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Drazepinone, a trisubstituted tetrahydronaphthofuroazepinone with herbicidal activity produced by *Drechslera siccans*

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Abstract

When grown in a minimal-defined medium, a strain of *Drechslera siccans*, a pathogenic fungus isolated from seeds of *Lolium perenne*, produced phytotoxic metabolites. This strain is one of the best toxin producers among several grass pathogenic fungal strains collected and tested to find phytotoxins to be used as natural herbicides of monocot weeds. From the culture filtrates of *D. siccans*, we isolated a new phytotoxic trisubstituted naphthofuroazepinone, named drazepinone, and characterised it as a 3,5,12a-trimethyl-2,5,5a,12a-tetrahydro-1*H*-naphtho[2′,3′:4,5]furo[2,3-*b*]azepin-2-one. Assayed at 2 μg μl^{-1} solution the novel metabolite proved to have broad-spectrum herbicidal properties, without antibacterial and antifungal activities, and low zootoxic activity. Its original chemical structure and the interesting biological properties make drazepinone a potential natural herbicide.

Keywords: Drazepinone; *Lolium perenne*; *Drechslera siccans*; Phytotoxins; Natural herbicide; Azepinones; Weed management

1. Introduction

In many countries, annual and perennial grasses are among the most problematic weeds for various crops (Holm et al., 1977). Such weeds are difficult to control because of their prodigious seed production, which is responsible for their reproduction and diffusion, their tolerance to the chemical herbicides available, and their growth habits that can enable them to escape from chemical and mechanical control practices.

Considering the increasing number of weed species that are tolerant or resistant to the use of herbicides (Naylor, 2002), and the difficulties in finding new chemical active compounds, biocontrol microorganisms and new herbicides from natural sources are receiving a renewed interest. Some promising fungal pathogens have been identified and their use as inundative agents has been proposed (Zhan and Watson, 1997; Chandramohan and Charudattan, 2001); furthermore, some fungal phytotoxins have been identified and considered as possible natural herbicides (Hallock et al., 1988; Kastanias and Tokousbalides, 2000).

Pathogenic fungi isolated from grass weeds were found in several fungal collections and many strains were collected (Fracchiolla, 2003). Such investigation was aimed at finding producers of toxic metabolites with herbicidal activities against grass weeds. Some of the selected fungal strains were able to produce highly phytotoxic culture filtrates, particularly one strain of *Drechslera siccans*, isolated from *Lolium perenne* L.

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This paper describes the isolation, structural elucidation, and biological characterization of the main toxic metabolite, named drazepinone (**1**), produced in liquid culture by *D. siccans*. Its structure was determined by extensive use of spectroscopic methods (essentially NMR and MS techniques).

2. Results and discussion

The culture filtrate (4 l) of *D. siccans*, showing high phytotoxicity on host plant leaves, was examined to ascertain the chemical nature of the phytotoxic metabolites. Preliminary in vitro experiments showed that the fungus produces lipophilic phytotoxins, which were exhaustively extracted with $CHCl_3$. The organic extract (667 mg) that showed, by TLC analysis, the presence of a main metabolite (R_f 0.45, silica gel, eluent D), was fractioned by combined CC and preparative silica gel chromatography (see Experimental for details), yielding the main toxin as a homogeneous oil (9.7 mg, 2.4 mg l^{-1}) withstanding crystallization. Based on its structural feature described below, the toxin was named drazepinone (**1**).

