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ALECTROL AND OROBANCHOL, GERMINATION STIMULANTS FOR *OROBANCHE MINOR*, FROM ITS HOST RED CLOVER

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Key Word Index—*Orobanche minor*; Orobanchaceae; clover broomrape; *Trifolium pratense*; Leguminosae; red clover; seed germination stimulant; alectrol; orobanchol; strigol.

Abstract—Three seed germination stimulants for a parasitic weed, clover broomrape (*Orobanche minor*), were isolated from the root exudate of its host, red clover (*Trifolium pratense*) and structures of the two of the stimulants were determined. One was identified as alectrol and the other was a novel strigol-related compound which was named orobanchol. Orobanchol was tentatively proposed to be a strigol isomer based on its MS and NMR spectral data. This is the first report on germination stimulants of *Orobanche* spp. from genuine host plants. GC-MS techniques to analyze strigolactones are also described.

INTRODUCTION

The broomrapes are root parasites belonging to the genus *Orobanche* in the family Orobanchaceae. Among the various species of the genus *Orobanche*, *O. ramosa*, *O. aegyptiaca*, *O. minor*, *O. cernua* and *O. crenata* are major crop parasites which are distributed mainly in Mediterranean countries, Western Asia and East Africa and cause severe damage to various crops [1–3]. The seeds of the parasites have special requirements for germination, i.e., after-ripening, conditioning and germination stimulants from host (or non-host) plants [4].

Up to the present, three germination stimulants collectively called strigolactones have been isolated from plant root exudates (Fig. 1): strigol as a germination stimulant of *Striga lutea* (*asiatica*) from cotton, a false host [5, 6], alectrol from *Vigna unguiculata*, a genuine host of *Alectra vogelii* Benth. and *S. gesnerioides* (Willd.) Vatke [7] and sorgolactone from a genuine host *Sorghum bicolor* of *S. asiatica* and *S. hermonthica* [8]. Recently, the structure of sorgolactone was unambiguously determined by its total synthesis [10]. Although the germination of *Orobanche* spp. has been known to be stimulated by strigolactones [4, 10, 11], nothing has been reported for the isolation of natural germination stimulants for *Orobanche* spp. from their hosts. The present paper reports the isolation and structural determination of two germination stimulants, alectrol and orobanchol, for *O. minor* Smith (clover broomrape) from red clover (*Trifolium pratense*).

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RESULTS AND DISCUSSION

Red clover was grown hydroponically using tap water under continuous fluorescent light. Germination stimulants were extracted from *ca.* 1700 l of the medium by using XAD-4. The eluate with MeOH was subjected to solvent partitioning to give a neutral EtOAc fraction. This was purified by Sephadex LH-20 chromatography and the active fraction was purified further by HPLC using a C_{18} column, resulting in the separation of three stimulants I, II and III which were eluted in Fraction 15, 19 and 22, respectively (Fig. 2). Although stimulant I was not further pursued because of its scarcity, stimulants II and III were purified to homogeneity by HPLC with silica and phenyl columns.

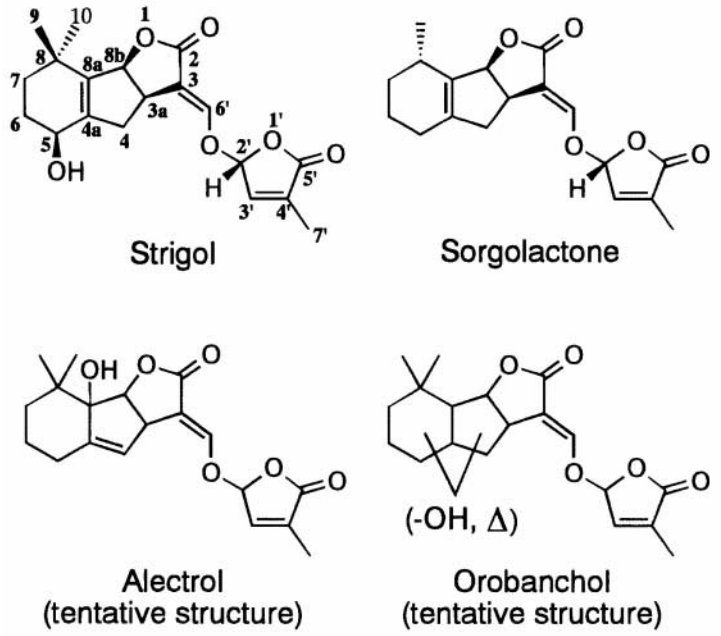

Fig. 1. Structures of root parasite seed germination stimulants (strigolactones).

