PERGAMON

Phytochemistry 53 (2000) 45–50

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Sunflower sesquiterpene lactone models induce *Orobanche cumana* seed germination

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Received 8 February 1999; accepted 9 September 1999

Abstract

Six sunflower sesquiterpene lactone models which share structural features of the lactone rings of strigol and its synthetic analogues, the GR family, with different conformational flexibilities were tested as *Orobanche cumana* germination stimulants. Among them, parthenolide and 3,5-dihydroxydehydrocostus-lactone significantly increased *O. cumana* germination, presenting higher activity than GR-24, used as a standard in the germination bioassay. The effect of these two compounds is species-specific, showing no germination stimulant activity on other *Orobanche* spp. tested (*O. crenata*, *O. ramosa* and *O. aegyptiaca*). Data presented are discussed in terms of a structure-activity relationship. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Orobanche* spp; *O. cumana*; *Orobanchaceae*; sunflower; Germination stimulants; Allelopathy; Sesquiterpene lactone; Parthenolides; Guaianolides

1. Introduction

The germination of root parasitic plant seeds depends on chemicals exuded from the roots of the host plant. The chemical nature of such germination stimulants is well known in the case of *Striga* (Butler, 1995), with no information available for other parasitic plants, with the exception of a recent report (Yokota, Sakai, Okuno, Yoneyama & Takeuchi, 1998), in which two germination stimulants for *Orobanche minor*, alectrol and orobanchol, have been isolated from the root exudate of its host *Trifolium pratense*. The *Striga* germination stimulants reported, isolated from both host and non-host-plants, belong to different chemical groups, most of them being sesquiterpenes. The largest group is collectively named strigolactones (Butler, 1995), strigol (**1**) being the most important representative and the first germination stimulant characterized. It was primarily isolated from cotton, a non-host plant (Cook et al., 1972) and then from other *Striga*-host plants like maize, proso millet and sorghum (Siame, Weerasuriya, Wood, Ejeta & Butler, 1993). Other compounds of the strigolactone group are sorgolactone, isolated from sorghum (Hauck, Müller & Schilknecht, 1992) and alectrol, produced by cowpea (Müller, Hauck & Schilknecht, 1992). Strigol has been the starting point for the development of the synthetic GR family (Fig. 1), used in studies on structure-activity relationships directed at establishing which part of the molecule constitutes the bioactiphore (Bergmann et al., 1993; Zwanenburg, 1998). One of the members, the GR-24, used as a standard in this paper, is able to induce the germination of all the parasitic plants tested (Pepperman & Bradow, 1988; Mangnus & Zwanenburg, 1992; Bergmann et al., 1993). Certain sesquiterpene lactones, which share structural features of the lactone rings of strigol and belong to the germacranolide and eudesmanolide skeletal type also induce *Striga* germination, although they have been reported in non host plants (Fischer, Weidenhamer & Bradow,

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0031-9422/00/$ - see front matter
PII: S0031-9422(99)00485-9

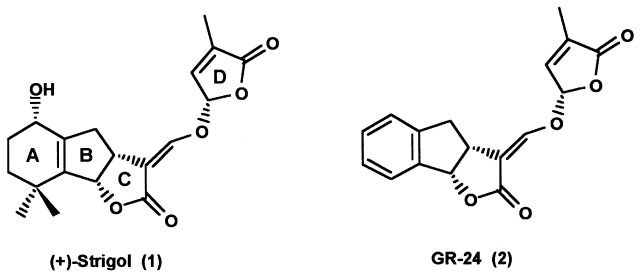

Fig. 1. Chemical structures of strigol and synthetic analogue GR-24.

1989; Fischer, Weidenhamer, Riopel, Quijano & Menelaou, 1990). *Striga* germination stimulants different from the above mentioned strigolactones include hydrobenzoquinones collectively called sorgoleones, the first germination stimulants isolated from a host plant, sorghum (Netzly, Riopel, Ejeta & Butler, 1988). On the basis of their limited water solubility, their rapid oxidation to inactive quinones and their production in different sorghum varieties, a minor role, if any, in controlling *Striga* germination has been hypothesized for sorgoleones (Butler, 1995).

