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Stimulation of *Orobanche ramosa* seed germination by fusicoccin derivatives: A structure–activity relationship study

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Abstract

A structure–activity relationship study was conducted assaying 25 natural analogues and derivatives of fusicoccin (FC), and cotylenol, the aglycone of cotylenins, for their ability to stimulate the seed germination of the parasitic species *Orobanche ramosa*. Some of the compounds tested proved to be highly active, being 8,9-isopropylidene of the corresponding FC aglycone and the dideacetyl derivative the most active FC derivatives. In both groups of glucosides and aglycones (including cotylenol), the most important structural feature to impart activity appears to be the presence of the primary hydroxy group at C-19. Furthermore, the functionalities and the conformation of the carbotricyclic ring proved to play a significant role. The dideacetyl derivative of FC, being easily and rapidly obtainable in high yield starting by FC, could be of interest for its practical application as a stimulant of *Orobanche ramosa* seed germination, inducing the "suicidal germination", an interesting approach for parasitic plant management.

Keywords: *Orobanche ramosa*; Fusicoccin; Cotylenol; Fusicoccin derivatives; Parasitic plant management; Seed germination; Structure–activity relationships

1. Introduction

Parasitic angiosperms of the genus *Orobanche* (broomrapes) are serious weed problems, being responsible for major losses to vegetable, legume and sunflower crops, by interfering with water and mineral intake and by affecting photosynthate partitioning.

The seeds germinate only if stimulated by host root exudates and start producing a tubercle only if they are near enough to the host roots. Furthermore, the parasites have a long underground phase, and by the time they emerge, much of the damage has already been produced. Due to its unusual life cycle and to the total dependence by the host, traditional control methods very often are impractical. Considering that the germination of seeds of parasitic plants depends on the presence of stimulating exudates produced by the roots of the host plant, an alternative approach for the management of parasitic weeds is the so called "suicidal germination". This latter consists in the induction of seed germination by the application of a germination stimulant to the soil, in the absence of the host. The parasite seeds will germinate but, in the absence of the host, will die, resulting in a reduction of the seed bank.

The chemical structure of some germination stimulants towards species of another parasitic genus, *Striga*, is known (Butler, 1995), but few information is available for *Orobanche* species, with the exception of alectrol and orobanchol (Yokota et al., 1998; Mori et al.,

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1999). Some natural compounds (strigol, xenognosin, dihydrosorgoleon, sorgolacton, strigol related compounds), isolated from species of both hosts and non hosts of *Striga* and *Orobanche,* are known as strigolactones (Cook et al., 1966, 1972; Lynn et al., 1981; Netzly et al., 1988; Hauck et al., 1992; Müller et al., 1992; Butler, 1995). Synthetic analogues of strigolactones, named "GR" family (Mangnus et al., 1992; Nefkens et al., 1997; Wigchert et al., 1999), have been developed and tested as well as several natural sesquiterpenes lactones (SL) (Fisher et al., 1990; Rugutt and Rugutt, 1997) and their derivatives. These were also used to carry out structure–activity relationships studies on *Orobanche cumana* seed germination (Galindo et al., 2002). However, the instability of strigolactones in the soil and their high cost for synthesis preclude their practical use under field conditions.

Among several fungal metabolites tested with the aim of finding new natural stimulants, Yoneyama et al. (1998) reported that fusicoccin (FC, **1**) and cotylenol (**19**) at concentrations 10^{-5} M induced seed germination of *Striga hermonthica* (Del.) Benth and *Orobanche minor* Smith.

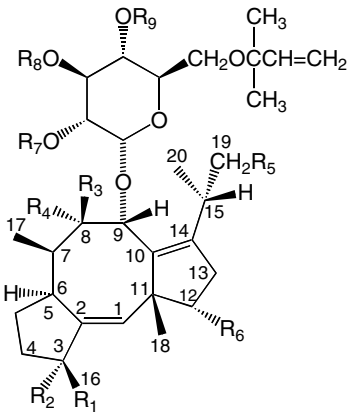
FC is the major toxic metabolite of *Fusicoccum amygdali* Delacr., the causative fungal agent of peach and almond canker, isolated in 1962 (Ballio et al., 1964) and structurally described in 1968 (Ballio et al., 1968a; Barrow et al., 1968). Many studies were carried out on the chemical, biosynthetic and biological properties of this toxin and on structure–activity relationships (SAR) (Ballio and Graniti, 1991; Marré, 1979; Ballio et al., 1991; Evidente et al., 1982).

Considering the efficacy of FC in stimulating seed germination of parasitic plants, and considering the availability in our laboratory of several derivatives and natural analogues of FC and its aglycone, as well as of cotylenol, due to previous works on the purification and identification of those compounds in our lab, we decided to carry out a structure–activity study using the seeds of another parasitic plant species, *O. ramosa*, which proved to be useful in a preliminary screening.

This paper reports the results of this SAR investigation and also the chemical characterization of one FC derivative and three derivatives of its aglycone never described.

2. Results and discussion

In this SAR study, a total of 25 compounds were used, 16 of them were glucosides and 9 were aglycones, having different biological activities (Ballio et al., 1981a,b). In particular, besides FC, the following compounds were tested for their ability to stimulate the germination of *O. ramosa* seeds: eight FC natural analogues (**3**, **4**, **6**–**10** and **16**) isolated from the culture filtrates of *F. amygdali*; seven FC derivatives (**2**, **5**, **11**–**15**) prepared from **1** by "ad hoc" chemical modifications; FC deacetyl aglycone (**17**), prepared by chemical degradation of the sugar moiety of **1**; some of its derivatives (**18**, **20**, **24** and **25**); three derivatives (**21**–**23**) of the 19-deoxydideacetyl FC (**3**); cotylenol (**19**), the aglycone of all cotylenins (CNs).