Among the three red clover stimulants, III was eluted most slowly in C_{18}- and phenyl-HPLC but moved fastest in silica HPLC (see Experimental), indicating that stimulant III has the lowest polarity. GC-MS analysis using a capillary column indicated that the molecular weight (346) of stimulant III (R_t, 6.88 min) is the same as strigol (R_t, 7.45 min). The fragment ions are also the same as those of strigol, but their relative intensities were clearly different from those of strigol (see Experimental). The major fragmentations are proposed as shown in Fig. 3. Stimulant III was finally identified as alectrol on the basis of ^{1}H NMR spectrum where major peaks were identical with those reported for alectrol (see Experimental). The structure tentatively proposed for alectrol earlier [7] is shown in Fig. 1.

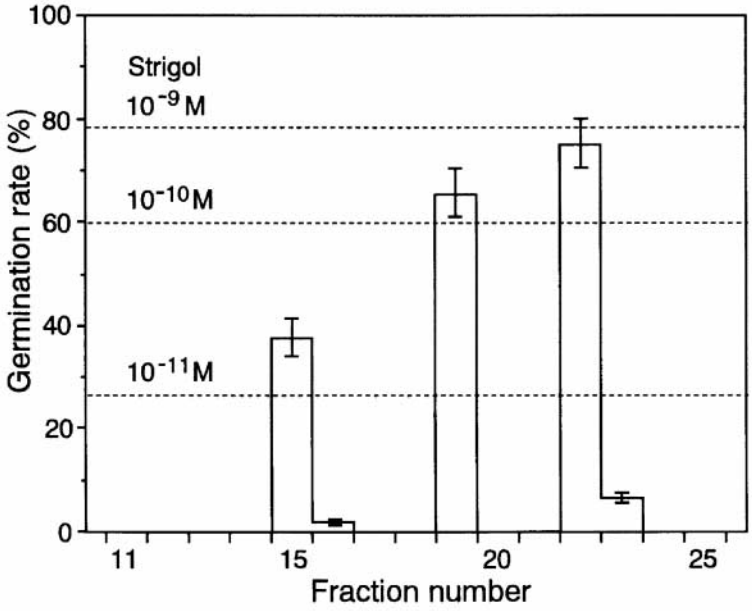

Fig. 2. Separation of *Orobanche minor* germination stimulants by revere-phase HPLC. Mobile phase was programmed by a 25 min gradient from MeOH–H_2O (4:6) to pure methanol. Fractions equivalent to 1 ml of culture medium were subjected to *Orobanche* germination bioassay. Bars indicate S.E. ($n = 6$).

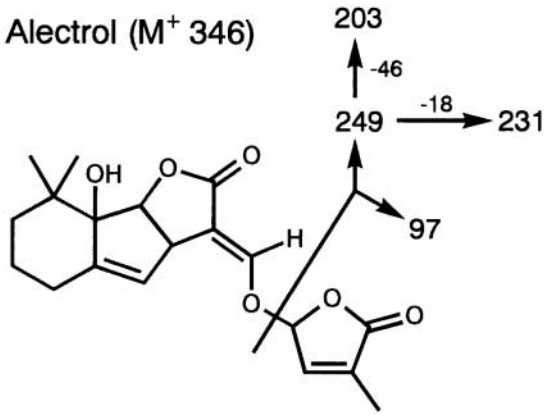

Fig. 3. Mass fragmentation of alectrol (germination stimulant III) isolated from red clover.