We are currently studying the sunflower-*Orobanche cumana* Wallr. (*Orobanche cernua* Loefl.; Pujadas & Thalouarn, 1998) interaction. Sunflower secondary metabolites are mainly terpenoids and phenolics (Rieseberg, Soltis & Arnold, 1987; Alfatafta & Mullin, 1992; Macías, Varela, Torres & Molinillo, 1993; Macías, Torres, Varela & Molinillo, 1996). Some of them, like cinnamic acids and 7-hydroxylated simple coumarins are root-excreted (Gutiérrez-Mellado, 1998; Gutiérrez-Mellado, Edwards, Tena, Cabello, Serghini & Jorrín, 1995). Sunflower plant phenolics (cinnamic acids, flavonoids and coumarins) do not affect *Orobanche* seed germination but, as in the case of scopoletin and ayapin, inhibit the GR-24 induced germination (Pérez de Luque, 1998; Jorrín et al., 1996; Jorrín et al., 1998).

Here we present data on the biological activity of six sunflower sesquiterpene lactone models (Fig. 2), both natural and synthetic, on the germination of different species and populations of *Orobanche*. Our main goal is first to characterize the sunflower natural germination stimulant for *O. cumana* and then develop agrochemicals which cause suicide germination of the parasitic weed seeds. With the appearance of new more virulent populations, *Orobanche* is now a serious problem for cultivated sunflower in Spain (Alonso, Fernández-Escobar, López, Rodríguez-Ojeda & Sallago, 1996).

With the exception of parthenolide (**4**), obtained from *Magnolia grandiflora*, the rest of the tested compounds have been synthesised from costunolide and dehydrocostuslactone, two abundant sesquiterpene lactones isolated from *Saussurea lappa* (Macías, Galindo & Massanet, 1992; Galindo, 1993). Compounds **3** (melampomagnolide A) (El-Feraly, 1983) and **6** (isozaluzanin C) (Bohlmann, Brindöpke & Rastogi, 1978) were previously isolated as natural products. A selection of model compounds was made, based on their similarity to those previously isolated from sunflower, and their relatively easy isolation or synthesis.

2. Results and discussion

Treatment of a dichloromethane (DCM) solution of santamarin with SeO2 (1:1) and *t*-butanol (2:1) under reflux yielded, after 6 h, the higher polarity compound **7** (40%). Compound **7** presented an EIMS with a molecular ion at *m*/*z* 264 according to the molecular for-

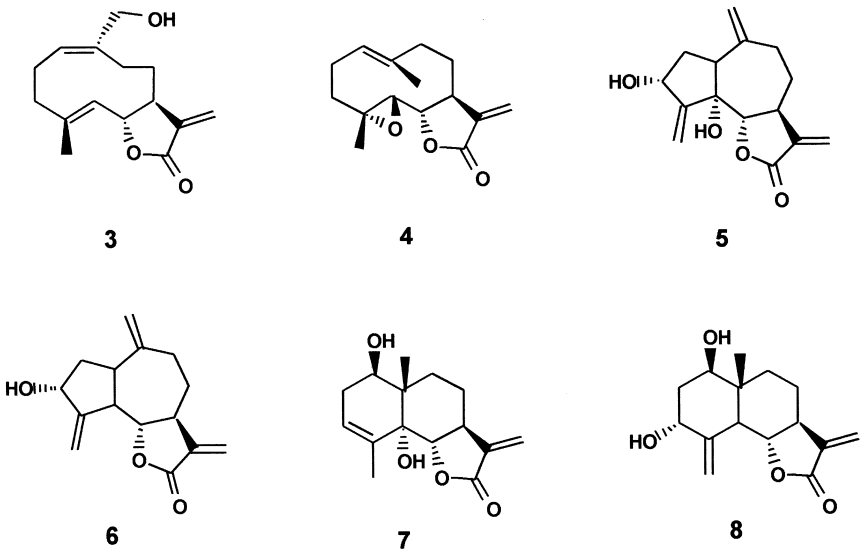

Fig. 2. Chemical structures of the sunflower sesquiterpene lactone models used in this study.

