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Trigoxazonane, a monosubstituted trioxazonane from *Trigonella foenum-graecum* root exudate, inhibits *Orobanche crenata* seed germination

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Abstract

Orobanche crenata is a major threat to grain legume production. Fenugreek (*Trigonella foenum-graecum*) is an annual legume that has been shown to effectively reduce *O. crenata* infection when intercropped with grain legumes. In this paper, we point that this can be attributed to allelopathy, through inhibition of the germination of *O. crenata* by fenugreek root exudates. The main inhibitory metabolite was isolated and characterized. Allelopathy was demonstrated in different bioassays, by inhibition of *O. crenata* seeds germination both by growing fenugreek and pea plants together (intercropped), and by application of fenugreek root exudates. Fenugreek root exudates were extracted with organic solvent and fractionated giving several fractions, two of which showed moderate (27%) and strong (54%) inhibition of *O. crenata* seed germination, respectively. The most active metabolite is a new monosubstituted trioxazonane, characterized by spectroscopic methods as the 2-butyl-[1,4,7,2]trioxazonane and named trigoxazonane.

Keywords: *Orobanche crenata*; Parasitic weed; *Trigonella foenum-graecum*; *Pisum sativum*; Germination inhibitor; Root exudate purification; Trigoxazonane; Trioxazonane

1. Introduction

Fenugreek (*Trigonella foenum-graecum* L.) is an annual legume known and used since ancient times for pharmaceutical, human food and animal feed purposes. Historically, fenugreek is one of the oldest known medicinal plants, used in Greek, Egyptian, Chinese and Arabian medicine (Petropoulos, 2002). At present, fenugreek is an important cash crop in India, China, Near East, East Africa and Mediterranean countries, with an important market for its seeds for curry powder and for flavouring agent for ruminant and pig feed (Fotopoulos, 2002). Fenugreek is also a popular forage and fodder crop known since ancient Greek time. Fenugreek is frequently intercropped with vetches or faba bean (Talelis, 1967).

The area of fenugreek cultivation coincides with the area of broomrape (*Orobanche* species) distribution. Broomrapes are parasitic achlorophyllus weeds totally dependent on the host for organic carbon, water and nitrogen (Joel et al., 2007). Crenate broomrape (*O. crenata* Forsk) has threatened legume crops since antiquity in the Mediterranean basin and the Middle East (Joel et al., 2007; Rubiales et al., 2006). A wide variety of approaches – physical, cultural, chemical and biological – have been explored but none of them has proven to be as effective, economical

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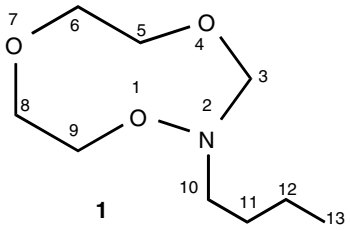


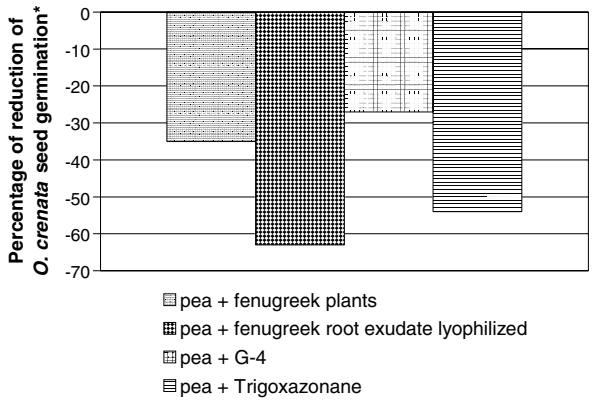
Fig. 1. Structure of trigoxazonane (**1**).

and applicable as desired (Parker, 1991; Joel et al., 2007). An interesting approach for controlling parasitic weeds is reducing soil seed bank by using trap and catch crops. An alternative approach is intercropping of susceptible host with inhibitory crops (Fernández-Aparicio et al., 2006b). In line with this approach, some studies have been done and suggested that intercropping fenugreek with faba bean (*Vicia faba* L.) can reduce crenate broomrape infection (Bakheit et al., 2002; Fernández-Aparicio et al., 2006a).

