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Germination Stimulants Produced by *Vigna unguiculata Walp* cv Saunders Upright

Sigrid Müller,* Christian Hauck, and Hermann Schildknecht

Organisch-Chemisches Institut, Universität Heidelberg, Im Neuenheimer Feld 270, W-6900 Heidelberg, FRG

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Abstract. A germination stimulant, alectrol, for the seeds of the angiospermous root parasites *Alectra vogelii Benth.* and *Striga gesnerioides (Willd.) Vatke* has been isolated from root exudates of its genuine host plant *Vigna unguiculata Walp* cv Saunders Upright. Its spectroscopic data lead to a chemical structure closely related to (+)-strigol. The compound is more active than strigol in stimulating seeds of the respective parasites, *Alectra vogelii* and *Striga gesnerioides*.

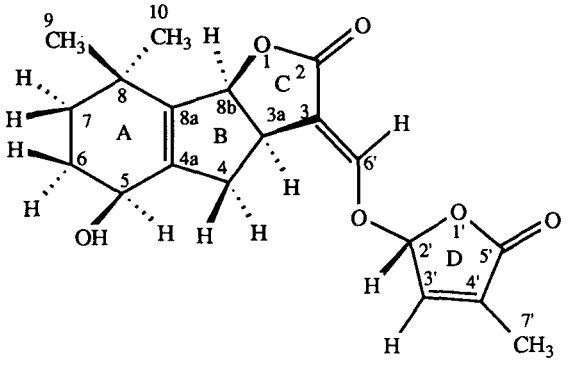
Root parasite flowering plants of the genera *Alectra, Striga* (Scrophulariaceae), and *Orobanche* (Orobanchaceae) have developed an interesting chemical ecology. Apparently, they have found an unchallenged ecophysiological and biochemical niche. The parasitic seeds are able to recognize their correct host plants, because they germinate only in their presence. Basic to this phenomenon is the chemical communication between parasite and host: seed germination is initiated by chemical substances occurring in the root exudates of their respective host plants. There seems to be a great variety of these so-called germination stimulants because different host plants produce different compositions of different stimulants, as demonstrated by chromatographic investigations (Sunderland 1960, Visser et al. 1974, 1987). The first stimulating compound, strigol (Fig. 1), was isolated from cotton, *Gossypium hirsutum*, a false host, which could not be attacked by the parasitic plants (Cook et al. 1966). Its structure and the absolute configuration have been determined (Cook et al. 1972, Brooks et al. 1985). Many strigol analogs have been synthesized since then; the most active are termed GR-compounds (GR, germination releaser) (Johnson et al. 1981). GR-7 (Fig. 1) was used as a standard in our laboratories. However, none of the synthetic germination stimulants were as active as the compound isolated from cotton. A 10^{-10} M solution of (+)-strigol stimulated 50% of the seeds of witchweed to germinate. Early attempts to isolate the stimulants from genuine host plants failed because of the extremely small amount of these unstable substances (Brown et al. 1951, 1952, Saunders 1933). Chang et al. (1986) identified quinones, so-called sorgoleons, as hydrophobic components in root exudates of sorghum as germination stimulants for witchweed. *Vigna* was found to produce at least four different germination stimulants with different chromatographic behavior (Brown et al. 1946, Visser et al. 1987). In the present study we report the first isolation of the most active germination stimulant, alectrol, from *Vigna unguiculata*, a genuine host for *Alectra vogelii* and *Striga gesnerioides* (Botha 1948, Visser 1975) and a false host for *Striga asiatica, Striga hermonthica*, and *Orobanche aegyptiaca* and propose a structure based on the spectroscopic data.

Abbreviations. AV, *Alectra vogelii;* OA, *Orobanche aegyptiaca;* SA, *Striga asiatica;* SG, *Striga gesnerioides;* SH, *Striga hermonthica;* TFA, trifluoroacetic acid.