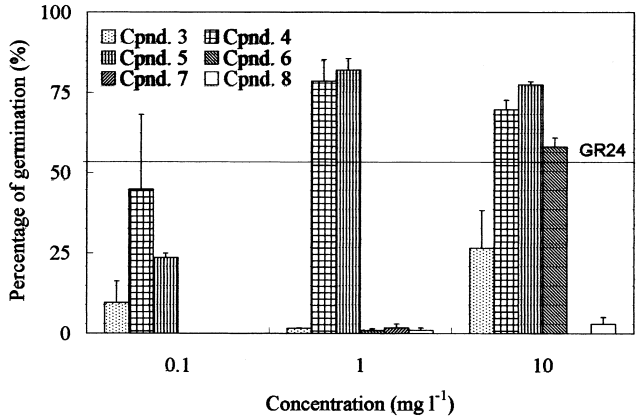

Fig. 3. Effects of compounds **3**–**8** on the germination percentage of *Orobanche cumana* Wallr. seeds. The horizontal line represent the germination level reached with 3.3 mg l^{-1} of GR-24. No germination was observed for seeds just incubated with water. Bars indicate standard error.

mula $C_{15}H_{20}O_4$. The ^{1}H-NMR spectrum was very close to that of santamarin, the starting product. Major differences arose from the signal corresponding at δ 4.08 ppm (1H, *d*, J = 11.2 Hz, H-6), and the deshielding effect observed for the signals at δ 3.33 ppm (1H, *m*, H-7) and δ 4.23 ppm (1H, *dd*, J = 6.8 Hz, J = 9.6 Hz, H-1). The assignation of these signals was confirmed by ^{1}H-NMR 2D COSY experiments and are in good accordance with the proposed stereochemistry of the 5-hydroxyl group. The same treatment as that described above was performed with reynosin, yielding 60% of compound **8**. The EIMS of this compound presented a molecular ion at *m*/*z* 264, according to the molecular formula $C_{15}H_{20}O_4$, and main losses at 246 $[M–H_2O]^+$ and 228 $[M–2H_2O]^+$. The ^{1}H-NMR spectrum showed major diagnostic signals like those at δ 4.35 ppm (1H, *dd*, J = 11.1 Hz, J = 4.4 Hz, H-3β) and δ 4.00 ppm (1H, *dd*, J = 11.0 Hz, H-6). CHOH–CH_2–CHOH system correlated in the ^{1}H-NMR 2D COSY spectrum along with the coupling constants of the H-3 proton signal permit us to confirm the proposed structure.

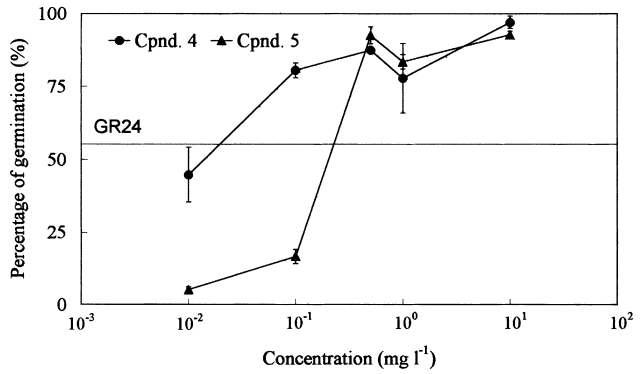

Fig. 4. Effects of compounds **4** and **5** on the germination percentage of *Orobanche cumana* Wallr. seeds. The horizontal line represent the germination level reached with 3.3 mg l^{-1} GR-24. No germination was observed for seeds just incubated with water. Bars indicate standard error.