1 R_1=CH_2OCH_3, R_2=R_4=R_7=R_9=H, R_3=R_6=OH, R_5=R_8=OAc

2 R_1=CH_2OCH_3, R_2=H, R_3+R_4=O, R_5=R_6=R_7=R_8=R_9=Ac

3 R_1=CH_2OCH_3, R_2=R_4=R_5=R_7=R_8=R_9=H, R_3=R_6=OH,

4 R_1=CH_2OCH_3, R_2=R_4=R_7=R_8=R_9=H, R_3=R_5=R_6=OH

5 R_1=CH_2OCH_3, R_2=R_4=R_7=R_8=R_9=H, R_3=R_6=OH, R_5=F

6 R_1=CH_2OCH_3, R_2=R_4=R_7=R_8=R_9=H, R_5=OAc, R_6=OH

7 R_1=CH_2OCH_3, R_2=OH, R_3=R_6=OH, R_4=R_5=R_7=R_8=R_9=H

8 R_1=CH_2OCH_3, R_4=R_7=R=$_8$=R_9=H, R_2=R_3=R_5=R_6=OH

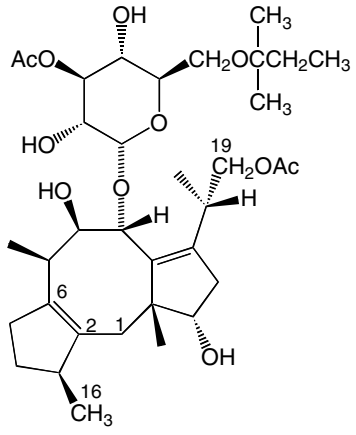
9 R_1=CH_2OCH_3, R_2=R_4=R_7=R_8=R_9=H, R_3=R_5=OH, R_6=OAc

10 R_1=R_4=R_5=R_7=R_8=R_9=H, R_2=CH_2OH, R_3=R_6=OH

For this purpose, one new FC derivative, 19-dehydroxy-19-fluorodideacetylFC (**5**), the FC true aglycone (**18**) and 12-epi- and 12-oxo-8,9-isopropylidene aglycone (**22** and **23**) of 19-deoxydideacetylFC (**3**) were prepared and characterised as reported in detail in Section 3. All the physical and spectroscopic data are consistent with the structure assigned to these new derivatives.

In our bioassay on *O. ramosa* seeds (Fig. 1), a different effect with FC and cotylenol was observed compared to that reported for *O. minor* (Yoneyama et al., 1998). The two compounds (**1** and **19**) were almost inactive on *O. ramosa*, against stimulation ranging between 56 and 86%, and 79 and 91% when assayed, respectively, at 10^{-4} and 10^{-5} M on *O. minor* seeds.

Also in case of deacetylaglycone of FC (**17**) the stimulation efficacy was lower on *O. ramosa* seeds (3–14%) compared to the effect observed on *O. minor* seeds.



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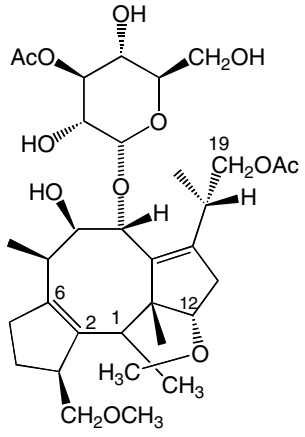

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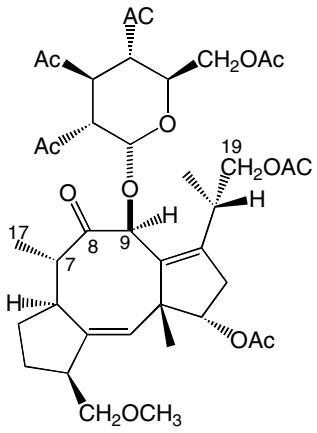

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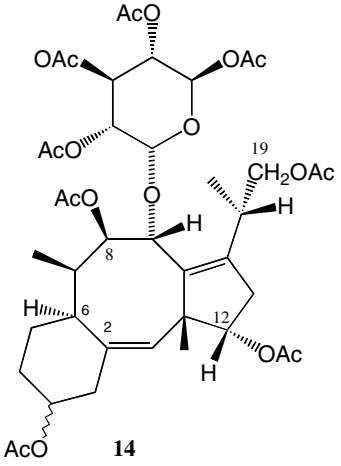

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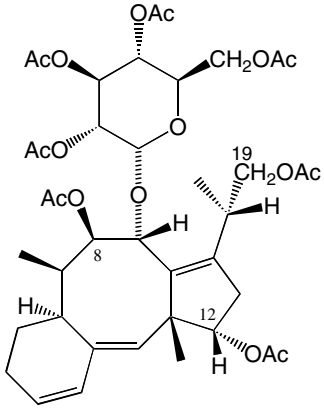

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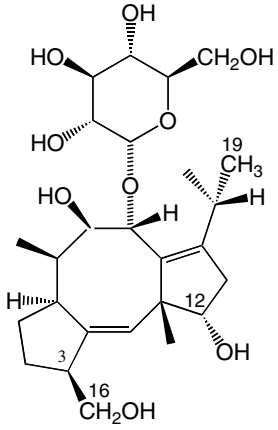

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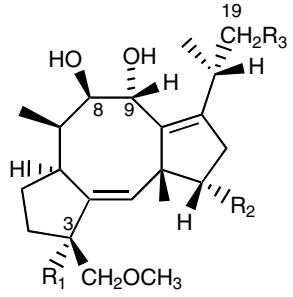
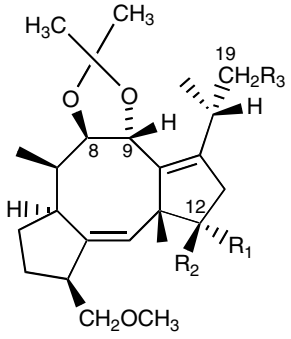
It is interesting to note that a marked decrease of the biological activity due to the substitution of the α-H on C-3, as in **19**, was already observed in previous SAR studies carried out on FCs and CLs (Ballio et al., 1981a,b; Pini et al., 1979) and confirmed with the lack of stimulation when **19** was tested to *O. ramosa* seed germination.