Drazepinone has a molecular formula of $C_{19}H_{19}NO_2$, corresponding to 11 degrees of unsaturations, as deduced from the molecular weight of 316.1324, measured by HR ESI mass spectrometry for its adduct with sodium. Considering the absence of the typical colour when its TLC chromatogram was sprayed with ninhydrin, its nitrogen atom does not have an amino nature. This is in agreement with the investigation of its ^1H NMR spectrum (Table 1) that showed the presence of a broad singlet at δ 12.4 (HN-1), typical of a secondary amide group (Pretsch et al., 2000). We also observed a broad singlet at δ 5.38 due to an olefinic proton (H-4), which in the COSY spectrum (Berger and Braun, 2004) coupled with a broad quartet ($J = 7.2$) at δ 2.90 (H-5). This in turn coupled with a geminal methyl group (Me-14), resonating as a doublet ($J = 7.2$) at δ 1.29, and with a broad doublet ($J = 1.3$ Hz) at δ 3.26 (H-5a). The latter also long-range coupled ($J < 1.0$ Hz) with both the vinyl methyl group at δ 1.72 (Me-13) and significantly, by a typical coupling across five bonds in an extended 'zig-zag' configuration, with the naphthalene proton appearing as a broad singlet at δ 7.30 (H-11) (Sternhell, 1969; Pretsch et al., 2000). The vinyl methyl group (Me-13), resonated at a very typical chemical shift as a broad singlet, being also allylic coupled with the olefinic proton (H-4) (Sternhell, 1969; Pretsch et al., 2000). The ^1H NMR spectrum also showed the presence of a further quaternary methyl group, appearing as a singlet at δ 1.65 (Me-15) and the typical signal pattern of a 2,3-disubstituted naphthalene moiety (Pretsch et al., 2000). The latter fragment generated the doublet ($J = 7.6$), the double doublet ($J = 7.6$, 7,6 Hz) (which both appeared as two double signals), the singlet and the broad singlet observed at the typical chemical shift values of δ 7.51, 7.38, 7.44 and 7.30 (H-7,10, H-8,9, H-11 and H-6, respectively). Based on these results we hypothesised the presence in **1** of one α,β-unsaturated lactam, one disubstituted naphthalene residue and, considering the total unsaturations, another ring. Their presence was corroborated by the typical secondary lactam (3358 and 1651 cm^{-1} for the NH and C=O groups, respectively), olefinic (1619 cm^{-1}), and aromatic (1599–1430 cm^{-1}) bands recorded in its IR spectrum (Nakanishi and Solomon, 1977), and the typical naphthalene absorption system (310, 275 and 215 nm) observed in the UV spectrum, with the maximum at 248 nm due to the α,β-unsaturated lactam (Scott, 1964). The HSQC correlations (Berger and Braun, 2004) further supported these partial structures. The conjugated trisubstituted olefinic and the vinyl methyl carbons resonated in the ^{13}C NMR spectrum (Table 1) at typical chemical shift values of δ 150.5, 126.3 and 14.8 (C-3, C-4 and C-13, respectively), and the other protonated carbons of the lactam ring appeared at δ 56.7 (C-5a) and 49.1 (C-5). The methyl groups bonded to the latter and to the quaternary C-12a carbon resonated at δ 20.3 and 26.3 (C-14 and C-15, respectively) (Breitmaier and Voelter, 1987). In the HSQC spectrum, the correlation of the 2,3-disubstituted-naphthalene residue was also observed. In the spectrum, the two double signals of the protonated carbons resonated at δ 128.5 and 127.6 (C-8,9 and C-7,10, respectively), and the other two at δ 133.8 and 127.2 (C-6 and C-11, respectively). The two quaternary bridgehead naphthalene carbons, the amidic carbonyl, and

Table 1
^1H and ^{13}C NMR data of drazepinone (**1**). The chemical shift are in δ values (ppm) from TMS[a]

C	δ[b]	δH	J (Hz)	HMBC
2	162.8 *s*			1.65
3	150.5 *s*			5.38, 3.26, 2.90, 1.72, 1.29
4	126.3 *d*	5.38 *br s*		3.26, 2.90, 1.72
5	49.1 *d*	2.90 *br q*	7.2	5.38, 3.26, 1.72, 1.29
5a	56.7 *d*	3.26 *br d*	1.3	5.38, 2.90, 1.65, 1.29
5b	111.2 *s*			3.26, 2.90
6	133.8 *d*	7.44 *s*		7.51
6a	113.5 *s*			7.38
7, 10	127.6 *d*	7.51 *d*	7.6	7.30
8, 9	128.5 *d*	7.38 *dd*	7.6, 7.6	
10a	133.6 *s*			7.44, 7.38
11	127.2 *d*	7.30 *br s*		
11a	164.9 *s*			7.44, 3.26
12a	103.8 *s*			5.38, 3.26, 2.90, 1.65
13	14.8 *q*	1.72 *br s*		5.38
14	20.3 *q*	1.29 *d*	7.2	3.26, 2.90
15	26.3 *q*	1.65 *s*		3.26
NH		12.4 *br s*		