The objectives of this present work were to study allelophatic effects of fenugreek root exudates on *O. crenata* germination. This paper describes the structural elucidation and biological activity of the monosubstituted trioxazonane isolated from the root exudates of *T. foenum-graecum* and named it trigoxazonane (**1**, Fig. 1). Its structure was determined by extensive use of spectroscopic methods (essentially NMR and MS techniques).

2. Results and discussion

The root exudate (15 l) of *T. foenum-graecum*, showing a strong inhibition of *O. crenata* seed germination (Fig. 2), was extracted with EtOAc and fractionated as described in Section 3, yielding trigoxazonane (**1**, Fig. 1), the main metabolite as a homogeneous compound. This compound has a molecular formula of $C_9H_{19}NO_3$ as deduced from HR EIMS spectrum consistent with 1 unsaturation. Preliminary ^1H and ^{13}C NMR investigations showed the presence of complex systems only in the oxygenated and/or nitrogenated methylene region, and the signals of a butyl side chain, the terminal methylene group of which was most likely linked to the tertiary nitrogen atom. These partial structures were confirmed by end absorption observed in the UV spectrum and the presence in the IR spectrum of bands due only to aliphatic carbon skeleton, which also indicated the ether and the tertiary amino nature of three oxygen and the nitrogen atoms, respectively (Nakanishi and Solomon, 1977). Based on these observations, the single unsaturation of **1** should be a ring. Detailed investigation of the ^1H NMR spectrum (Table 1) showed the presence of four oxygenated methylene groups all appearing as triplet ($J = 4.6$ Hz) at δ3.58, 3.64, 3.66 and 3.71, typical of oxygenated primary carbons (Pretsch et al., 2000). The methylene protons at δ3.71 (CH_2-5) coupled in the COSY spectrum (Berger and Braun, 2004) with those at δ3.66 (CH_2-6), while those at δ3.64 (CH_2-8) coupled with those at δ3.58 (CH_2-9). As expected in the same spectrum the triplet ($J = 7.4$ Hz) of the terminal methyl group of the side chain at δ0.91 (CH_3-13) coupled with the adjacent methylene protons resonating as a multiplet ($J = 7.4$ Hz) at δ1.35 (CH_2-12). The latter coupled with the adjacent methylene protons observed as quintet ($J = 7.4$ Hz) at δ1.56 (CH_2-11) and this in turn coupled with the triplet ($J = 7.4$ Hz) of the methylene group at δ3.45 (CH_2-10), which is probably linked to the tertiary nitrogen atom (Pretsch et al., 2000). A structure having a nitrogenated butyl side chain and the trioxygenated ring was consistent with the couplings observed in the TOCSY spectrum (Berger and Braun, 2004) and the signals observed in the ^{13}C NMR spectrum (Table 1). The ^{13}C NMR spectrum showed the presence of four ether methylene carbons at the typical chemical shift values of δ70.6, 70.5, 70.0 and 66.9 (Breitmaier and Voelter, 1987), which, based on the coupling observed in the HSQC spectrum (Berger and Braun, 2004), were assigned to C-8, C-6, C-9 and C-5, respectively. The butyl side chain carbon signals observed at δ71.2, 31.7,



*Reduction of *O. crenata* seed germination relative to the positive control (55% germination, established as 0% reduction)
All values are statistically different from positive control (Tukey test, $p < 0,05$)

Fig. 2. Inhibition of *O. crenata* seed germination in presence fenugreek root exudate, chromatographic fraction and trigoxazonane (**1**).