17 R_1=H, R_2=OH, R_3=OH

18 R_1=H, R_2=OH, R_3=OAc

19 R_1=OH, R_2=R_3=H



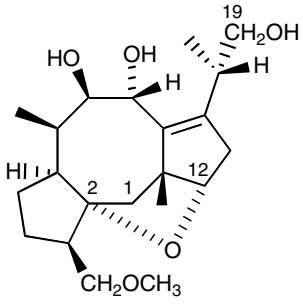
20 R_1=OH, R_2=H, R_3=OH

21 R_1=OH, R_2=H, R_3=H

22 R_1+R_2=O, R_3=H

23 R_1=H, R_2=OH, R_3=H

24 R_1+R_2=O, R_3= $OC(Ph)_3$



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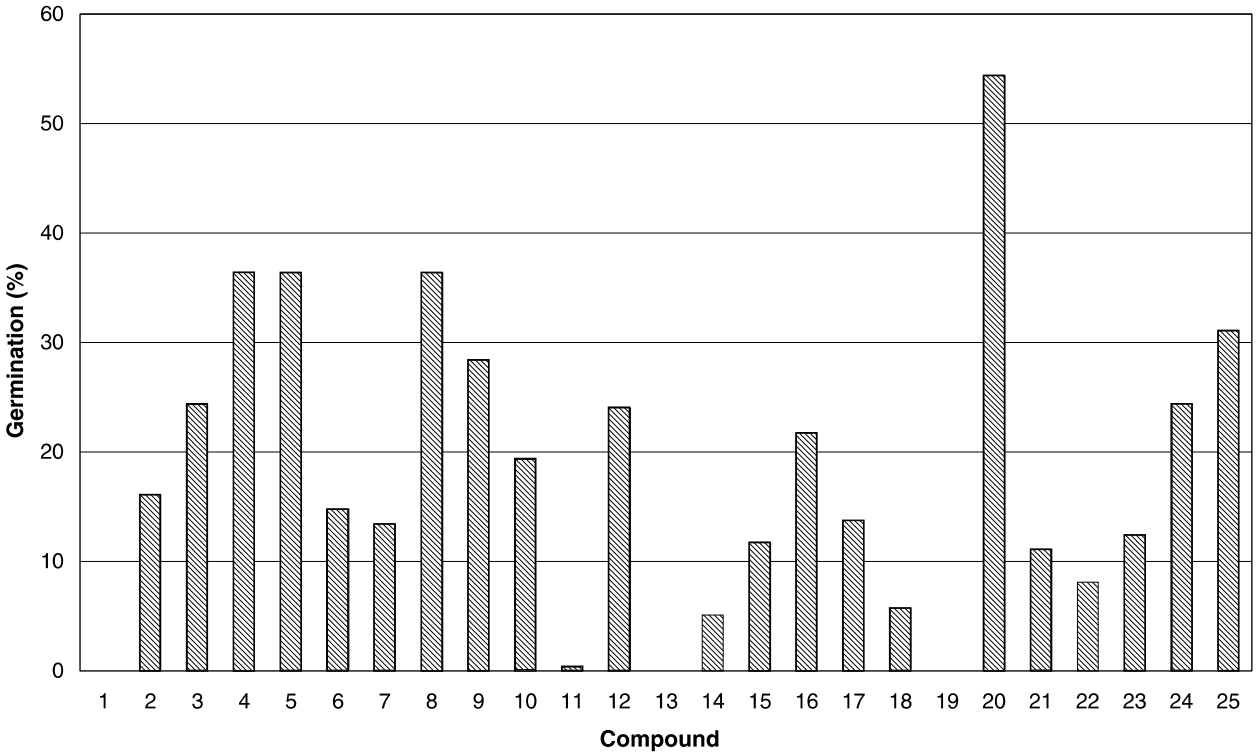

Fig. 1. Effect of fusicoccin derivatives and natural analogues on *O. ramosa* seed germination. Data were processed by analysis of variance, according to a complete randomized experimental design. Significant means were tested by LSD test ($p < 0.05$). LSD = 6.9.

Some FC natural analogues and derivatives showed a higher activity compared to **1**, that appears to be modulated by chemical modifications, essentially in the functionalities and/or the conformation of the carbotricyclic diterpenoid ring.

Among the glucosides (**1**–**16**), noteworthy differences in the activity were noted. The most active (36%) compound appeared to be dideacetylFC (**4**). This could have an interest for a practical application because it can be prepared easily and rapidly from **1** in high yield. When the activity of **4** was compared to that of the other glucosides, the importance of the presence of a free primary hydroxy group at C-19 appeared evident. In fact, FC and some natural analogues, i.e. 19-deoxydideacetylFC, 19-monoacetyldideacetylFC, 19-deoxy-3α-hydroxydideacetylFC, 16-*O*-demethyl-19-deoxydideacetyl-3-epiFC, de-*t*-pentenyl-16-*O*-demethyl-19-deoxydideacetylFC (**1**, **3**, **6**, **7**, **10** and **16**, respectively) showed a significant decrease (between 14% and 24 %) of the stimulation activity because they bear HO–CH_2-19 acetylated or converted to a methyl group. The latter three derivatives (**7**, **10**, **16**) also showed substantial modification of functionalities and/or conformation of the carbotricyclic ring. Contrary to, some other glucosides, i.e. 3α-hydroxydideacetylFC and 12-monoacetyldideacetylFC (**8** and **9**), having a free hydroxy group linked to CH_2-19 and any substantial modifications of the carbotricyclic ring, showed the same weakly reduced stimulation activity (36% and 28%, respectively). Furthermore, 19-dehydroxy-19-fluorodideacetylFC (**5**) showed the same activity (36%) of **4** and this is probably due to the presence of an F atom linked to CH_2-19, which has a similar electronegativity of the hydroxy group. The remaining glucosides (**2**, **11**–**15**) are derivatives prepared by FC, and showed the acetylation of all hydroxy groups (including that at CH_2-19) and other significant modifications of functionalities and conformation of the carbotricyclic ring. In fact, **2** showed the oxidation to a ketone of the hydroxy group at C-8; **11** exhibited the hyrogenolysis of the methoxymethylene group on C-3 accompanied by the isomerisation of the double bond from 1,2 to 2,6; **12** showed the same isomerisation of double bond as consequence of the formation of a pseudoacetonide ring between the hydroxy group at C-12 and C-1; **13** showed the same oxidation of the C(8)–OH as in **2** and the epimerisation of both C-7 and C-9; finally **14** and **15** showed the cleavage of the ether bond and the expansion of the cyclopentane to a cyclohexane ring. Furthermore, the derivative **12**–**15** showed the lacking of the *t*-pentenyl residue at C-6 of glucosyl residue while **11** showed a *t*-pentyl group in the same position. These glucosides derivatives showed a marked decrease of the stimulant activity (2–18%; for **11** and **15** this activity has been measured at 10^{-4} M) as consequence of the acetylation of the hydroxy group at CH_2-19 and of the other modifications described above.

