experimental data revealed a planar structure of **5** to be 4-*O*-acetylrobanchol as in Fig. 2. The structure of **5** closely resembled that of (+)-orobanchol (**3**) (Yokota et al. 1998) except for the resonances due to those derived from an acetyl moiety ( $\delta$  169.7 and 21.0 in  $^{13}\text{C}$ -NMR,  $\delta$  2.05 in  $^1\text{H}$ -NMR, Tables 1 and 2). The above experimental determination that **5** should be acetylrobanchol was supported due to **3** having the molecular ion of  $m/z$  346. Although the resonance of  $\delta$  4.56–4.60 (H-4) of (+)-orobanchol (**3**) in  $^1\text{H}$ -NMR was not observed in that of **5**, there was a resonance of  $\delta$  5.73 (H-4) in that of **5** as a downfield peak shift. From the observation of the down field shifted

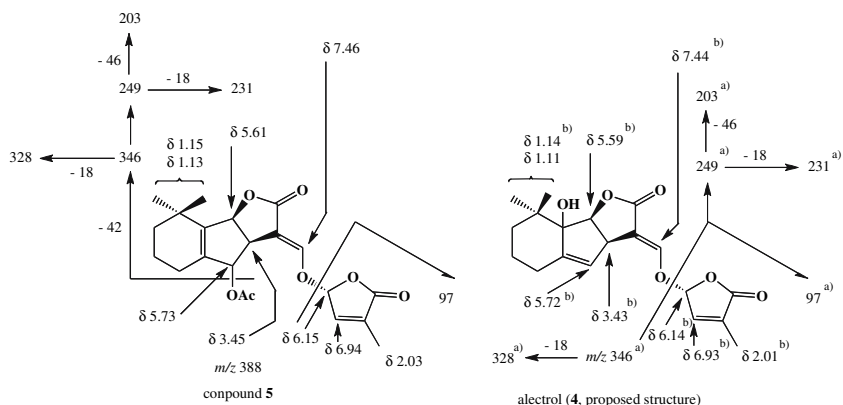


**Fig. 2** Structure of **5** isolated from the eluate of the root of *Vigna unguiculata* and its important correlations in  $^1\text{H}$ - $^1\text{H}$  COSY and NOE experiments

resonance, the attached position of the acetyl moiety in **5** was substantiated to be C-4. Since there were remarkable differences in the resonances of H-9 between **5** ( $\delta$  7.46) and (+)-2-epiorobanchol (**6**, Fig. 1) ( $\delta$  7.50–7.52), which was synthesized (Hirayama and Mori 1999), but not between **5** and **3**, the structure of **5** was determined to be (+)-4-*O*-acetylrobanchol (Fig. 2). The absolute configuration of (+)-orobanchol (**3**) was revealed as shown in Fig. 1, and its  $[\alpha]_D$  was reported to be  $+158^\circ$  ( $c$  0.38,  $\text{CHCl}_3$ ) (Hirayama and Mori 1999). Although the optical rotation of **5** showed  $[\alpha]_D +20^\circ$  ( $c$  0.30,  $\text{CHCl}_3$ ), the absolute configuration of **5** could not be determined due to the structural difference between **3** and **5**.

To investigate the germination activity of **5**, the seeds of *S. gesnerioides* were independently incubated with **5**, GR-24 (**7**, Fig. 1) that was one of the synthetic stiolgolactone analogues, and  $\text{H}_2\text{O}$ . When the application of GR-24 (**7**,  $0.57 \times 10^{-10}$  mol/disk) showed 80% germination rate, that of **5** ( $0.35 \times 10^{-9}$  mol/disk) showed 69% germination rate. In the same time, the  $\text{H}_2\text{O}$ -treated control showed 1% germination rate. The biological activity of **5** was determined to be almost one tenth of that of GR-24 (**7**). Although we could compare the ability of releasing seed dormancy of *S. gesnerioides* between (+)-4-*O*-acetylrobanchol (**5**) and GR-24 (**7**), we could not perform the test using strigol (**1**), sorgolactone (**2**) and orobanchol (**3**) due to no authentic samples of **1**–**3**.

**Fig. 3** EI-MS and GC-EI-MS fragmentation patterns and partial assignments ( $\delta$ ) of  $^1\text{H-NMR}$  for **5** and **4**. (a) The cleaved ions reported by Yokota et al. (1998), (b) the resonances reported by Yokota et al. (1998)



In this paper, we reported **5** as the active ingredient to stimulate the germination of *S. gesnerioides*. Since alectrol (**4**) had been reported as a germination release factor in cowpea with its molecular weight as  $m/z$  346 (Müller et al. 1992), it could be concluded that compound **5** was not alectrol (**4**) due to the difference in molecular weight between **4** and **5**. But there is some possibility that the reported molecular weight ( $m/z$  346) by Müller et al. (1992) and Yokota et al. (1998) might be the cleavage derived from the original compound. In the reports of Müller et al. (1992) and Yokota et al. (1998), they used desorption chemical ionization and GC-EI to determine the molecular weight of alectrol (**4**,  $m/z$  346), respectively. In our experiments, molecular ion peak of **5** could not be found by EI-MS, which gave a cleavage ion of  $m/z$  346 together with similar pattern of cleaved ions (Fig. 3) with those of alectrol (**4**) reported by Yokota et al. (1998). Instead of using EI-MS, the pseudomolecular ion peak ( $m/z$  389) could be observed by employing the measurement of FD-MS, which was milder ionization method than EI-MS, but, even if the FD-MS measurement, the cleavages were found. It seemed that the measurement of MS for **5** should be needed subtle approach. Due to the observation of the ion,  $m/z$  346, in our EI-MS and previous experiments by Müller et al. (1992) and Yokota et al. (1998), good accordance of  $^1\text{H-NMR}$  resonances between **5** and alectrol (**4**) (Fig. 3), and the evidence of natural occurrence of 5-*O*-acetylstrigol (Strigil acetate, Cook et al. 1972; Sato et al. 2005), it is not too far from the truth to suppose that the real chemical structure of alectrol (**4**) might be (+)-4-*O*-acetyl-orobanchol (**5**). Since at present we have no access to the natural product itself, we conclude that

reisolation of alectrol (**4**) must be carried out in order to resolve this uncertainty.

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