Table 1
^1H and ^{13}C NMR data of trigoxazonane (**1**)[a]

C	δC[b]	δH	J (Hz)	HMBC
3	95.6 *t*	4.74 *s*		3.71
5	66.9 *t*	3.71 *t*	4.6	4.74, 3.66, 3.64
6	70.5 *t*	3.66 *t*	4.6	3.71,3.64
8	70.6 *t*	3.64 *t*	4.6	3.66, 3.58
9	70.0 *t*	3.58 *t*	4.6	3.64, 3.45
10	71.2 *t*	3.45 *t*	7.4	1.56, 1.35
11	31.7 *t*	1.56 *quin*	7.4	3.45, 0.91
12	19.2 *t*	1.35 *m*	7.4	3.45, 1.56, 0.91
13	13.9 *q*	0.91 *t*	7.4	1.56, 1.35

[a] The chemical shifts are in δ values (ppm) from TMS. 2D ^1H,^1H (COSY, TOCSY) ^{13}C, ^1H (HSQC) NMR experiments delineated the correlations of all protons and the corresponding carbons.
[b] Multiplicities determined by DEPT spectrum.

19.2 and 13.9 were assigned to C-10, C-11, C-12 and C-13, respectively (Breitmaier and Voelter, 1987). In the same spectrum was also observed the signals of an additional methylene group at δ95.6 (C-3). This methylene carbon in the HSQC spectrum coupled with the singlet resonating δ4.74 (CH_2-3), a typical chemical shift value for a proton linked to both an oxygen and a nitrogen atom. This methylene group served as the linker for ring closure. On the basis of these results trigoxazonane can be formulated as the 2-butyl[1,4,7,2]trioxazonane (**1**, Fig. 1).

The structure of **1** was supported by several correlations, as recorded in the HMBC (Table 1) and NOESY (Table 2) (Berger and Braun, 2004) spectra. The HMBC spectrum showed the coupling of the H_2-3 protons with C-5, the H_2-5 protons with both C-3 and C-6, the H_2-6 protons with both C-5 and C-8, the H_2-8 protons with C-5, C-6 and C-9, the H_2-9 protons with C-8, the protons of the terminal methyl group (Me-13) of the butyl side with both C-11 and C-12, the H_2-12 protons with both C-10 and Me-13, the H_2-11 protons with C-10, C-12 and Me-13, the H_2-10 protons with C-9, C-11 and C-12. As expected, the NOESY spectrum showed couplings between the protons of the terminal methyl (Me-13) group and those of the α-methylene group (CH_2-12) of butyl side chain, between those of the latter group and those of CH_2-11, and in turn those of CH_2-11 with the protons of the methylene group (CH_2-10) linked to the nitrogen of the trioxazonane ring. Furthermore, the same spectrum showed the significant effects between the protons of the nitrogen-linked methylene group of the butyl side chain at δ3.45 (CH_2-10) with those of the methylene group of the trioxazonane ring at δ3.58 (CH_2-9), and those of the other methylene group of the same ring at δ3.71 (CH_2-5) with the methylene group at δ4.74 (CH_2-3). Considering the inversion and the high flexibility of the oxazonane ring (Fülöp et al., 1997; Riddell et al., 1978) and the butyl side chain, these latter two nOe-effects indicate that the ring probably assumes a folded conformation with the CH_2-9 pointing towards the butyl side chain.

The HR EIMS spectra, recorded in positive mode, in addition to the pseudomolecular ion $[M+2H]^+$ at *m/z* 191.2691, probably due to the opening of the trioxazonane ring (Porter, 1985; Pretsch et al., 2000), showed typical fragmentation peaks characteristic of a tertiary amine and oxazolidine and oxazine derivatives (Pretsch et al., 2000; Porter, 1985). In fact, the pseudomolecular ion $[M+2H]^+$, frequently found (Fülöp et al., 1997), generated the ions at *m/z* 161, 131, 103 by consecutive losses two CH_2O and CO molecules. Furthermore, the molecular ion, by losses of CH_2 followed alternatively by CH_2O or butene produced the ions at *m/z* 175 and 145 or 119, respectively. The same spectrum showed the significant peaks produced from the butylamino residue at *m/z* 73 and 57 due to the ions $[C_4H_9–NH_2]^+$ and $[C_4H_9]^+$, respectively. ESI MS, recorded in positive mode, showed the sodium cluster of the open ring compound at *m/z* 214.