Preliminary data on germination (see Fig. 3) with a population of *Orobanche cumana* showed a great difference between compounds **3**, **6**, **7** and **8** and compounds **4** and **5**. Compounds **3**, **7** and **8** were inactive as germination stimulants, while compound **6** caused a 60% induction only at the highest concentration tested (10 mg l^{-1}). Compounds **4** and **5** stimulated *Orobanche* germination at concentrations of as low as 0.1 mg l^{-1}, the percentage of germinated seeds being higher than that obtained in the presence of GR-24.

A second dose-response bioassay was performed with compounds **4** and **5** at concentrations of 0.01–10 mg l^{-1} (see Fig. 4). Germination percentages near to 100% of the seeds were obtained for both compounds, with no differences between them, at concentrations of from 0.5 to 10 mg l^{-1}, while less than 60% germination was observed in the presence of 3.3 mg l^{-1} of GR-24. Differences in the germination capacity between both compounds were present at the lower concentrations tested (0.01–0.1 mg l^{-1}). The germacranolide parthenolide (**4**) presented activity at 0.01 mg l^{-1} (germination percentage of 44%); to the contrary, only a slight germination percentage was observed for the guaianolide (**5**) at 0.01 and 0.1 mg l^{-1} (lower than 10%). Similar results in terms of the relative percentage of germination induced by the different compounds have been obtained with other *O. cumana* populations, although the percentage of germination depended on the population (data not shown).

Similar bioassays performed with *O. crenata*, *O. ramosa*, and *O. aegyptiaca* with compounds **4** and **5** resulted in no induction of the germination. In the same bioassays, 45–50% of induction was obtained with GR-24 3.3 mg l^{-1}.

Sesquiterpene lactones have been reported previously as germination inductors of parasitic witchweed (*Striga asiatica*). The eudesmanolides santamarin and reynosin, structurally close to compounds **7** and **8**, showed higher germination-inducing activities than strigol (Fischer et al., 1990). Several *trans*, *trans*-germacranolides, parthenolide (**4**) and dihydroparthenolide among them, also presented high levels of activity, which only were slightly lower than strigol (Fischer et al., 1990; Rugutt & Rugutt, 1998). No significant activities were found when pseudoguaianolides were tested. From a structural point of view, the activity has been related to the presence of a γ-lactone ring and the spatial disposition of the rest of the molecule (called 'double crown'), very close to that of strigol (Fischer et al., 1989, 1990).

In the present case, the eudesmanolides **7** and **8**, structurally close to reynosin and santamarin, but with

the difference of an additional hydroxyl group, did not present any activity even at 10 mg l^{-1}. Actually, GR-24 has a very flat structure in the ABC ring system. Thus, the necessary requirements to exert any induction on the germination of *Orobanche cumana* have to be very different from those in witchweed.

Recently Zwanenburg (1998) supports the idea that the real bioactiphore for the induction of the germination of *Orobanche* resides in the enol ether part of the strigolactones. In this study we have tested six molecules where none has such a functionalization in the molecule. However, compounds **4** and **5** showed higher levels of activity than those of GR-24. It is obvious that, at least in these families of natural compounds, different structural requirements are needed to exert germination.

A comparison of the activities shown with **5** and **6** 1 mg l^{-1} treatments resulted in a drastic change. While compound **6** was inactive, the introduction of another hydroxyl group caused compound **5** to be more active than the standard GR-24. Compounds **7** and **8** also presented two hydroxyl groups in their structures. As a consequence, the number of hydroxyl substituents cannot be referred to as a requirement to show any activity. Other requisites, such as their relative spatial distribution could also be more important.

Parthenolide (**4**) presented higher levels of activity at the lower concentrations which is in good accordance with the previous results for *Striga* (Fischer et al., 1990). The spatial distribution of the skeleton is very close to those of compounds **7**, **8**, strigol and the eudesmanolides reynosin and santamarin. Therefore, this requirement cannot be seen as being responsible for the activity in the case of *Orobanche*.