Stimulant II was a new compound and named orobanchol. It had similar R_ts to those of strigol on C_{18}- and silica-HPLC although it was eluted earlier than strigol on phenyl-HPLC (see Experimental). The UV spectrum of orobanchol determined in 25% aq. MeCN ($\lambda_{max} = 243$ nm) was similar to that of strigol ($\lambda_{max} = 239$ nm), suggesting that orobanchol carries the same chromophore as strigol. When analyzed by GC-MS, TMSi-orobanchol gave rise to two ion peaks at R_ts 4.75 and 3.43 min. The former ($[M]^+$ m/z 418, composition not determined) was found to be the intact molecule, while the latter, a degradation product ($[M]^+$ m/z 394.1974, calc. for $C_{20}H_{34}O_4Si_2$: 394.1976). The formation of the degradation product was due to the loss of the D ring moiety which seemed to occur when the sample was retained in the top of the GC column used in a splitless mode. TMSi-strigol similarly gave rise to two peaks with R_ts of 4.98 ($[M]^+$ 418.1887, calc. for $C_{22}H_{30}O_6Si$: 418.1880) and 3.55 min ($[M]^+$ 394.1981, calc. for $C_{20}H_{34}O_4Si_2$: 394.1983), these molecular weights being identical to those of TMSi-orobanchol and its degradation product, respectively. Possible fragmentations of these compounds are proposed on the basis of high resolution MS data on TMSi-strigol, its degradation product and TMSi-orobanchol (Fig. 4). Fragmentations due to the fission of the B-ring and D-ring of TMSi-orobanchol and its degradation product were basically similar to those of TMSi-strigol and its degradation product. However, A-ring fragmentations were different between them. The C6–C7 and C8–C8a bonds appear to be cleaved in TMSi-strigol and its degradation product, while the C5–C6 and C8–C8a bonds, in TMSi-orobanchol and its degradation product (Fig. 4). Thus, there is the possibility that orobanchol may have a double bond at C4–C4a which is allylic to and hence may facilitate the splitting of both the C5–C6 and C8–C8a bonds. The chromatographic behaviors or polarity of orobanchol are similar to those of strigol but clearly distinct from those of alectrol, suggesting that orobanchol may have a hydroxyl at the same position (C5) as strigol does. We obtained only an incomplete ^1H NMR spectrum of orobanchol because of the extremely low recovery in the final HPLC procedure. The signals at δ 7.45 (1H, vinyl ether proton, H-6′) and δ 2.03 (3H, Me on D ring, H-7) confirm that orobanchol and strigol have the same D ring structure. The signals of geminal Me groups on the A ring appeared at about δ 1.1 quite similar to the fields strigol (δ 1.10, 1.18) and alectrol (δ 1.11, 1.14). However, no further structural information was obtained from the ^1H NMR spectral data. Thus we could not reach an unambiguous conclusion regarding the structure of orobanchol (Fig. 1). A synthetic approach to determine the structure of orobanchol is now being attempted.

The present work suggests that alectrol, orobanchol and an unknown stimulant which are excreted from the root of the genuine host, red clover may be involved in the parasitism of *Orobanche minor*. Thus, strigolactones are likely to be natural germination stimulants not only for *Striga* spp. but also for *Orobanche* spp, although there is no indication how these compounds behave in the soil under the natural conditions.

The *Orobanche minor* germination-stimulatory activity was detected in both the root exudate and root tissue extract from hydroponically-grown red clover (Table 1). Further, we could observe significant germination-stimulatory activity in the root, but not in the aerial tissue of naturally-grown red clover (Table 2). These observations suggest that

Table 1. Effects of the extracts from the root and culture medium of 11-week-old hydroponically-grown red clover on the germination rate of *Orobanche minor* seed

	Sampling amount from one growth tray		
	$1/10^3$	$1/10^4$	$1/10^5$
Root	69 ± 3.4	15 ± 1.8	0.0 ± 0.0
Culture medium	71 ± 5.0	53 ± 6.2	0.0 ± 0.0

Data are expressed as % ± S.E. ($n = 6$). Control, 0.0 ± 0.0; Strigol 10^{-10} M, 96 ± 2.2; 10^{-11} M, 75 ± 4.4. From one growth tray, *ca.* 9 g of roots and 1 l of medium was obtained.

Table 2. Effects of the extracts from the root and shoot of naturally-grown red clover on the germination rate of *Orobanche minor* seed

	Fraction tested (0.2 g fr. wt equivalent)		
	neutral EtOAc fraction	acidic EtOAc fraction	aqueous residue
Root	49 ± 4.4	0.0 ± 0.0	5.0 ± 2.3
Shoot	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 1.3

Data are expressed as % ± S.E. Control, 0.0 ± 0.0; Strigol 10^{-8} M, 83 ± 1.8.