[a] 2D ^1H, ^1H (COSY) and 2D ^{13}C, ^1H (HSQC) NMR experiments delineated the correlations of all protons and the corresponding carbons.
[b] Multiplicities determined by DEPT spectrum.

the quaternary carbons of the lactam ring appeared in the ^{13}C NMR spectrum at δ 133.6 and 113.5 (C-10a and C-6a), and at δ 162.8 and 103.8 (C-2 and C-12a), respectively. The latter is probably bonded to an oxygen atom in agreement with the molecular formula of $C_{19}H_{19}NO_2$, which also supports the presence of another ring that justifies the 11 unsaturations. Such a ring appears to be a trisubstituted dihydrofuran located between the naphthalene and the azepinone residues, whose bridgehead carbons are C-5 and C-12a and two naphthalene quaternary carbons, resonating in the ^{13}C NMR spectrum at typical chemical shift values of 164.9 (C-11a) and 111.2 (C-5b) (Breitmaier and Voelter, 1987).

On the basis of these results, and the direct correlations observed in the COSY, HSQC and those observed essentially for the quaternary carbons in the HMBC spectra (Berger and Braun, 2004), the chemical shifts of all the protons and the corresponding carbons were assigned as reported in Table 1, and the 3,5,12a-trimethyl-2,5,5a,12a-tetrahydro-1*H*-naphtho[2′,3′:4,5]furo-[2,3-*b*]azepin-2-one structure was attributed to drazepinone. It was supported by several $^{1}H,^{13}C$ long-range correlations recorded for **1** in the HMBC spectrum (Table 1), and by EI and ESI MS data. The latter, in addition to the sodium clustered $[M + Na]^+$ ion at *m/z* 316.1324, showed the potassium $[M + K]^+$ clustered and the pseudomolecular ions at *m/z* 332 and 294, respectively. Beside the molecular ion at *m/z* 293, the EI MS showed the ions generated by fragmentation mechanisms typical of α-alkylsubstituted furan rings (Pretsch et al., 2000). In fact, the molecular ion by the alternative loss of hydrogen and methyl residue generated the ions at *m/z* 292 and 278, respectively.

The relative stereochemistry of drazepinone was based on NOESY correlations (Berger and Braun, 2004) whose intensities are reported in Table 2. Among them (Fig. 1), the following correlations determine the spatial arrangement of the azepinone ring. A strong NOE effect was observed between the H-5a and the Me-15, both linked to the bridgehead carbons between the dihydrofuran and the azepinone rings, which consequently should be a *cis*-jointed. The strong interaction between H-5a and the adjacent methyl group Me-14 confirms the *cis*-configuration. Furthermore, the strong effect between H-5 and H-5a justifies the small value observed for their coupling in the ^{1}H NMR spectrum. Finally, significant effects were observed between NH-1 and naphthalene H-6, and the H-4 and Me-15.

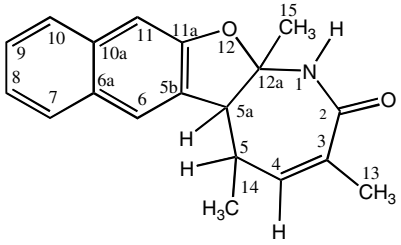
All of the observed NOEs were used for molecular mechanics and dynamics calculations, based on a distance calibration (r^{-6}, two-spin approximation) to convert the volumes of the most intense and significant NOESY cross-peaks of the azepinone ring into internuclear distances. All the calculated structures show that the azepinone ring takes up a boat-like conformation with the amidic proton pointing toward the naphthalene ring, therefore justifying the observed NOE effects between NH-1 and H-6, and H-4 and Me-15. The pattern of the found NOEs ruled out the other possible boat-like conformation, with the amidic proton pointing away of the naphthalene ring.