The same SAR were obtained by testing the aglycones. Among them, the most active (54%) proved to be the isopropylidene derivative (**20**) followed by the deacetylFC-aglycone (**17**, 14%). In fact, both compounds have a free

hydroxy group at CH_2-19. When the other isopropylidene derivatives (**21**–**24**) were compared to **20**, a marked decrease of stimulation (8–24%) was observed and this could be attributed to the lack of the hydroxy at CH_2-19, which became a methyl group in **21**–**23** with the latter two derivatives showing another modification of the functionalities of the carbotricyclic ring with the oxidation (**22**) and the epimerisation (**23**) of the hydroxy group at C-12. The derivative **24** showed an *O*-trityl group linked to CH_2-19. The total absence of stimulation activity of cotylenol (**19**), as compared to deacetylaglyconeFC-aglycone (**17**) (14%), is due to the same modification observed in the above described derivatives as the C(19)H_2OH was transformed to a methyl group. The cotylenol, as previously cited, also showed the α-hydroxylation of the C-3. The derivative **25**, compared to **20**, showed a less marked reduction of the stimulant activity (32%), having a similar structure with an ethereal pentacyclic ring between C-12 and C-2, while **20** showed an isopropylidene ring between C-8 and C-9 but both derivatives have a free hydroxy group at $CH_2$19. Finally, the true aglycone of fusicoccin (**18**) compared to **17** showed a marked reduction of the stimulant activity (6%) and this was due to the acetylation of the hydroxy group at H_2C-19.

In conclusion, the most important feature to impart the stimulation of germination *O. ramosa* seeds in FCs seems to be the presence of the hydroxy group on CH_2-19 both in the glucosides and aglycones. The alteration of the functionalities and conformation of the carbotricyclic ring further induce the decrease of activity.

These results are in agreement with older SAR studies (Ballio et al., 1981a,b; Pini et al., 1979) whose main conclusions are that the conformation of the carbotricyclic framework plays a discriminating role for the selection of a class of compounds that may display affinity toward specific FC-receptors. Compounds that do not have the required conformation or that cannot be easily deflected into conformation as in to that of FC cannot be expected to display affinity for FC receptors (Ballio et al., 1991).

The results presented support the idea of using fungal metabolites to induce the ''suicidal germination'' of parasitic plant seeds, in the absence of the host, in order to reduce the seed bank and allow long-term management of those noxious plants.

3. Experimental

3.1. General

The optical rotations were measured on a JASCO P1010 digital polarimeter; IR and UV spectra were determined as neat and in MeCN solution, respectively, on a Perkin–Elmer Spectrum ONE FT-IR Spectrometer and a Lambda 25 UV–Vis spectrophotometer. ^{1}H, ^{13}C, COSY-45, HMQC, and HMBC NMR spectra were recorded at 500, 400, 125, and 100 MHz, respectively, in $CDCl_3$, on Bruker and Varian spectrometers of the Centro Interdipartimentale di Metodologie Chimico-Fisiche, dell'Universitá di Napoli Federico II. The same solvent was used as internal standard. COSY-45, HMQC and HMBC NMR spectra were recorded using Bruker and Varian microprograms. EI and HREI MS were taken at 70 eV on a Fisons Trio-2000 and a Fison ProSpec spectrometer, respectively. Electrospray ionization MS were recorded on a Perkin–Elmer API 100 LC–MS; a probed voltage of 5300 V and a declustering potential of 50 V were used. Analytical and preparative TLC were performed on silica gel (Merck, Kieselgel 60 F_{254}, 0.25 and 0.50 mm, respectively) plates; the spots were visualised by exposure to UV radiation and/or by spraying with 10% H_2SO_4 in methanol and then with 5% phosphomolybdic acid in methanol, followed by heating at 110 °C for 10 min. Column chromatography was carried out on silica gel (Merck, Kieselgel 60, 0.063–0.20 mm). Solvent systems: (A) $CHCl_3$-*iso*-PrOH (9.1), (B) $CHCl_3$-*iso*-PrOH (4:1), (C) petrol-Me_2CO (2.3:1). The Al_2O_3 Wolm neutral and the Amberlite IRA 900 F^- form were purchased from Merck and Fluka, respectively. The petrol ether used had the b.p. 40–70 °C.

3.2. Plant material

Seeds of *O. ramosa* were obtaining by collecting mature capsules from fields of tobacco and tomato heavily infested by the broomrapes, in Southern Italy. Capsules were air dried and opened, allowing seeds extrusion. The material was then sifted through thin sieves to separate seeds from other vegetable residues, and finally clean seeds were collected and stored in plastic vials at 5 °C until their use.