The only common feature between GR-24 and active compounds is the presence of the α,β-unsaturated lactone ring. However, the inactive compounds also presented this feature. Therefore, only two possibilities can be considered: (a) the lactone ring does not exert any influence on the activity; (b) a more plausible one, in which not only a specific functional constituent must be considered for the activity, but also the accessibility of the bioactiphore to a putative receptor, associated with the spatial arrangement of the rest of the molecule. This hypothesis has been previously suggested (Fischer et al., 1990).

In summary, the following order of activity can be observed for *Orobanche* induced germination: guaianolides > *trans,trans*-germacranolides > GR-24 > melampolides > eudesmanolides.

Sesquiterpene lactones belonging mainly to the guaianolide and germacranolide types have been previously isolated from sunflower. They have been detected in the aerial part of the plant and so far there are no studies indicating their presence in roots or root exudates. However, a number of lactones, the so-called pseudoguaianolides, fairly similar to the parthenolide used here, have been detected in root exudates of *Parthenium hysterophorus* (Kanchan & Jayachandra, 1980). These compounds inhibit germination and growth of lettuce, and, parthenin induces *Striga asiatica* germination. It is important to test the activity of the guaianolides isolated from roots on the germination of *Orobanche*. Although, if positive, it would also be necessary to isolate them in the rhizosphere of sunflower prior to confirming any hypothesis of a role as biocommunicators in the sunflower-*Orobanche cumana* system. Since none of the lactones tested were able to induce the germination of other *Orobanche* species, such a role would explain why sunflower is the target for the subspecies *O. cumana* and not for the others.

3. Experimental

Melampomagnolide A (**3**), was obtained from costunolide, while 5α-hydroxy-isozaluzanin C (**5**) and *iso*zaluzanin C (**6**) were prepared from dehydrocostuslactone following the methodology previously described (Macías et al., 1992). Reynosin and santamarin (starting materials for the synthesis of **7** and **8**) were obtained by cyclization of costunolide with MCPBA as previously described (Parodi, Fronczek & Fischer, 1989). Parthenolide (**4**) was obtained from *Magnolia grandiflora* leaf extract and purified by CC and recrystallization from hexane:EtOAc mixed.

5α-Hydroxysantamarin (**7**): 71 mg of santamarin were dissolved in dichloromethane (DCM) and refluxed with 33 mg SeO_2 (1:1) and 7.2 ml *t*-Butanol during 12 h. Purification of the reaction mixed with CC yielded 30% of **7**.

3α-Hydroxyreynosin (**8**): 25 mg of reynosin was treated under the same conditions as above, yielding 60% of **8**.

Structures were verified by comparison (when available) and discussion of their spectroscopic data (MS, IR, ^{1}H- and ^{13}C-NMR) with those reported in the literature.

3.1. 5α-Hydroxysantamarin (7)

$C_{15}H_{20}O_4$, powder. EIMS (70 eV) *m/z* (rel. int.): 264 $[M]^+$ (2), 246 $[M–H_2O]^+$ (5), 246 $[M–2H_2O]^+$ (6); IR $\lambda_{max}^{KBr,\ neat}$ (cm^{-1}): 3432 (–OH), 2882 (C–H), 1755 (γ-lactone), 1662 (C═C); ^{1}H-NMR (399.952 MHz, $CDCl_3$): δ 6.08 (1H, *d*, $J_{7,\ 13b} = 3$ Hz, H-13b), δ 5.48 (1H, *brs*, H-3), δ 5.40 (1H, *d*, $J_{7,\ 13a} = 3$ Hz, H-13a), δ 4.23 (1H, *dd*, $J_{1,\ 2\alpha} = 7$ Hz, $J_{1,\ 2\beta} = 10$ Hz, H-1α), δ 4.08 (1H, *d*, $J_{6,\ 7} = 11$ Hz, H-6), 3.33 (1H, *m*, H-7), δ 2.45 (1H,

brdd, $J_{1,\,2\alpha} = 7$ Hz, $J_{1,\,2\beta} = 10$ Hz, H-2β), δ 1.97 (1H, *m*, H-8β), δ 1.94 (1H, *m*, H-2α), δ 1.90 (3H, *brs*, H-15), δ 1.60 (1H, *m*, H-8β), δ 0.92 (3H, *s*, H-14).