Drazepinone is structurally related in part to a group of naturally occurring compounds that are broadly distributed in nature as plant and marine organism metabolites. Most of them show interesting biological activity

Table 2
^{1}H NOESY data and internuclear distances obtained for drazepirone (**1**)

Hydrogens	Observed signal intensity[a]	Numerical values of NOE integrals[b]	Average internuclear distance[c] (Å)
H1–H6	*m*	0.75	3.32
H1–Me15	*w*	0.62	3.93
H4–H5	*w*	0.66	3.76
H4–H5a	*w*	0.68	3.68
H4–Me13	*s*	0.80	3.09
H4–Me15	*s*	0.78	3.18
H5–H5a	*s*	0.76	3.23
H5–Me13	*w*	0.62	3.91
H5–Me14	*s*	0.90	2.77
H5a–Me14	*s*	0.89	2.81
H5a–Me15	*s*	0.82	2.99

[a] *s*, strong (2.8–3.2 Å); *m*, medium (3.2–3.6 Å); *w* (3.6–4.0 Å).
[b] Integrals are referred to the NOE observed for the H6–H7 pair, whose intensity was taken as internal reference.
[c] Determined for the calculated structures.



1

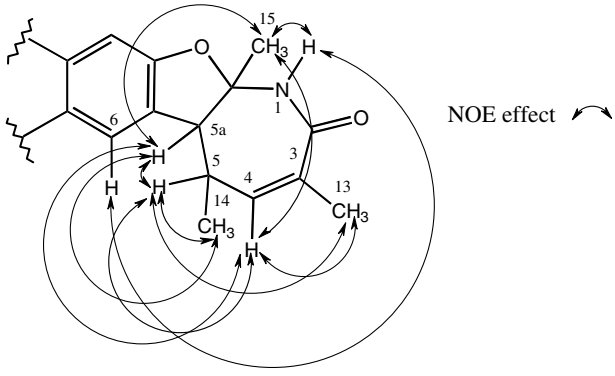


Fig. 1. Structure and NOE correlations of drazepinone (**1**).

(Mattia et al., 1982; Sekine et al., 1989; Xu et al., 1997; Baudoin et al., 2002) and the total synthesis of some of them has been achieved (Xu et al., 1997; Baudoin et al., 2002). Natural compounds containing the naphthoazepin skeleton have not been reported yet, and those having furoazepine are described only as synthetic derivatives with important pharmacological activity (Cortes et al., 1994; Cho et al., 2004). Therefore, drazepinone is the first natural compound presenting all three moieties jointed in a new and interesting bioactive fungal metabolite.

The fungal culture filtrate proved to be particularly effective, when applied by infiltration both to the host and non-host plant tested, causing quick chlorosis in the injected leaf tissues, followed by wide necrosis along the leaves. The culture filtrate produced by *D. siccans*, assayed with this screening procedure, proved to be one of the most toxic filtrates among those produced by tens of grass pathogens, collected and tested with the aim to find producers of toxic metabolites having herbicidal activity (Fracchiolla, 2003).

Applied to wounded leaves, the toxin caused necrosis on almost all the species tested (Table 3). Necrosis severity ranged from very wide, as in the case of *Urtica dioica*, to small ones as those observable applying the toxin to *Setaria viridis* and *L. perenne* leaves. The necrosis on *Euphorbia helioscopia* and *Mercurialis annua* leaves, both Euphorbiaceae, and *Chenopodium album* were also interesting. On the opposite, *Amaranthus retroflexus* and *Bromus* sp. were completely unaffected by the toxin. The symptoms caused by the pure drazepinone and by the culture filtrate appear to be almost the same, both in term of speed of appearance and size of necrosis, although the concentration of drazepinone in the culture filtrate is much lower with respect to the pure solution. This could mean that, besides drazepinone, the main toxin in the culture extracts, the fungus could produce other bioactive compounds. Their possible presence and role are under ascertainment.

Assayed up to 50 μg/disk on *Geotrichum candidum*, drazepinone showed a weak fungistatic activity, causing only a slight reduction of the fungal growth. The toxin proved to be completely inactive when tested up to 50 μg/disk on *Pseudomonas syringae* and *Lactobacillus plantarum* (a Gram-negative and a Gram-positive bacterium, respectively). Assayed for zootoxic activity at 10^{-3} M, the metabolite caused the total mortality of shrimp larvae, which decreased to 81% and 12% when assayed at 10^{-4} and 10^{-5} M, respectively.