3.3. Chemical

Fusicoccin (**1**) was produced by *F. amygdali* as reported Ballio et al. (1968b). The crystalline sample of **1** obtained as previously reported (Ballio et al., 1968a) preserved at −20 °C under dark for about 26 years showed by TLC (eluent A) and ^{1}H NMR analyses the presence of some minor alteration products, that probably are the well known isomer formed by the shift of the acetyl group from the C-3 to C-2 and C-4 of the glucosyl residue, respectively, (*allo*- and *iso*-FC) (Ballio et al., 1972a) of the sugar moiety. So that the sample was purified by a column chromatography (eluent A). The corresponding dideacetyl derivative (**4**) was prepared by alkaline hydrolysis of **1** according to the procedure previously reported (Ballio et al., 1970) and purified by preparative TLC (eluent B). This latter was also used to prepare the deacetylaglyconeFC (**17**) through the sugar oxidation followed by a β-elimination reaction as previously reported (Ballio et al., 1968a). The crude sample was purified by preparative TLC (eluent B). The purity sample of **1**, **4** and **17** were checked by TLC and ^{1}H NMR analysis.

The other FC derivatives and analogues, whose purity was ascertained by TLC and ^{1}H NMR, were prepared

according to the references listed below: **2** and **13** (Ballio et al., 1981); **3** and **21** (Ballio et al., 1974); **6** (Ballio et al., 1972b); **7** (Ballio et al., 1975a); **8** (Randazzo et al., 1980); **9** (Ballio et al., 1972a); **10** (Ballio et al., 1975b); **11** (Evidente et al., 1984); **12** (Capasso et al., 1977); **14** and **15** (Chiosi et al., 1983) **16** (Barrow et al., 1973); **17** (Ballio et al., 1968a); **19** (Sassa, 1972; Sassa et al., 1975) was a gift of Prof. T. Sassa (Yamagata University, Japan); **20** (Ballio et al., 1968a); **24** (Randazzo et al., 1979); **25** (Casinovi et al., 1974).

3.4. 19-Fluoro-19-dehydroxydideacetylfusicoccin (*5*)

The dideacetylFC (**4**) obtained from FC (**1**) as previously reported (Ballio et al., 1970) was converted into the corresponding 19-monotosylate by reaction with one equivalent of toluene-*p*-sulphonyl chloride in dry pyridine (20 h at room temperature) and purified and characterised as previously reported (Ballio et al., 1974). Half mequivalent (375 mg) of monotosylate dissolved in *n*-pentane (10 ml) was treated with 2 mequivalent of Amberlite IRA 900 F^- form. The reaction was carried out at room temperature for 20 h according to Cainelli and Manescalchi (1976) procedure. The reaction was stopped by filtration of the resin and the clear solution was evaporated under reduced pressure. The oily residue was purified by column chromatography eluted with eluent A affording the 19-fluoro-19-dehydroxydideacetylfusiccoin (**5**) as a homogeneous oily compound (232 mg). **5** had: $[\alpha]_D^{25}$ +5.4 (*c* 0.2); IR ν_{max} cm^{-1} 3387, 1642; UV $\lambda_{max}(\log\varepsilon)$ nm: <220; ^1H NMR spectrum differed from that of FC (Ballio et al., 1991) for the following signals, δ: 4.38 (2 H, *dd*, $^2J_{H\text{-}19,F} = 47.4$ and $^3J_{H\text{-}15,F} = 6.9$ Hz, CH_2F-19), 3.50 (1H, *m*, H-15) (Pretsch et al., 2000). ^{13}C NMR spectrum differed from that of dideacetyl fusicoccin (Radics et al., 1975) for the following signals, δ: 140.3 (*d*, $^3J = 3.4$ Hz, C-14), 86.5 (*d*, $^1J = 171.8$ Hz, C-19), 34.2 (*d*, $^2J = 19.9$ Hz, C-15), 13.9 (*d*, $^3J = 7.4$ Hz, C-20) (Breitmaier and Voelter, 1987; Pretsch et al., 2000); the ^1H and ^{13}C NMR data were assigned also in agreement with COSY, HMQC and HMBC spectra; HR EIMS (rel. int.) *m/z*: 598.3527 [$C_{32}H_{51}FO_9$, calcd. 598.3517, M^+] (0.5), 580 $[M - H_2O]^+$ (0.1), 567 $[M - MeO]^+$ (0.1), 448 $[M - H_2O - Me - C_5H_6 - CH_2O]^+$ (2), 369 [aglicone + H]$^+$ (1), 354 [aglicone + H − Me]$^+$ (1), 257 [*t*-pentenylglucosyl]$^+$ (6), 239 [*t*-pentenylglucosyl − H_2O]$^+$ (7), 229 [*t*-pentenylglucosyl − CO]$^+$ (10), 227 [*t*-pentenylglucosyl − CH_2O]$^+$ (9), 170 [glucosyl − H_2O]$^+$ (24), 148 (100); ESI-MS (+) *m/z*: 637 $[M + K]^+$, 621 $[M + Na]^+$, 599 $[M + H]^+$.

3.5. Aglycone of fusicoccin (*18*)

DeacetylaglyconeFC (**17**, 106 mg) prepared by FC (**1**) as previously reported (Ballio et al., 1968a) was dissolved in EtOAc (42 ml) and treated with Al_2O_3 (Wolm neutral, 14.6 g) the reaction was carried out at reflux of the solvent for 3 days. The reaction was stopped by filtration of Al_2O_3 and the clear solution evaporated under reduced pressure. The residue (86 mg) was purified by column chromatography (eluent A) to give FC-aglycone (**18**) as a homogenous compound (13 mg) and another fraction containing pure not reacted **17** (58 mg). **18** had: $[\alpha]_D^{25}$ +6.9 (*c* 0.2); IR ν_{max} cm^{-1} 3424, 1721, 1647; UV $\lambda_{max}(\log\varepsilon)$ nm: <220; ^1H NMR spectrum differed from that FC-aglycone (Ballio et al., 1991) for the following signals, δ: 4.10 and 3.80 (1H each, *dd*, $J_{19,19'} = 10.5$ and $J_{19,15} = 10.0$ and $J_{19,19'} = 10.5$ and $J_{19',15} = 4.6$ Hz, H-19 and H-19′, respectively), 3.40 (1H, *m*, H-15), 2.04 (3H, *s*, MeCO). HR EIMS (rel. int.) *m/z*: 408.2521 [$C_{23}H_{36}O_6$, calcd. 408.2512, M^+] (5), 391$[M + H - H_2O]^+$ (6), 373 $[M + H - 2H_2O]^+$ (4), 331 $[M + H - H_2O - AcOH]^+$ (3), 330 $[M^+ - H_2O - AcOH]^+$ (7), 312 $[M^+ - 2H_2O - AcOH]^+$ (5), 300 $[M + H - H_2O - AcOH - MeO]^+$ (6), 299 $[M - H_2O - AcOH - MeO]^+$ (5), 285 $[M + H - H_2O - AcOH - MeO - Me]^+$ (12), 281 $[M^+ - 2H_2O - AcOH - MeO]^+$ (24), 147 (100); ESI-MS (+) *m/z*: 447 $[M + K]^+$, 431 $[M + Na]^+$, 409 $[M + H]^+$.