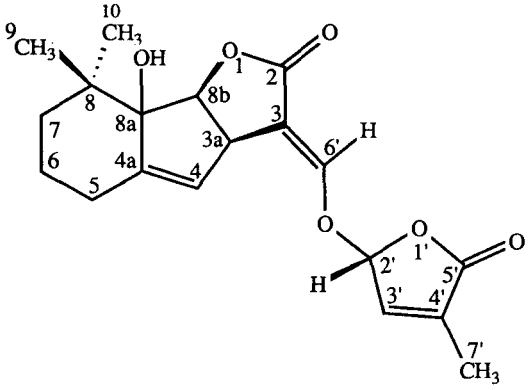
Materials and Methods

Plant Materials

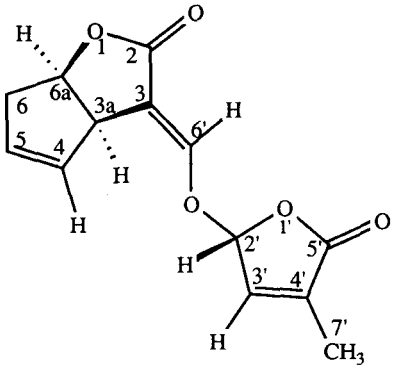
Host plants were commercial *Vigna unguiculata* Walp cv Saunders Upright. Seeds of *Alectra vogelii, Orobanche aegyptiaca, Striga asiatica, Striga gesnerioides,* and *Striga hermonthica* were harvested in 1981, 1975, 1986, 1987, and 1980, respectively.



(+)-Strigol



Alectrol (proposed structure)



GR-7

Fig. 1. Structures of the germination stimulants (+)-strigol, GR-7, and alectrol.

Strigol

Racemic (±)-strigol was synthesized in 1974 (Heather et al. 1976). It was separated into its enantiomers by high-performance liquid chromatography (HPLC) on cellulose triacetate (Hauck et al. 1990).

Bioassay

The germination test was performed in accordance with Visser (1975); seeds of the parasites were preconditioned at 30°C (AV, SA, SG, and SH) and 25°C (OA) in a water-saturated atmosphere for 6 days (AV, SG) and 10 days (SA, SH, and OA). Germination tests were determined 24 (SA, SH), 48 (AV, SG), or 72 (OA) h after application of the test solution. Each test was replicated at least three times. Where possible, data were subjected to statistical treatment by calculating standard deviations.

Recovery of Stimulants

Plants were cultivated in Stellenbosch, South Africa, in stainless steel trays, 250 × 100 × 10 cm deep, as described previously (Visser et al. 1987). About 15,000 *Vigna unguiculata* plants were cultivated in each tray. Drainage water with a flow of 10 L/h was recycled through a column filled with about 500 g of XAD-4 resin (Serva, Heidelberg, FRG), which was replaced every 2 weeks. After 2 months new host plants were cultivated. About 20 batches were collected and desorbed with methanol in a Soxhlet extractor under reduced pressure. During the whole period of isolation the temperature of the solutions containing the stimulants didn't rise above 40°C. At temperatures above 40°C a decrease in activity was found. The crude extract (35.75 g) was dissolved in 1 L methanol/water 70/30 and extracted for 24 h with 250 ml dichloromethane by a continuous liquid–liquid extractor. The extract was evaporated to dryness, yielding 13.7 g.

Chromatography

A glass column (111 × 3 cm) was filled with Florisil 100–200 mesh (Serva) and equilibrated with 5% methanol in dichloromethane. The extract (13.7 g) was dissolved in 1 ml of the 5% methanol solution and chromatographed on Florisil with 100 ml of dichloromethane (A), 200 ml of methanol/dichloromethane 80/20 (B), and 100 ml of methanol (C). The three eluents were col-

lected separately and evaporated to dryness with a yield of 3.4 g A, 2.5 g B, and 7.8 g C. The activity was found in fraction B.

HPLC

HPLC systems of different manufacturers were used with variable wavelength UV detectors and with a photodiode array detector. Different detection wavelengths were applied, but 240 nm proved to be ideal. Different columns manufactured by Macherey & Nagel were used: Nucleosil, C-18, 250 × 16 mm; Nucleosil, C-18, 5 μ, 250 × 8 mm; Nucleosil, C-18, 5 μ, 250 × 4 mm; Polygosil, C-18, 250 × 30 mm; Aminex HPX 87H, 300 × 7.8; and Nucleosil, Phenyl, C-18, 5 μ, 250 × 8 mm. Flow rates were 0.5, 2.0, 8.0, and 24 ml/min with column diameters of 4, 8, 16, and 30 mm, respectively. Fractions were collected in test tubes and diluted 1:10 repeatedly for bioassays. Active fractions, which were not separated from inactive fractions, were combined, evaporated to dryness, and used for further separation.