Fenugreek plants, when cultivated alone, incited very little germination of *O. crenata* seeds (5%), compared to the susceptible pea (*Pisum sativum* L.) cv. Messire used as positive control (55% established at 100%). When fenugreek was intercropped with pea, germination of *O. crenata* seeds in the vicinity of the pea roots suffered a 35% reduction compared to the positive control (Fig. 2). This is in agreement with published reports on reduction broomrape infection of faba bean (*Vicia faba*) intercropped with fenugreek under field conditions (Bakheit et al., 2002; Kharrat and Halila, 2005).

The lyophilized root exudate of fenugreek seedlings, when applied to plates with pea roots induced 63% reduction of *O. crenata* seed germination. The germination reduction was by 27% when fraction 4 (G4) of the initial chromatographic column of the ethyl acetate extract of fenugreek root exudates was applied, and by 54% when **1** was applied. No significant reduction of *O. crenata* seed germination was observed with other fractions (data not shown). The difference observed in the inhibition of *O. crenata* seeds germination caused by the fenugreek root exudates compared to that of **1** is probably due to the presence former of other metabolites (see the results of G4 fraction) that may have contributed to the total inhibitory effect observed.

In conclusion, considering that isoxazolidine, oxazolidine and oxazines containing compounds are well known as synthetic compounds but rare as natural substances (Eckstein and Urbanski, 1963; Castillo et al., 1999; Xiaoyong et al., 2003) and oxazonane is reported as a synthetic compound (Ullmann and van der Schalk, 1912), **1** is the first example of a trioxazonane as a naturally occurring compound. This was confirmed by the presence of **1** in the ethyl acetate extract as ascertained by HPLC, with a retention time of 11.4 min, and LC–MS analysis carried out in comparison with an authetich sample of **1.** The only example of close related compound is 1.4.7.13-pentaoxa-2-azacyclopentadecene a synthetic crown compound (Okahara and Ikeda, 1980).

Table 2
2D ^{1}H NOE (NOESY) data obtained for trigoxazonane (**1**)

Considered	Effects	Considered	Effects
4.74 (H_2-3)	3.71 (H_2-5)	1.56 (H_2-11)	3.45 (H_2-9), 1.35 (H_2-12),
3.71 (H_2-5)	4.74 (H_2-3)	1.35 (H_2-12)	1.56 (H_2-11), 0.91 (H_3-13)
3.58 (H_2-9)	3.45 (H_2-10)	0.91 (H_3-13)	1.35 (H_2-12)
3.45 (H_2-10)	3.58 (H_2-9), 1.56 (H_2-11)		

3. Experimental

3.1. General

IR spectrum was recorded as neat on a Perkin–Elmer Spectrum One FT–IR Spectrometer and UV spectrum was taken in MeCN solution on a Perkin–Elmer Lambda

25 UV–Vis spectrophotometer. ^{1}H spectrum was recorded at 600, 400 MHz, in $CDCl_3$ on Bruker spectrometers. The ^{13}C NMR spectrum was recorded at 150, 100 and 75 MHz, in the same solvent and using the same instruments. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT experiment (Berger and Braun, 2004). DEPT, COSY-45, TOCSY, HSQC, HMBC and NOESY experiments (Berger and Braun, 2004) were performed using Bruker microprograms. EI and HR EI MS were taken at 70 eV on a QP 5050 Shimadzu and Fison Prospec spectrometer, respectively. Electrospray (ESI) MS were recorded on a Perkin–Elmer API 100 LC–MS instrument, a probed voltage of 5300 V and a declustering potential of 50 V were used. HR ESI MS spectrum was recorded on Micromass Q-TOF Micro instrument. Analytical and preparative TLC were performed on silica gel (Merck, Kieselgel 60 F_{254}, 0.25 and 0.50 mm, respectively) or reverse phase (Whatman, KC18 F_{254}, 0.20 mm) plates; the spots were visualized by exposure to UV light and I_2 vapours or by spraying first with 10% H_2SO_4 in methanol and then with 5% phosphomolybdic acid in ethanol, followed by heating at 110 °C for 10 min. CC: silica gel (Merck, Kieselgel 60, 0.063-0.200 mm). Solvent systems: (A) $CHCl_3$-*iso*-PrOH (19:1); (B) $CHCl_3$-*iso*-PrOH (9:1); (C) $CHCl_3$-*iso*-PrOH (7:3); (D) H_2O-EtOH (2:3).