3.6. Preparation of 12-oxo- and 12-epi-19-deoxy8,9-isopropylidene aglycone of *3* (*22* and *23*)

8,9-Isopropylidene aglycone of the 19-deoxydideacetylaglycone of FC (**21**) was prepared from the natural minor metabolite **3**, isolated from the culture filtrate of *F. amygdali*, according to the procedure previously reported (Ballio et al., 1974). A solution of the amorphous acetonide (**21,** 102 mg) in anhydrous CH_2Cl_2 (4 ml) was treated with pyridinium chlorochromate (Corey and Suggs, 1975) (1.8 g) at room temperature under stirring. The reaction was controlled by TLC (eluent C) and stopped with anhydrous Et_2O (8 ml) after 3 h. The residue, after usual work-up, was purified by preparative TLC (eluent C) to give the derivative **22** as an amorphous solid (98.8 mg). **22** had: $[\alpha]_D^{25}$ −134.2 (*c* 0.4); IR ν_{max} cm^{-1} 1746, 1648; UV λ_{max} $(\log\varepsilon)$ nm: <220; ^1H NMR spectrum differed from that FC aglycone (Ballio et al., 1991) for the following signals, δ: 3.52 (1H, *sept*, $J_{15,19} = J_{15,20} = 6.8$ Hz, H-15), 3.02 and 2.97 (1H each, *d*, $J_{13,13'} = 23.4$ Hz, H-13 and H, 13′, respectively), 1.54 and 1.39 (3H each, *s*, two Me of isopropylidene group), 1.07 and 1.06 (3H each, *d*, $J_{15,19} = J_{15,20} = 6.8$ Hz, Me-19 and Me-20); the assignments were confirmed by COSY spectrum. HR EIMS (rel. int.) *m/z*: 390.2781 [$C_{24}H_{38}O_4$, calcd. 390.2771, M^+] (5), 389 $[M + H]^+$ (100), 374 $[M + H - Me]^+$ (21), 361 $[M + H - CO]^+$ (9), 357 $[M - MeO]^+$ (12), 332 $[M - CH_2{=}CH_2 - CO]^+$ (39), 330 $[M - Me_2(CO)]^+$ (38), 299 $[M - Me_2(CO) - MeO]^+$ (26). ESI-MS (+) *m/z*: 427 $[M + K]^+$, 411 $[M + Na]^+$, 389 $[M + H]^+$. The derivative **22** (52 mg) in MeOH (5 ml) was stirred with $NaBH_4$ (103 mg) at room temperature. TLC (eluent A) showed that after 30 min all the starting material had been reduced. After decomposition with 0.1 N HCl of borohydride excess, the mixture was diluted with water (100 ml) and extracted with CH_2Cl_2 (3 × 60 ml). The combined extracts were washed with water and dried (Na_2SO_4).

Evaporation of the solvent gave an oily residue consisting of **20** and its 12-epiderivative **23**. Column chromatography of the mixture, eluted with eluent A, afforded **20** (14.5 mg) followed by **23** (24 mg) obtained as an amorphous solid. **23** had: $[\alpha]_D^{25}$ −41.2 (*c* 0.4); IR ν_{max} cm^{-1}: 3268, 1646; UV λ_{max} (log ε) nm: <220; ^1H NMR spectrum differed from that FC aglycone (Ballio et al., 1991) for the following signals, δ: 3.98 (1H, *m* H-12), 2.55 (1H, *dd*, $J_{13,13'} = 15.6$ and $J_{12,13} = 7.3$ Hz, H-13) and 2.09 (1H, *dd*, $J_{13,13'} = 15.6$ and $J_{12,13} = 7.6$ Hz, H-13′), which are similar to the values previously reported for the $HOCH(12)CH_2$ (13) system in the 12-epi-8,9-isopropylidene aglycone of FC (Evidente et al., 1982), 3.44 (1H, *m*, H-15), 1.51 and 1.36 (3H each, *s*, two Me of isopropylidene group), 1.00 and 0.99 (3H each, *d*, $J_{15,19} = J_{15,20} = 6.8$ Hz, Me-19 and Me-20); the assignments were confirmed by COSY spectrum. HR EIMS (rel. int.) *m/z*: 391 $[M + H]^+$ (100), 392.2929 [$C_{24}H_{40}O_4$, calcd. 392.2928, M^+] (80), 376 $[M + H - Me]^+$ (36), 348 $[M + HMe - CO]^+$ (15), 333 $[M + H - Me_2(CO)]^+$ (15), 332 $[M - Me_2(CO)]^+$ (9), 315 $[M + H - Me_2(CO) - H_2O]^+$ (30), 300 $[M - Me_2(CO) - MeOH]^+$ (84). ESI-MS (+) *m/z*: 429 $[M + K]^+$, 413 $[M + Na]^+$, 391 $[M + H]^+$.