Spectroscopic and Spectrometric Methods

UV spectra were recorded with a photodiode array detector Merck-Hitachi L-3000 or with a UV-spectrometer Hitachi U 3200 in 40% acetonitrile in water. Circular dichroism spectra were recorded with Yobin Yvon Mark III circular dichrograph in 40% acetonitrile in water. Mass spectra were recorded with a VG ZAB high-resolution mass spectrometer, source temperature 220°C, electron energy 70 eV with polyfluorokerosene (PFK) as reference substance. Infrared spectra in KBr were recorded with a Bruker IFS-86 spectrometer. ^{1}H-Nuclear magnetic resonance spectra were recorded with Bruker AM 500 spectrometer, with stimulants dissolved in $CDCl_3$ and C_6D_6.

Results

For the detection of the germination stimulants a highly sensitive bioassay has been performed as described previously (Visser 1975). In order to optimize this bioassay, the optimal germination conditions for the respective seeds of the angiospermous flowering plants were determined. An important factor in the germination process is the so-called conditioning of the seeds, a treatment which sensitizes the seeds prior to germination by exposing them to a water-saturated atmosphere for some time. According to previous observations (Botha 1948, Visser 1989) the optimal temperatures for both processes, conditioning and germination, were 30°C for the genera *Alectra* and *Striga* and 20–25°C for the genera *Orobanche*. In order to obtain the optimal time of conditioning for the respective seeds, we determined their germination rates after application of 10 μl of a standard solution of the synthetic stimulant GR-7, 10^{-5}–10^{-8} M, to the seeds at different conditioning times (Fig. 2). As shown in Fig. 2, the seeds of AV were sensitive to germination after 4–5 days and 10–11 days of conditioning at the low concentration. The seeds of SG germinated after 4–7 days. Strong decreases of germination of AV and SG after 15 days were observed. The seeds of SA attained the maximum after 9 days and a continuous maximal plateau was characteristic. The seeds of SH germinated after 11–14 days. The seeds of OA showed two maxima after 5 and 11 days, respectively. Both seeds showed the gently descending slope of the curve. According to these results germination tests were preconditioned for 6 (AV, SG) and 10 (SA, SH, OA) days. During the isolation of the germination stimulants all separation steps were monitored by bioassays, performed as described above: fractions were diluted 1:10 repeatedly and tested with seeds of different parasitic flowering plants.

The XAD-4 resin has been shown to be efficient in absorbing the germination stimulants released by the roots of *Vigna unguiculata* (Tang and Young 1982, Visser et al. 1987). The desorption of an active fraction with methanol and the extraction of the methanol phase with dichloromethane separated the stimulants from more polar and inactive compounds. By chromatography on Florisil the activity was effectively recovered in the second midpolar fraction. Further separation of the isolated peak was done by HPLC. Since the activity was lost after silica gel chromatography, only reversed-phase columns were used. For analytical HPLC separation (Nucleosil C-18), a solvent gradient from 40% methanol in water to 100% methanol eluted all injected compounds. In order to test the ability of the collected fractions to stimulate the germination of different parasitic seeds, the fractions were diluted 1:10 repeatedly. Figure 3 displays two zones of activity, S and S-3, which were more active in stimulating seeds of *Alectra vogelii* and *Striga gesnerioides*, the respective genuine parasite plants of *Vigna unguiculata*, than seeds of *Striga asiatica* and *S. hermonthica*. We also found response by the seeds of *Orobanche aegyptiaca*, although they required higher concentrations of the stimulants. For preparative isolation we focused our attention on the most active fraction S-3. Fraction S turned out to consist of three active compounds, which were separated by HPLC, but the final yield (<1 μg each) was too small for further structural investigation (Müller 1991). For the isolation of the pure germination stimulant from S-3, six separation steps under various HPLC conditions were necessary, as listed in Table 1.