3.2. 3α-Hydroxyreynosin (*8*)

$C_{15}H_{20}O_4$, powder. EIMS (70 eV) *m/z* (rel. int.): 264 $[M]^+$ (2), 246 $[M–H_2O]^+$ (3), 246 $[M–2H_2O]^+$ (5); IR $\lambda_{max}^{KBr,\,neat}$ (cm^{-1}): 3310 (–OH), 2871 (C–H), 1755 (γ-lactone), 1662 (C═C); ^{1}H-NMR (399.952 MHz, $CDCl_3$): δ 6.08 (1H, *d*, $J_{7,\,13b} = 3$ Hz, H-13b), δ 5.41 (1H, *d*, $J_{7,\,13a} = 3$ Hz, H-13a), δ 5.16 (1H, *brs*, H-15), δ 5.01 (1H, *brs*, H-15′), δ 4.35 (1H, *dd*, $J_{2\beta,\,3\beta} = 11$ Hz, $J_{2\alpha,\,3\beta} = 4$ Hz, H-3β), δ 3.93 (1H, *dd*, $J_{1,\,2\alpha} = J_{1,\,2\beta} = 3$ Hz, H-1α), δ 2.74 (1H, *d*, $J_{2,\,6} = 11$ Hz, H-5), δ 2.56 (1H, *m*, H-7), δ 0.77 (3H, *s*, H-14); ^{13}C-NMR (100.577 MHz, $CDCl_3$): δ 171.3 (C-12), δ 144.7 (C-4), δ 138.9 (C-11), δ 117.1 (C-13), δ 112.0 (C-15), δ 171.3 (C-12), δ 79.7 (C-6), δ 72.7* (C-3), δ 72.3* (C-12), δ 49.4 (C-7), δ 47.9 (C-2), δ 43.0 (C-5), δ 37.3 (C-10), δ 35.2 (C-9), δ 21.1 (C-8), δ 10.3 (C-14).

3.3. Plant material

Seeds of *O. cumana* and *O. crenata* were collected in 1994 and 1995 in experimental fields in Andalusia (South of Spain). Seeds of *O. ramosa* and *O. aegyptiaca* were provided by Dr. D. M. Joel (Department of Weed Research, Agricultural Research Organisation, Newe-Ya'ar Research Centre, Israel).

3.4. Orobanche seed-germination assay

Fifty seeds of *O. cumana* were placed homogeneously dispersed in a Petri dish (ϕ 55 mm) on Whatman GF/A paper. For preconditioning, 1 ml of a solution of 0.3 mM of 2-[*N*-Morpholino]ethanesulfonic acid was added to the filter, and the petri dishes sealed to prevent drying and incubated in darkness at 20°C for 11 days. After this period, 250 μl of an aqueous solution of GR-24 or compounds **3**–**8** plus 500 μl water were added to every Petri and dishes sealed with parafilm and incubated for 4 days in darkness at 20°. Germination was observed under a microscope (30×) and the germinated seeds expressed as a percentage of the total seeds. A seed was considered to be germinated when the radicle was at least 0.2 mm long.

Acknowledgements

This research has been supported by Comisión Interministerial de Ciencia y Tecnología, Spain (CICYT; project AGF97-1230-C02) and the Consejería de Educación y Ciencia de la Junta de Andalucía (Grupo de Investigación Bioquímica Vegetal y Agrícola, AGF97-1230-CO2). Thank you to Dr Joel for providing us with the *Orobanche* seeds.

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