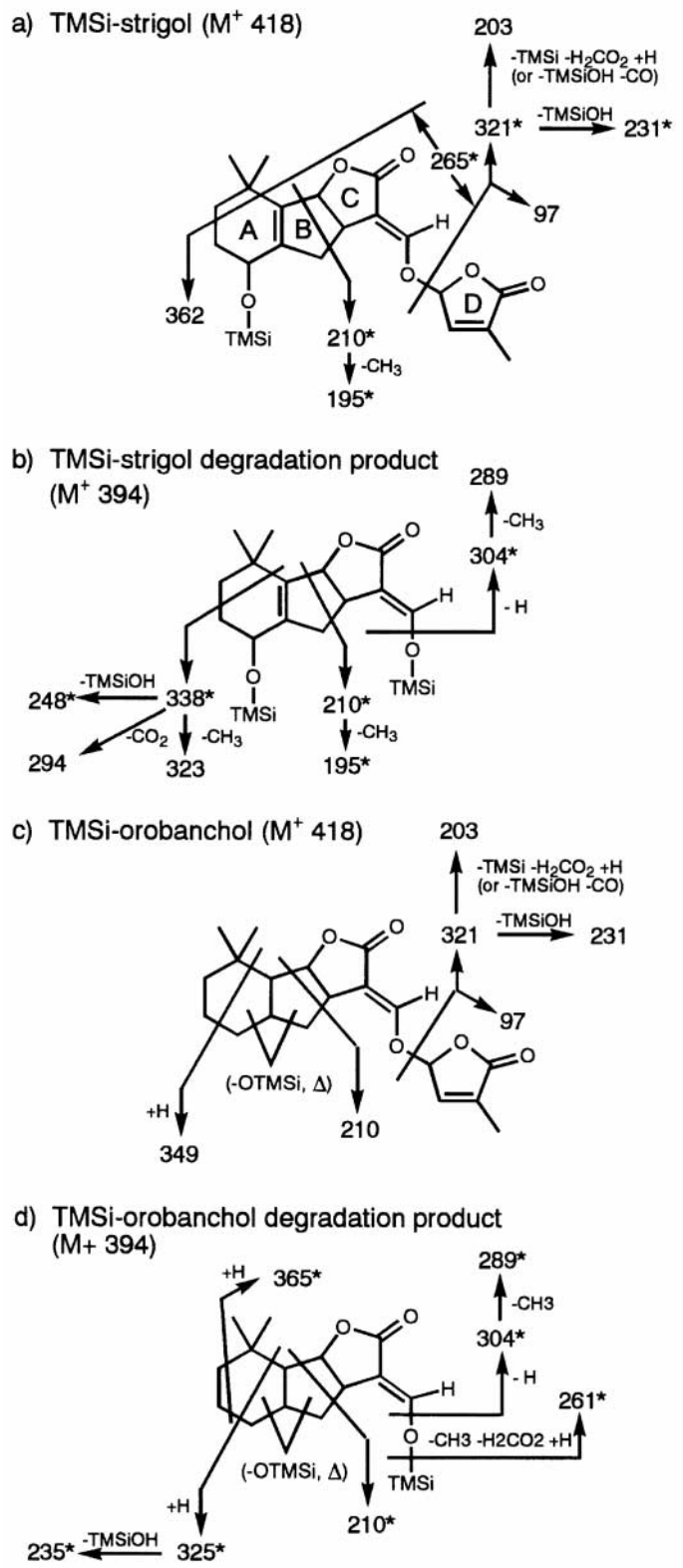

Fig. 4. Mass fragmentations of TMSi derivatives of strigol and orobanchol (germination stimulant II isolated from red clover) as well as their degradation products formed during GC. *High resolution data were obtained for these ions.

strigolactones are synthesized in and excreted from the root both in hydroponic culture and under natural conditions.