3.7. Seed germination tests

O. ramosa seeds were sterilised for 10 min in 1% sodium hypochlorite, containing a drop of a wetting agent (Tween 80). Seeds were rinsed with sterile tap water and placed on wet glass microfibre filters (GF/A Whatman) in Petri dishes. Seeds were kept at 26 °C in the dark for 3 weeks. Filters were then cut in small pieces, each containing around 100 seeds. The pieces were placed on another filter moistened with 2 ml of the assay solution and kept at 25 °C in the dark. All the metabolites were assayed at 10^{-4} and 10^{-5} M, dissolving them first in methanol in order to have a final concentration of methanol not exceeding 0.5%. The synthetic stimulant (GR24) was used as positive control at 5 ppm (percentage of germination: 54–66%), and distilled water as negative control (percentage of germination: 0–9%). After 4 days, the percentage of seed germination was determined. Data were processed by analysis of variance, according to a complete randomized experimental design. Significant means were tested by LSD ($p < 0.05$).

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References

Ballio, A., Brufani, M., Casinovi, C.G., Cerrini, S., Fedeli, W., Pellicciari, R., Santurbano, B., Vaciago, A., 1968a. Structure of fusicoccin A. Experientia 24, 631–635.

Ballio, A., Carilli, A., Santurbano, B., Tuttobello, L., 1968b. Produzione di fusicoccina in scala pilota. Ann. Ist. Super. Sanità 4, 317–332.

Ballio, A., Casinovi, C.G., Capasso, R., Ferrara, A., Randazzo, G., 1981. Structure revision of the ketone formed by Jones oxidation of fusicoccin triacetate and preparation of 9-epifusicoccins. Gazz. Chim. Ital. 111, 129–132.

Ballio, A., Casinovi, C.G., D'Alessio, V., Grandolini, G., Grandazzo, G., Rossi, C., 1974. 19-Deoxydideacetylfusicoccin, a minor metabolite of *Fusicoccum amygdali*. Experientia 30, 844–845.

Ballio, A., Casinovi, C.G., Framondino, M., Grandolini, G., Randazzo, G., Rossi, C., 1972a. Structure of three isomers of monodeacetylfusicoccin. Experientia 28, 1150–1151.

Ballio, A., Casinovi, C.G., Framondino, M., Rossi, C., Menichini, F., Randazzo, G., Rossi, C., 1972b. Structures of isofusicoccin and allofusicoccin. Experientia 28, 126–127.

Ballio, A., Casinovi, C.G., Grandolini, G., Marta, M., Randazzo, G., 1975a. 19-Deoxy-3α-hydroxydideacetylfusicoccin. Gazz. Chim. Ital. 105, 1325–1328.

Ballio, A., Casinovi, C.G., Grandolini, G., Pomponi, M., Randazzo, G., Rossi, C., 1975b. 16-*O*-demethyl-19-deoxydideacetyl-3-epifusicoccin. Gazz. Chim. Ital. 105, 647–650.

Ballio, A., Casinovi, C.G., Randazzo, G., Rossi, C., 1970. Characterization of by-products of fusicoccin in culture filtrates of *Fusicoccum amygdali*. Experentia 26, 349–351.

Ballio, A., Castellano, S., Cerrini, S., Evidente, A., Randazzo, G., Segre, A.L., 1991. ^1H NMR conformational study of fusicoccin and related compounds: molecular comformation and biological activity. Phytochemistry 30, 137–146.

Ballio, A., Chain, E.B., De Leo, P., Erlanger, B.F., Mauri, M., Tonolo, A., 1964. Fusiccocin: a new wilting toxin produced by *Fusicoccum amygdali* Del. Nature 203, 297.

Ballio, A., De Michelis, M.I., Lado, P., Randazzo, G., 1981a. Fusicoccin structure–activity relationships: stimulation of growth by cell enlargement and promotion of germination. Physiol. Plant. 52, 471–475.

Ballio, A., Federico, R., Scalorbi, D., 1981b. Fusicoccin structure–activity relationships. In vitro binding to microsomal preparations of maize coleoptiles. Physiol. Plant. 52, 476–481.

Ballio, A., Graniti, A., 1991. Phytotoxins and their involvement in plant diseases. Experientia 47, 751–864.

Barrow, K.D., Barton, D.H.R., Chain, E., Ohnsorge, U.F.W., Sharma, R.P., 1973. The structure of fusicoccin H. J. Chem. Soc. C, 1590–1599.

Barrow, K.D., Barton, D.H.R., Chain, E., Ohnsorge, U.F.W., Thomas, R., 1968. The constitution of fusicoccin. Chem. Commun., 1198–1200.

Breitmaier, E., Voelter, W., 1987. Carbon-13 NMR Spectroscopy. VCH, Weinheim, pp. 205–206.

Butler, L.G., 1995. Chemical communication between the parasitic weed *Striga* and its crop host – a new dimension in allelochemistry. In: Inderjit Dakshini, K.M.M., Einhellig, F.A. (Eds.), Allepathy: Organisms, Processes and Application, ACS Symposium Series 582. American Chemical Society, Washington, DC, pp. 158–168.

Cainelli, G., Manescalchi, F., 1976. Polymer-supported reagents. The use of anion exchange resins in the synthesis of primary and secondary alkyl fluoride from alkyl halides or alkyl methanesulfonates. Synthesis, 472–473.

Capasso, R., Evidente, A., Lasaponara, M., Randazzo, G., 1977. Una insolita reazione dell'acetone sulla fusicoccina. Rend. Accad. Sci. Fis. Mat. Napoli 44, 1–5.

Casinovi, C.G., Santurbano, B., Conti, G., Malorni, A., Randazzo, G., 1974. Structure and spectroscopic properties of an isomer of fusicoccin deacetylaglycone and some derivatives. Gazz. Chim. Ital. 104, 679–691.

Chiosi, S., Evidente, A., Randazzo, G., Casinovi, C.G., Segre, A.L., Ballio, A., 1983. The reaction of fusicoccin with Fritz and Schenk reagent. Gazz. Chim. Ital. 113, 717–720.

Corey, E.J., Suggs, J.W., 1975. Pyridinium chlorochromate. Efficient reagent for oxidation of primary and secondary alcohols to carbonyl compounds. Tetrahedron Lett. 31, 2647–2650.