The isolated compound is comparable to strigol in both its chromatographic properties and UV spectrum (λ max = 236 nm). But the different biological activities—strigol was most active in stimulating the seeds of witchweed (SA), whereas the stimulant of

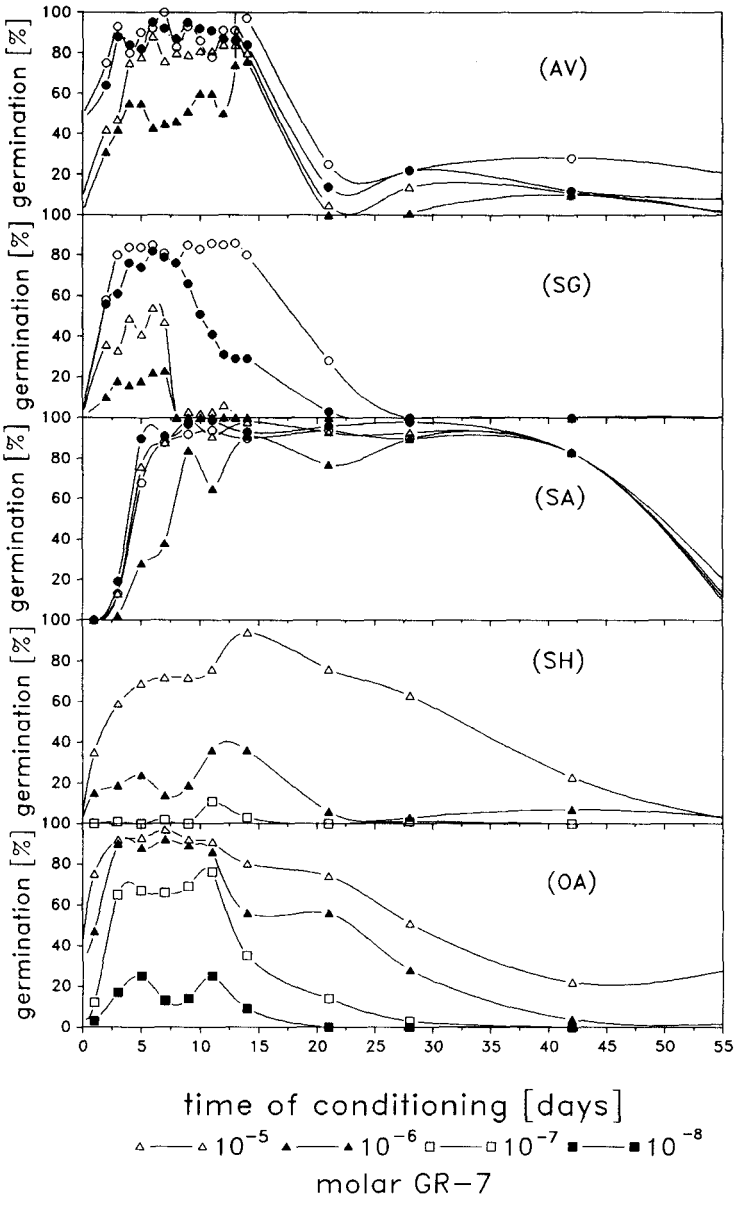


Fig. 2. Procentual germination of the seeds of *Alectra vogelii* (AV), *Striga gesnerioides* (SG), *Striga asiatica* (SA), *Striga hermonthica* (SH), and *Orobanche aegyptiaca* (OA), respectively, preconditioned at different times.

Vigna unguiculata favored *Alectra vogelii*—and the different retention times under various HPLC conditions, as listed in Table 1, gave evidence of two different compounds. The new compound was termed alectrol because of its high germination activity for seeds of *Alectra vogelii* and the presence of an alcoholic functional group in its structure, as described below.

The absolute structure of (+)-strigol is known from its determination by x-ray crystallographic analysis (Brooks et al. 1985). To elucidate the chemical structure of alectrol, we compared the spectroscopic and spectrometric data with those of (+)-strigol.

UV spectra of alectrol and strigol were identical with λ max = 236 nm, indicating similar chromophores. Using the molar extinction coefficient of 17,700 of strigol (Cook et al. 1972) the isolated amount of alectrol was calculated to be about 300 µg.

The circular dichroism spectrum of alectrol showed a positive Cotton effect at 220 nm with an absorption shoulder at 230 nm. Therefore, it differed significantly from the CD spectrum of (+)-strigol. The latter showed a positive Cotton effect in the region of the UV absorption maximum of about 236 nm.