We have also first demonstrated that strigolactones can be analyzed by GC-EIMS with or without trimethylsilylation using a capillary column where a short column (4 m) rather than a longer one (15 m) was effective. Strigol has been identified from root exudates of maize, sorghum and proso millet by its R_ts in HPLC and negative ion chemical ionization mass spectrometry [12]. However, negative ion chemical ionization mass spectrometry gave rise to simple mass spectrum with suppressed fragmentations, which may be insufficient to specify structurally similar compounds. Furthermore, the present work showed that it is difficult to differentiate orobanchol from strigol by means of R_ts in HPLC. Thus, it will be important to apply the GC-EIMS techniques for analysis of natural strigolactones in future studies.

EXPERIMENTAL

Instrumental analyses

UV spectral data were obtained by an on-flow photodiode array detector attached to a HPLC system. GC-EIMS was conducted on a DB-5 capillary column (4 m × 0.25 mm) using a helium carrier gas. Samples were introduced in a splitless mode. To analyze TMSi derivatives which were prepared by reacting with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide at the room temp. for 10 min, the column temp. was kept at 130° for the first 1.5 min, elevated to 220° by a 32° min^{-1} gradient, then to 270° by a 16° min^{-1} gradient and finally kept at 270° for 5 min. For the GC-MS analysis of underivatized samples, the column temp. was kept at 130° for the first 1.5 min, elevated to 270° for 16° min^{-1} and kept at 270° for 5 min. In GC-high resolution MS, elemental compositions were determined within 7 millimass errors.

Chemicals

Authentic strigol was synthesized according to Refs [14] and [15].

Orobanche seed germination assay

Seeds of *Orobanche minor* were collected from the parasite attached to naturally-grown red clover (*Trifolium pratense*) in mid-June in the Watarase area, Tochigi, Japan and stored at 4° until use for germination assay [13]. The seeds (*ca.* 12 seeds/disk) were dispersed on glass fiber disks (6 mm ∅) which were put on a wet filter paper placed in a glass petri dish (90 mm $\phi \times 20$ mm). The petri dishes were sealed to prevent drying and incubated in the dark at 27° for 10 days. Three glass fiber disks carrying the conditioned seeds were transferred to a plastic petri dish (50 mm $\phi \times 11$ mm) in which a filter paper wetted with 1 ml of 10^{-4} M GA_3 solution is placed. Beforehand, the filter paper had been impregnated with 200 μl of acetone solution of a test sample and air-dried. The plastic dishes were sealed and incubated in the dark at 27° for 4 days. Germination rates were determined using a microscope (×40).

Hydroponical culture of red clover

Red clover seeds (*ca.* 1200/tray) were sown on two layers of gauze in 60 plastic growth-trays (38 × 29 × 10 cm) with a meshed bottom. The trays were placed in plastic vessels (42 × 29.5 × 6 cm) and filled with tap water (1 l) so as to moisten the gauze. Seedlings were grown under continuous fluorescent light (*ca.* 3000 lux) at *ca.* 25°. From 4 weeks after sowing, the medium liquid was collected weekly for about 10 weeks. In total 1700 l of the medium was collected and processed as described below.

Purification of germination stimulants from the culture medium

The culture medium was collected and passed through an XAD-4 column (*ca.* 1 l in volume). MeOH washing was concentrated, extracted with EtOAc and the EtOAc soln was washed with 0.2 M K_2HPO_4 (pH 9). The EtOAc soln was dried over Na_2SO_4 and concentrated to give a neutral fraction which was chromatographed on a Sephadex LH-20 column (bed volume, 1 l) using MeOH–$CHCl_3$ (4:1) as a mobile phase. The active fraction (434 mg) was further purified in two portions by HPLC on a Senshu-Pak C_{18} column (250 mm × 20 mm) using a 25 min gradient from MeOH–H_2O (4:6) to pure MeOH at a flow rate of 9.9 ml min^{-1} and fractions were collected every min, resulting in the separation of three active fractions 15 (10 mg), 18 (6 mg) and 22 (10 mg) as shown in Fig. 2. The R_t of authentic strigol was 18.1 min. Fraction 15 (stimulant I) was not further pursued.