Drechslera is a well-known genus producing phytotoxic metabolites. Most of those pathogens and their toxins have been deeply studied being agents of very severe diseases of cropped cereals (Tatum, 1971; Padmanabhan, 1973; Strobel et al., 1988). Some species were also isolated from grass weeds (Chandramohan and Charudattan, 2001), and their toxins proposed as potential natural herbicides (Kastanias and Tokousbalides, 2000; Kenfield et al., 1989a,b). Toxins with structure completely different from drazepinone were previously isolated from other strains of the same fungus, such as de-*O*-methyldiaporthin (Hallock et al., 1998) and siccanol (Lim et al., 1996), an isocoumarin and a bicyclic sesterterpene, respectively. Siccanol completely inhibited the root of Italian ryegrass (*L. multiflorum* Lam.) seedlings at a level of 100 ppm (Lim et al., 1996). De-*O*-methyldiaporthin was almost inactive when assayed on host plants (*L. perenne* L. and *A. sativa* L.), whereas it was toxic when assayed on corn, crabgrass, and soybean (at 4 mmol), and on Barnyard grass and spiny amaranth (8 and 21 nmol, respectively) (Hallock et al., 1998), with a toxicity resembling that caused by drazepinone.

Table 3
Toxicity of drazepinone (**1**) in the leaf-puncture assay

Common name	Scientific name	Family	Toxicity[a]
Alligator weed	*Alternanthera philoxeroides* (Mart.) Griseb.	Amaranthaceae	+
Annual mercury	*Mercurialis annua* L.	Euphorbiaceae	+++
Brome	*Bromus* sp.	Poaceae	–
Common chickweed	*Stellaria media* L.	Caryophyllaceae	++
Common mallow	*Malva silvestris* L.	Malvaceae	+
Durum wheat	*Triticum durum* Desf.	Poaceae	++
Fat hen	*Chenopodium album* L.	Chenopodiaceae	+++
Green bristlegrass	*Setaria viridis* L. Beauv.	Poaceae	+
Madwoman's milk	*Euphorbia helioscopia* L.	Euphorbiaceae	+++
Perennial ryegrass	*Lolium perenne* L.	Poaceae	+
Pigweed	*Amaranthus retroflexus* L.	Amaranthaceae	–
Sowthistle	*Sonchus arvensis* L.	Asteraceae	++
Stinging nettle	*Urtica dioica* L.	Urticaceae	++++

+, necrosis with diameter 1–2 mm;
++, necrosis 2–3 mm;
+++, necrosis 3–5 mm;
++++, wider necrosis.
[a] Toxicity scale: – no symptoms;

The original chemical structure of drazepinone, the interesting phytotoxic activity, the low activity against fungi and bacteria, and the relatively low zootoxicity, suggest further studies for its use as an environmentally friendly and safe herbicide (Evidente and Motta, 2001).

3. Experimental

3.1. General

Optical rotation was measured in $CHCl_3$ solution on a JASCOP1010 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. ^1H and ^{13}C NMR spectra were recorded at 600, 400, and at 150, 100 and 75 MHz, respectively, in $CDCl_3$ on the Bruker spectrometers at the NMR Service of Istituto di Chimica Biomolecolare. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra (Braun et al., 1998). DEPT, COSY-45, HSQC, HMBC and NOESY experiments (Braun et al., 1998) were performed using Bruker microprograms. EI MS were taken at 70 eV on a Fisons Trio-2000. HR Electrospray and ESI MS were recorded on a Cetaf Micromass and 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) or reverse phase (Whatman, KC18 F_{254}, 0.20 mm) plates; the spots were visualised by exposure to UV radiation and/or iodine vapours and by spraying 0.5% ninhydrin in Me_2CO and/or chromosulphuric acid followed by heating at 110 °C for 10 min. CC: silica gel (Merck, Kieselgel 60, 0.040–0.063 mm). Solvent systems: (A) $CHCl_3$–*iso*-PrOH (9:1); (B) EtOAc; (C) EtOAc–MeOH (4:1); (D) EtOAc–MeOH (19:1) and (E) EtOH–H_2O (1.5:1)

3.2. Fungal strain, culture medium and growth conditions

A strain of *D. siccans* (Drechsler) Shoemaker, isolated from diseased seeds of *Lolium perenne* was kindly supplied by Dr. József Bakonyi, Plant Protection Institute, Hungarian Academy of Science, Budapest, and stored as a single spore culture (ITEM 6217) in the Collection of the Istituto di Scienze delle Produzioni Alimentari, CNR, Bari, Italy. The fungus was maintained on potato-dextrose agar medium. For the production of toxic metabolites Roux bottles (1 l) containing a mineral-defined medium (200 ml; Pinkerton and Strobel, 1976) were seeded with mycelium fragments of actively growing colonies. The cultures were incubated under static conditions at 25 °C in the dark for 4 weeks, then filtered, assayed for phytotoxic activity and lyophilised for the successive purification steps.