The infrared spectrum of alectrol differed slightly

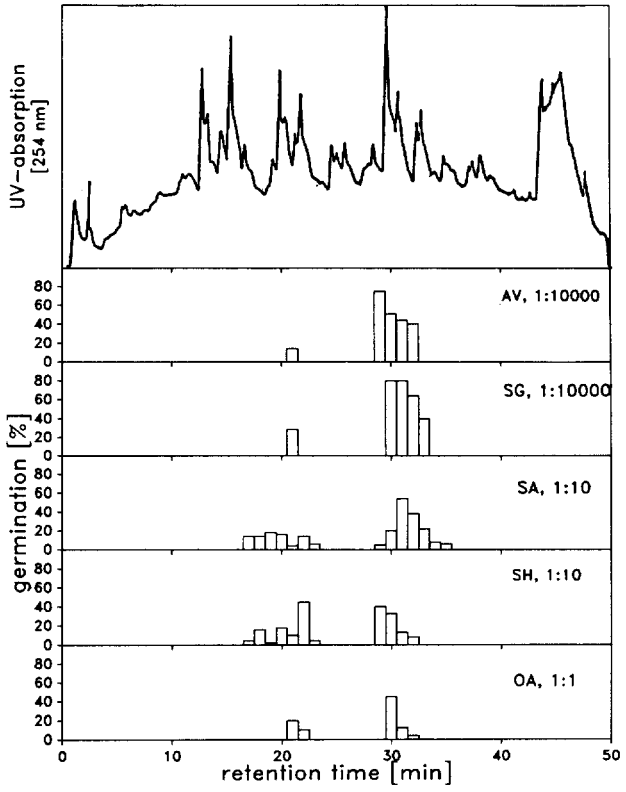


Fig. 3. Analytical HPLC separation of *Vigna* root exudates on Nucleosil C-18, 250 × 8 mm, solvent gradient 40% methanol in water to 100% methanol for 40 min, 100% methanol for 10 min, flow rate 2 ml/min, UV detection wavelength 254 nm. Germination rates of seeds of *Alectra vogelii* (AV), *Striga gesnerioides* (SG), *S. asiatica* (SA), *S. hermonthica* (SH), and *Orobanche aegyptiaca* (OA) with fractions diluted 1:10,000, 1:10, and undiluted.

Table 1. Subsequent preparative HPLC separations to isolate the germination stimulant, alectrol, from *Vigna* root exudates. The retention times of alectrol are compared to those of strigol.

Column	Solvent flow: 2 ml/min	Retention time (min) strigol	Retention time (min) alectrol	Yield (mg)
C-18	70% methanol	8.9	13.3	276
C-18	60% acetonitrile	8	14.4	18.6
Aminex HPX 87H	28% acetonitrile/ 4 mM TFA	11 16	15	10
Phenyl	40% acetonitrile/ 2 mM TFA	10.3	36.5	<1
Phenyl	63% methanol	11	15.5	0.3

in the fingerprint region below 1000 cm^{-1}, where alectrol showed two bands at 840 and 750 cm^{-1}. The most intense absorption bands, however, were identical: 2930 and 2865 cm^{-1}, 1783 and 1740 cm^{-1}, 1680 cm^{-1}, and bands around 1190, 1095, 1020, and 955 cm^{-1}.

The mass spectra of alectrol and strigol were almost identical with the base peak at m/z 97 ($C_5H_5O_2$) and a molecular peak at m/z 346. A DCI-spectra of alectrol with methane as the reactant gas generated ions of m/z 347 and proved m/z 346 as the molecular mass of alectrol. Both spectra indicated cleavages from m/z 346 ($C_{19}H_{22}O_6$) to peaks at m/z 249 ($M^+-C_5H_5O_2$), m/z 231 ($M^+-C_5H_5O_2-H_2O$), m/z 203 ($M^+-C_5H_5O_2-H_2O-CO$), which indicated a hydroxyl group in the structure of alectrol.

The proton-NMR spectrum in $CDCl_3$ of alectrol showed some differences compared to the spectrum of strigol (Cook et al. 1972). The signals belonging to the protons of ring C and D were identical in both spectra. But the signals in the spectrum of strigol at δ 2.67 and δ 4.9, belonging to the protons at C-4 and C-5, respectively, were absent in the spectrum of alectrol. New peaks appeared at δ 5.7 and δ 2.9. The signals of the methyl groups were intense enough to be assigned unambiguously. The signals appeared at δ 1.99, δ 1.16, and δ 1.08 in the spectrum of strigol in $CDCl_3$. In the spectrum of alectrol, the peak at δ 1.1 was shifted slightly to δ 1.14.