Fraction 18 (stimulant II) was purified by HPLC on a Senshu-Pak Aquasil column (200 mm × 6 mm) using a 30-min gradient from $CHCl_3$–hexane (3:7, half-satd with water) to EtOAc–$CHCl_3$ (1:1, half-satd with water) at a flow rate of 3 ml min^{-1}, giving rise to an active fraction (R_t 25–27 min). The R_t of authentic strigol was 26.3 min. This fraction was purified by HPLC using a Senshu-Pak Phenyl column (150 mm × 4.6 mm) using MeOH–H_2O (45:55) at a flow rate of 0.5 ml min^{-1}, giving rise to an active fraction (R_t 10.5–12 min). Stimulant II was detected at 10.6 min, while strigol at 13.5 min. The active fraction was further purified on the same

phenyl column under the same conditions as above except the use of MeCN–H_2O (1:3) as a mobile phase, affording stimulant II (R_t 20.8 min) in an active fraction (R_t 20.5–22 min). The R_t of authentic strigol was 22.1 min.

Fraction 22 (stimulant III) was similarly purified on the above Aquasil column under the same conditions except using a 25-min gradient from $CHCl_3$–hexane (3:7, half-satd with water) to pure $CHCl_3$ (half-satd with water) followed by a 10-min gradient up to EtOAc–$CHCl_3$ (1:1, half-satd with water), giving rise to an active fraction (R_t 26–27 min). The R_t of authentic strigol was 37.0 min. The active fraction was purified on the above Phenyl column under the same conditions except using a 30-min gradient from MeCN–H_2O (3:7) to MeCN–H_2O (1:1), affording stimulant III (R_t 23.1 min) in an active fraction (R_t 23–24 min).

Orobanchol (stimulant II)

^{1}H NMR (600 MHz) δ: *ca.* 1.1 (3H, *s*, H-9 or H-10), *ca.* 1.1 (3H, *s*, H-10 or H-9), 2.03 (3H, *s*, H-7′), 7.45 (1H, H-6′). GC-EIMS of TMSi-orobanchol, 70 eV, *m*/*z* (rel. int): 418 $[M]^+$ (11), 403 (3), 349 (5), 321 (27), 300 (7), 285 (12), 275 (21), 231 (100), 210 (39), 203 (21), 97 (38), 73 (48). GC-EIMS of TMSi-orobanchol degradation product, 70 eV, *m*/*z* (rel. int): 394 $[M]^+$ ($C_{20}H_{34}O_4Si_2$, 62), 379 ($C_{19}H_{31}O_4Si_2$, 31), 365 ($C_{18}H_{29}O_4Si_2$, 11), 350 (7), 325 ($C_{15}H_{25}O_4Si_2$, 45), 304 ($C_{17}H_{24}O_3Si$, 31), 289 ($C_{16}H_{21}O_3Si$, 34), 276 (14), 261 ($C_{15}H_{21}O_2Si$), 235 ($C_{12}H_{15}O_3Si$, 11), 210 (9), 73 (100).

Alectrol (stimulant III)

^{1}H NMR (600 MHz) δ: 1.11 (3H, *s*, H-9 or 10), 1.14 (3H *s*, H-10 or 9), 2.01 (3H, *s*, H-7′) 3.43 (1H, *d* of multiplet, J = 6.6 Hz, H-3a), 5.59 (1H, *d*, J = 6.6 Hz, H-8b), 5.72 (1H, *s*, H-4), 6.14 (1H, *s*, H-2′), 6.93 (1H, *s*, H-3′), 7.44 (1H, *d*, H-6′, J = 2.4 Hz). GC-EIMS, 70 eV, *m*/*z* (rel. int): 346 $[M]^+$ (19), 328 (19), 249 (18), 232 (91), 231 (100), 217 (24), 214 (21), 203 (29), 97 (90).