3.2. Production, extraction and purification of trigoxazonane (1) from root exudate

Approximately 4800 seedling plants of *T. foenum-graecum* were grown for 25 days in perlite in plastic trays. Then, perlite was carefully removed and seedlings were grown for two days in hydroponic sterile deionised water allowing for the release the root exudate into water. The water was collected, filtered with sterile gauze and lyophilised. The lyophilized root exudates (1.6 g, from 15 l) was dissolved in Milli-Q water, centrifuged and extracted with EtOAc (3×800 ml). An aliquot of the organic extract (68 mg, from 13 l) was purified by silica gel column chromatography eluted sequentially with the solvent system A, B, C and then washed with MeOH, obtaining 10 groups (G1-G10) of homogeneous fractions. The residue of the fifth fraction (G5, 32 mg, 2.5 mg l^{-1}) appeared to be a homogeneous oily compound (R_f 0.60 and 0.35, silica gel eluent A and reverse phase eluent D, respectively) and was named trigoxazonane (**1**).

3.3. Trigoxazonane (*1*)

Compound **1** had: IR ν_{max} 2957–2867 cm^{-1}; UV λ_{max} nm < 200 nm; ^{1}H and ^{13}C NMR spectra: see Table 1; EI–MS (rel. int) *m/z*: 191.1530 [$C_9H_{21}NO_3$, calcd. 191.1521, $M + 2H]^+$(5), 175 $[M–CH_2]^+$(7), 161 $[M + 2H–CH_2O]^+$ (11), 145 $[M–CH_2–CH_2O]^+$(6), 131$[M + 2H\text{-}2xCH_2O]^+$ (5), 119 $[M–CH_2–CH_2{=}CH–CH_2–CH_3]^+$(10), 103 $[M + 2H–2xCH_2O–CO]^+$ (58) 73$[CH_3CH_2CH_2CH_2NH_2]^+$(45), 57 $[CH_3CH_2CH_2CH_2]^+$(100), HR ESI–MS (+) *m/z*: 214 $[M + 2H + Na]^+$.

3.4. LC–MS analyses

Analytical and HPLC grade solvents for chromatographic use were purchased from Carlo Erba. Water was HPLC quality, purified in a Milli-Q system. Disposable syringe filters, Anotop 10–0.2 μm, were purchased from Whatman. Chromatographic separation was performed using a HPLC apparatus equipped with two Micropumps Series 200 (Perkin–Elmer), an UV–VIS series 200 (Perkin–Elmer) detector setted at 200 nm and a Macherey–Nagel high-density reversed-phase Nucleosil 100-5 C_{18} HD column (250×4.6 mm i.d.; 5 μm) provided with an in-line guard column from Alltech. Aliquots of the samples (20 μl) were injected for analysis, having the mobile phase of 55:45, v/v MeCN and water at a flow rate of 1 ml/min. The trigoxazonane and the ethyl acetate extract of fenugreek root exudates samples were prepared dissolving 1.0 and 7.0 mg, respectively, in 1 ml of MeOH. The trigoxazonane standard sample was purified and identified from fenugreek root exudate as previously described. MS analysis was performed an API 3000 triple quadruple mass spectrometer (Applied Biosystems) equipped with a Turbo-Ionspray source working in the positive ion mode. The analyses were performed using the following settings: drying gas (air) was heated to 450 °C, capillary voltage (IS) was setted to +5500 V and the declustering potential was setted at +50 V. Acquisition was performed in scan in the range *m/z* 150–450 a.m.u.