Discussion

Host plants produce a mixture of different compounds which stimulate the seed germination of their respective parasitic flowering plants (Sunderland 1960, Visser and Botha 1974). The seeds of the parasites are most effectively stimulated by one major compound. The most active compound in the root exudates of the leguminous plant *Vigna unguiculata* is a highly potent germination stimulant for *Alectra vogelii* and *Striga gesnerioides*. As is shown in Fig. 4 alectrol still stimulated 50% of the seeds of *Alectra vogelii* and *Striga gesnerioides* at a dilution of 10^{-12} M [(+)-strigol 10^{-9} M]. To stimulate the seeds of witchweed *(Striga asiatica)* a 10-fold more concentrated solution (10^{-9} M) was necessary compared to (+)-strigol (10^{-10} M, Fig. 4). For the germination of *Orobanche aegyptiaca* the value was the same: both stimulants germinated 50% of its seeds with a 10^{-8} M [(+)-strigol $10^{-8.5}$] concentrated solution (Fig. 4). Therefore, alectrol had a typical activity profile and could be easily distinguished from strigol, the germination stimulant for witchweed.

At the same time of this investigation, we also isolated a highly active germination stimulant from *Sorghum bicolor* and termed it sorgolactone (Hauck et al., in press). The three compounds, sorgolac-

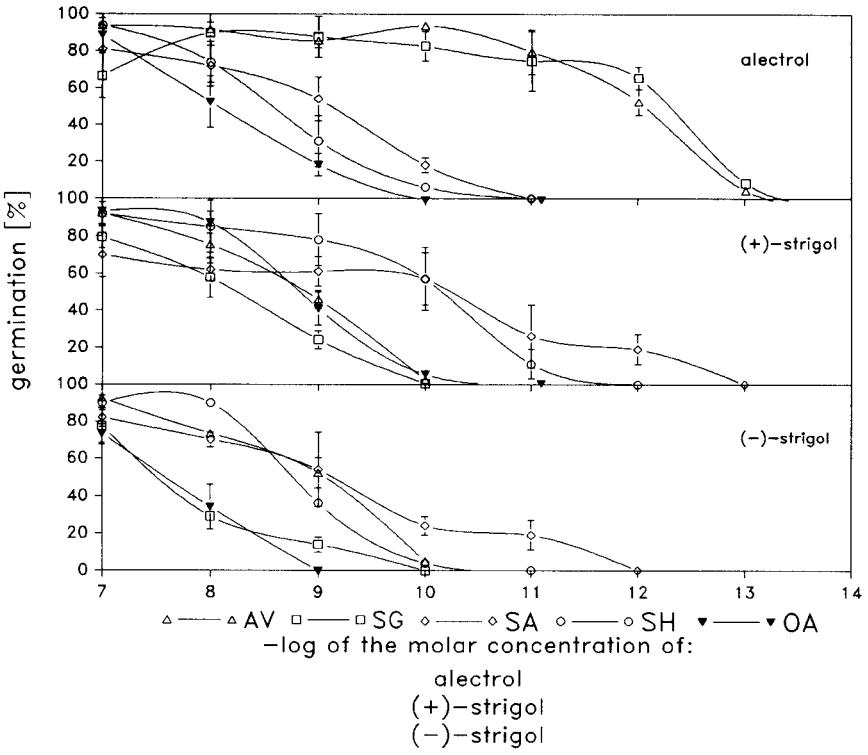


Fig. 4. Procentual germination of seeds of *Alectra vogelii* (AV), *Striga gesnerioides* (SG), *S. asiatica* (SA), *S. hermonthica* (SH), and *Orobanche aegyptiaca* (OA) after application of different concentrated solutions of alectrol, (+)-strigol, and (−)-strigol.

tone, alectrol, and strigol, seem to form a new class of plant hormones, as has been proposed previously (Cook et al. 1966). It is likely that the other germination stimulants occurring in the root exudates of *Vigna unguiculata* are highly related to strigol as we have found for alectrol. The possible effect of strigol and its analogous compounds as regulators in the germination/nongermination equilibrium has been emphasized by many authors (Hsiao et al. 1981, Kust 1966). This could explain the inhibition effect of highly concentrated solutions of strigol, alectrol, or GR-7 which we have observed repeatedly. The curves obtained with a larger amount of added stimulant differed slightly from those obtained with lower GR-7 concentrations (Fig. 2). The latter showed at least two maxima, which passed into a maximal plateau with increasing stimulant concentration. This observation agrees with a previous hypothesis (Whitney 1979, Worsham 1987) [i.e., as a possible result of a delicate balance between stimulants (promoters) and inhibitors, which is dependent on their respective concentrations]. The inhibitors are proposed to occur in the seed coat and are leached out during the conditioning period (Pavlista et al. 1979). The requirement of a conditioning period, however, has been interpreted in different ways by various workers, all still speculative at the moment (overview given by Visser 1989).