Strigol

GC-EIMS, 70 eV, *m*/*z* (rel. int): 346 $[M]^+$ (6), 328 (6), 249 (15), 231 (59), 217 (6), 214 (8), 203 (18), 97 (100). GC-EIMS of TMSi-strigol, 70 eV, *m*/*z* (rel int): 418 $[M]^+$ ($C_{22}H_{30}O_6Si$, 72), 403 ($C_{21}H_{27}O_6Si$, 14), 362 (18), 321 ($C_{17}H_{25}O_4Si$, 100), 303 (18), 265 ($C_{13}H_{17}O_4Si$, 47), 247 (23), 231 ($C_{14}H_{15}O_3$, 47), 210 ($C_{12}H_{22}OSi$, 33), 203 (23), 195 ($C_{11}H_{19}OSi$, 29), 185 (16), 97 (82), 73 (71). GC-EIMS of TMSi-strigol degradation product, 70 eV, *m*/*z* (rel. int). 394 $[M]^+$ ($C_{20}H_{34}O_4Si_2$, 100), 379 (9), 350 ($C_{19}H_{34}O_2Si_2$, 11), 338 ($C_{16}H_{26}O_4Si_2$, 23), 323 (15), 304 ($C_{17}H_{24}O_3Si$, 9), 294 (18), 289 (10), 248 ($C_{13}H_{16}O_3Si$, 33), 210 ($C_{12}H_{22}OSi$, 31), 195 ($C_{11}H_{19}OSi$, 34).

Extraction of the roots and culture medium of red clover grown hydroponically

Red clover was grown hydroponically as described above. Roots (*ca.* 9 g) were harvested from 11-week-old plants grown in one growth tray and extracted with MeOH. The MeOH extract together with the neutral EtOAc fraction obtained from the culture medium in the same way as stated above were subjected to *Orobanche* seed germination assay (Table 1).

Extraction of the roots and shoots of red clover grown naturally

Roots (10 kg fr. wt.) and shoots (40.5 kg fr. wt) of red clover were separated from the plants which were harvested in April at National Grassland Research Institute, Nishinasuno, Tochigi, Japan. Roots and shoots (each 100 g fr. wt) were extracted with MeOH. After evaporation of the MeOH, the aq. residue was acidified to pH 3 by 1 N HCl and extracted with EtOAc. The EtOAc extract was separated into neutral and acidic fractions using a Pi buffer, pH 9. The neutral and acidic EtOAc fractions as well as the aq. residue were concentrated to dryness prior to bioassay (Table 2).

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REFERENCES

1. Foy, C. L., Jain, R. and Jacobsohn, R., *Rev. Weed Sci.*, 1989, **4,** 123.
2. Visser, J. H., *Naturwissenschaften*, 1989, **76,** 253.
3. Parker, C. and Riches, C. R., *Parasitic Weeds of the World.* CAB International, Wallingford, U.K., 1993, p. 111.
4. Joel, D. M., Steffens, J. C. and Matthews, D. E., in *Seed Development and Germination*, ed. N. Kigel and G. Galili. Marcel Dekker, New York, 1995, p. 567.
5. Cook, C. E., Whichard, L. P., Turner, B., Wall, M. E. and Egley, G. H., *Science*, 1966, **154,** 1189.
6. Cook, C. E., Whichard, L. P., Wall, M. E., Egley, G. H., Coggon, P., Luhan, P. A. and McPhail, A. T., *J. Am. Chem. Soc.*, 1972, **94,** 6198.

7. Müller, S., Hauck, C. and Schildknect, H., *J. Plant Growth Regul*., 1992, **11,** 77.
8. Hauck, C., Müller, S. and Schildknecht, H., *J. Plant Physiol*., 1992, **139,** 474.
9. Sugimoto, Y., Wigchert, S. C. M., Thuring, J. W. J. F. and Zwanenburg, B., *Tetrahedron Lett*., 1997, **13,** 2321.
10. Mori, K., Matsui, J., Bando, M., Kido, M. and Takeuchi, Y., *Tetrahedron Lett*., 1997, **38,** 2507.
11. Nefkens, G. H. L., Thuring, J. W. J. F., Beenakkers, M. F. M. and Zwanenburg, B., *J. Agric. Food Chem*., 1997, **45,** 2273.
12. Siame, B. A., Weerasuriya, Y., Wood, K., Ejeta, G. and Butler, L. G., *J. Agric. Food Chem*., 1993, **41,** 1486.
13. Takeuchi, Y., Omigawa, Y., Ogasawara, M., Yoneyama, K., Konnai, M. and Worsham, A. D., *Plant Growth Regul*., 1995, **16,** 153.
14. Brooks, D. W., Bevinakatti, H. S., Kennedy, E. and Hathaway, J., *J. Org. Chem*., 1985, **50,** 628.
15. Dailey, O. D., Jr., *J. Org. Chem*., 1987, **52,** 1984.