3.5. Bioassays on Orobanche crenata germination

In a first experiment inhibition of *O. crenata* seed germination by fenugreek was studied in a minirhizotron experiment consisting of 15×15 cm square Petri dishes with 8 mg of *Orobanche* seeds spread over a glass microfibre filter paper sheet (GFFP) (Fernández-Aparicio et al., 2006a). Pea and fenugreek seeds were germinated in Petri dishes on wet glass-fibre filter papers and kept in the dark at 20 °C for 5 days. When the radicle reached 4–5 cm length, seedlings plants were transferred to new dishes, in which pea and fenugreek plants were grown separately (1 pea or 1 fenugreek plant per Petri dish, simulating a monocrop), or together (1 pea plant + 1 fenugreek plant per Petri dish, simulating an intercrop). Six Petri dishes were established per combination. *O. crenata* seeds were preconditioned in Petri dishes with filter paper wet with deionised water for 11 days in the dark at 20 °C before use. *O. crenata* seeds (8 mg) were previously spread on the paper, after being disinfected with ethanol (70%, for 30 s) followed by sodium hypochlorite (1%, for 20 min) and placed in darkness at 20 °C for 10 days for preconditioning (Pérez-de-Luque et al., 2005). Dishes were sealed with parafilm, covered with aluminium foil to exclude light and were placed vertically, the germinating host plant upwards, in trays. Test plants

were maintained in a growth chamber at 20 °C with a 14 h photoperiod.

Four hundred seeds located close (<3 mm) to the distal part of young plant root were observed per Petri dish under a stereoscopic microscope at 30× magnification and the number of those germinated counted and expressed as percentage of the total. Seeds having an emerged radicle were scored as germinated.

The inhibitory effect of the lyophilized exudate, the residue of the organic extract, the corresponding aqueous phase residue and all the chromatographic fraction groups and pure **1** was tested *in vitro* on conditioned seeds of *O. crenata* that were stimulated to germinate being in close proximity with pea roots (Pérez-de-Luque et al., 2005) and exogenous application of the synthetic germination stimulant GR24 (Zhou et al., 2004). *O. crenata* seeds (2.3 mg) were spread on 9 cm GFFP disks and placed in wet conditions in the dark at 20 °C for eleven days conditioning. The GFPP were then placed on Petri dishes of 9 cm diameter filled previously with perlite. A seedling of pea was placed in each plate, carefully extending the roots over GFPP. Each Petri dish was irrigated with 2 ml of GR24 10^{-4}M dissolved in 0.3 mM MES (2-morpholinoethansulfonic acid monohydrate, Merck). Simultaneously, the lyophilized exudate was applied on the GFFP at a concentration of 0.45 mg/cm^2. The organic extract (21.94 μg/cm^2), corresponding aqueous phase residue and all the chromatographic fraction groups were applied on separate GFFP plates in amounts equivalent to that of the organic extract, in accordance with the proportions in which the fractions were separated by the column chromatography. Pure trigoxazonane was applied at a concentration of 10.32 μg/cm^2. Each treatment consisted of four replications in a completely randomized design. To dissolve the test samples for each treatment 0.7% of methanol (14 μl) was used and then mixed with 0.3 mM MES (2 ml) and applied to the GFFP dishes, with the conditioned seeds of *O. crenata*. Petri dishes were kept in a growth chamber at 20 °C with 12 h of supplemental light and were irrigated with sterile deionised water twice per week. Germination of *O. crenata* seed in the proximity of the pea roots (< 3 mm) was scored six days later. Percent germination was determined as indicated above. Petri dishes with pea seedling and GR24 (dissolved in MES) irrigated with 2 ml of sterile deionised water containing 0.7% methanol were used as positive control. Plates with deionised water with 0.7% methanol without pea plants nor GR24 were used as negative control. Percentage *O. crenata* seed germination for each treatment was expressed relative to the germination obtained in the positive control (Fig. 2) (55% germination, established as 0% inhibition). Statistical analyses were performed using Statistix 8.0 statistical package (Analytical Software, Tallahase, USA). Percentage data were transformed to angles ($y = \text{arcsine}\sqrt{\times}/100$) and again checked before applying analysis of variance. Comparisons of means were Tukey tests. Null hypotheses were rejected when $P \leqslant 0.05$.

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