A provisional chemical structure for alectrol is given here. It is an isomer of strigol with the elementary composition $C_{19}H_{22}O_6$. The mass- and IR-spectra of the isomers are almost identical. Therefore, rings C and D bridged by the enol ether should be identical in both molecules. These facts are supported by the proton-NMR data. The proton at C-2′ and C-3′, respectively, appeared as a pentet at δ 6.9 (J = 1.5 Hz) and δ 6.1 (J = 1.5 Hz). They are coupled to each other and to the vinylic methyl group, CH_3-7′ (δ 2.0, t) with the same coupling constant (1.5 Hz) as in strigol. These data together with the positive Cotton effect in the CD spectrum of alectrol indicate the same absolute configuration at C-2′ as in (+)-strigol. The vinylic proton at C-6′ (δ 7.45, d, J = 2.5 Hz) may be compared with the analogous, E-configurated proton in (+)-strigol, since coupling constant and chemical shift are the same. The CD and NMR spectra showed significant differences close to chromophores and functional groups. The typical NMR signals in the spectrum of strigol, for the two hydrogen atoms at C-4, disappeared in the spectrum of alectrol. A new NMR signal at δ 5.75 could be assigned to an olefinic proton at C-4, which is coupled to H-3a (δ 3.45) and H-8b (δ 5.6) with coupling constants of 3 Hz, as proved by COSY-NMR. The position of H-3a is defined by its coupling to H-6′ (J = 2.5 Hz). The strong coupling between H-3a and H-8b (J = 7.2

Hz) identified the latter and contributes to the observed peak pattern of H-3a (quartet of doublet), the result of three couplings, one less than in strigol. This fact supported the data obtained by COSY-NMR and led to the assignment of the signal at δ 5.75 to H-4. The similar chemical shifts and couplings of H-8b (δ 5.6) and H-3a (δ 3.45) in alectrol compared to those in strigol (δ 5.5 and δ 3.6, respectively) indicated that ring C is the same. The double bond in alectrol has moved one position counterclockwise into ring B compared to strigol, leading to a cyclohexyl ring A in alectrol. As there was no peak close to δ 4.12, alectrol has no secondary hydroxy group at C-5 like strigol. The hydroxy group, the presence of which is proved by high-resolution mass spectral data and by a broad signal at δ 2.85 in the NMR (D_2O exchange), must have moved to the tertiary position C-8a. The absolute configuration at this center, however, is unknown.

The positions of the geminal methyl groups in the cyclohexyl ring A of alectrol were not assigned unambiguously. The COSY-NMR data in the region between δ 2 and δ 1 could not be interpreted well because of the chemical noise due to the small amount of alectrol. That is also the reason why weak couplings ($J < 3$ Hz) could not be identified in the COSY-NMR. The peak pattern of H-8b (δ 5.6) showed a triplet of a doublet, caused by a coupling to H-3a (δ 3.45) and to H-4 (δ 5.75), and an unknown third proton. The third coupling could not be identified properly. There are three reasonable coupling partners: protons at C-8, C-5 (analogous to the long range coupling, $J_{8b,5} = 2$ Hz, in strigol), or the hydroxy group at C-8a. The coupling with protons at C-8 leads to a structure with the geminal methyl groups at position C-5; the other two possibilities result in a structure with the methyl groups positioned at C-8. The latter possibility is more likely, considering the chemical shifts of the methyl groups in alectrol (δ 1.14 and δ 1.16). They showed a similar influence of the lactone ring oxygen, which was responsible for the shifted position of the protons at C-9 (δ 1.18) in the spectrum of strigol. The slight shifting of the protons at C-10 in alectrol could be due to the influence of the OH- group at C-8a. Therefore, we suggest their position at C-8, as in strigol, as the more likely one, resulting in a proposed structure for alectrol as displayed by Fig. 1.

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