Cook, C.E., Whichard, L.P., Turner, B., Wall, M.E., Egley, G.H., 1966. Germination of witchweed (*Striga lutea*): isolation and properties of a potent stimulant. Science 154, 1189–1190.

Cook, C.E., Whichard, L.P., Wall, M.E., Egley, G.H., Coggon, P., Luhan, P.A., McPhail, A.T., 1972. Germination stimulants II. The structure of strigol – a potent seed germination stimulant of witchweed (*Striga lutea* Lour). J. Am. Chem. Soc. 94, 6198–6199.

Evidente, A., Randazzo, G., Ballio, A., 1982. The biosynthetic origin of fusicoccin hydroxyisopropyl group. Tetrahedron 38, 3169–3172.

Evidente, A., Randazzo, G., Casinovi, C.G., Ballio, A., 1984. Catalytic hydrogenation of fusicoccin. Gazz. Chim. Ital. 114, 61–64.

Fisher, N.H., Weidenhamer, J.D., Riopel, J.L., Quijano, L., Menelaou, M.A., 1990. Stimulation of witchweed germination by sesquiterpene lactones: a structure–activity study. Phytochemistry 29, 2479–2483.

Galindo, J.C.G., De Luque, A.P., Jorrín, J., Macias, F.A., 2002. SAR studies of sesquiterpene lactones as *Orobanche cumana* seed germination stimulants. J. Agric. Food Chem. 50, 1911–1917.

Hauck, C., Müller, S., Schildknecht, H., 1992. A germination stimulant for parasitic flowering plants from *Sorghum bicolor*, a genuine host plant. J. Plant Physiol. 139, 474–478.

Lynn, D.G., Steffens, J.C., Kamut, V.S., Graden, D.W., Shabanowitz, J., Riopel, J.L., 1981. Isolation and characterization of the first recognition substance for parasitic angiosperm. J. Am. Chem. Soc. 103, 1868–1870.

Mangnus, E.M., Dommerholt, F.J., De Jong, R.L.P., Zwanenburg, B., 1992. Improved synthesis of strigol analogue GR 24 and evaluation of the biological activity of its diastereomers. J. Agric. Food Chem. 40, 1230–1235.

Marré, E., 1979. Fusiccocin: a tool in plant physiology. Annu. Rev. Plant Physiol. 30, 273–288.

Mori, K., Matsui, J., Yokohota, T., Sakai, H., Bo, M., Takeuchi, Y., 1999. Structures and synthesis of orobanchol, the germination stimulant of *Orobanche minor*. Tetrahedron Lett. 40, 943–946.

Müller, S., Hauck, C., Schildknecht, H., 1992. Germination stimulants produced *by Vigna unguiculata* Walp cv Sunders Upright. J. Plant Growth Regul. 11, 77–84.

Nefkens, G.H.L., Thuring, J.W.J.F., Beenakkers, M.F.M., Zwanenburg, B., 1997. Synthesis of phthaloylglycine-derived strigol analogue and its germination stimulatory activity towards seeds of parasitic weeds *Striga hermonthica* and *Orobanche crenata*. J. Agric. Food Chem. 45, 2273–2277.

Netzly, D.H., Riopel, J.L., Ejeta, G., Butler, L.G., 1988. Germination stimulants of witchweed (*Striga asiatica*) from hydrophobic root exudate of sorghum (*Sorghum bicolor*). Weed Sci. 36, 441–446.

Pretsch, E., Bühlmann, P., Affolter, C., 2000. Structure Determination of Organic Compounds – Tables of Spectral Data. Springer, Berlin, pp. 112–113, 198.

Pini, C., Vicari, G., Ballio, A., Federico, R., Evidente, A., Randazzo, G., 1979. Antibodies for fusicoccins. Plant Sci. Lett. 16, 343–353.

Radics, L., Kajtár-Peredi, M., Casinovi, C.G., Grandolini, G., Rossi, C., 1975. Carbon-13 NMR spectra of fusicoccin and derivatives. Org. Magn. Res. 7, 137–142.

Randazzo, G., Evidente, A., Capasso, R., Chiosi, C.G., Casinovi, C.G., Ballio, A., 1980. A new 3α-hydroxyfusicoccin from culture filtrates of *Fusicoccum amygdali* Del.. Pyhtopathol. Mediterr. 19, 181–182.

Randazzo, G., Evidente, A., Capasso, R., Colantuoni, F., Tuttobello, L., Ballio, A., 1979. Further experiments on the biosynthesis of fusicoccin. Gazz. Chim. Ital. 109, 101–104.

Rugutt, J.K., Rugutt, K.J., 1997. Stimulation of *Striga hermonthica* seed germination by 11b,13-dihydroparthenolide. J. Agric. Food Chem. 45, 4845–4849.

Sassa, T., 1972. Structure of cotylenol, the aglycone of cotylenins leaf growth substances. Agric. Biol. Chem. 36, 2037–2039.

Sassa, T., Takahama, A., Shindo, T., 1975. The stereostructure of cotylenol, the aglycone of cotylenins leaf growth substances. Agric. Biol. Chem. 39, 1729–1734.

Wigchert, S.C.M., Kuiper, E., Boelhouwer, G.J., Nefkens, G.H.L., Verkleif, J.A.C., Zwanenburg, B., 1999. Dose-response of seeds of the parasitic weeds *Striga* and *Orobanche* towards the synthetic germination stimulants GR 24 and Nijmegen 1. J. Agric. Food Chem. 47, 1705–1710.

Yokota, T., Sakai, H., Okuno, K., Yoneyama, K., Takeuchi, Y., 1998. Alectrol and orobanchol, germination stimulant for *Orobanche minor*, from its host red clover. Phytochemistry 49, 1967–1973.

Yoneyama, K., Takeuchi, Y., Ogasawara, M., Konnai, M., Sugimoto, Y., Sassa, T., 1998. Cotylenins and fusicoccins stimulate seed germination of *Striga hermonthica* (Del.) Benth and *Orobanche minor* Smith. J. Agric. Food Chem. 46, 1583–1586.