3.3. Purification of drazepinone (*1*)

The lyophilised residue corresponding to 4 l of culture filtrate was dissolved in 400 ml of ultrapure water and extracted with $CHCl_3$ (4×400 ml). The combined organic extracts were dehydrated (Na_2SO_4) and evaporated under reduced pressure to give a phytotoxic red-brown oil residue (667 mg), which was fractionated by column chromatography, eluted with the solvent system A. Fractions were collected and pooled on the basis of their TLC profiles to yield 11 major fraction groups. Among them only the residue from fractions 6 (16.2 mg) and 7 (79.8 mg), containing the main metabolite, showed high phytotoxicity. These latter were gathered together and applied to a silica gel column, eluted first with solvent B and then with solvent C. Six groups of homogeneous fractions were collected. The phytotoxic activity was concentrated in the fifth fraction, which contained the main metabolite as also proved by TLC analysis (R_f 0.45, silica gel, eluent D). The residue of this latter fraction (33.9 mg) was further purified by prep. TLC, carried out in the same conditions, yielding a homogeneous oily toxic metabolite (9.7 mg, 2.4 mg l^{-1}) resistant to crystallization [R_f 0.45, and 0.23, by silica gel and reversed-phase TLC, eluent systems D and E, respectively], which was named drazepinone (**1**).

3.4. Drazepinone (*1*)

Compound **1** had: $[\alpha]_D^{25} + 7.1$ (ca. 0.2); IR ν_{max} cm^{-1} 3358, 1651, 1619, 1599, 1499, 1455, 1430; UV λ_{max} (log ε) nm: 310 (sh), 275 (sh) 248 (4.05), 215 (sh); ^1H and ^{13}C NMR: Table 1; HR ESI MS (+) *m/z*: 332 $[M + K]^+$, 316.1324 ($C_{19}H_{19}NO_2Na$, calcd. 316.1313) $[M + Na]^+$, 294 $[MH]^+$; EI MS (rel. int., %) *m/z*: 293 $[M]^+$ (41) 292 $[M - H]^+$ (54) 278 $[M - Me]^+$ (18), 57 (100).

3.5. Biological assays

3.5.1. Injection assay

The phytotoxic activity of the culture filtrate was preliminarily assessed using an infiltration assay on fully expanded leaves of *Triticum durum* var. *simeto* and *L. perenne* plants grown in greenhouse. Leaves were injected with aliquots of the culture filtrate using adapted medical pliers for hypodermal injections, having two rubber plugs to gently hold the leaf, one of them bearing the tip of a syringe needle that allows filtrate injection into the leaf mesophill. After injection, plants were kept in the greenhouse at natural light condition for 5–7 days, with daily checks for symptoms appearance.

3.5.2. Leaf puncture assay

Culture filtrates, their chromatographic fractions, and pure drazepinone were assayed on *L. perenne* leaves using a puncture assay. Drazepinone was also assayed on several monocot and dicot species, both weeds and cultivated plants (Table 3). All the leaves were detached from young plants grown in greenhouse. The toxin, as well as the fractions, was dissolved in a small amount of methanol or dimethylsulfoxide and diluted to the desired final concentration with distilled water (final concentration of solvents: 1%). The toxin was assayed at a final concentration of 2 μg μl^{-1} solution. Droplets (15 μl) of the assay solutions were applied on punctured detached leaves stored in moistened chambers, kept at 25 °C under continuous fluorescent lights. Symptoms appearance was observed 2 or 3 days after application.

3.5.3. Antibiotic and zootoxic assays

The antifungal activity of the toxins was checked on *Geotrichum candidum*, whereas the antibiotic activity was assayed on *Pseudomonas syringae* van Hall (Gram-negative) and on *Lactobacillus plantarum* (Orla-Jensen) Bergey (Gram-positive), as already described (Bottalico et al., 1990), up to 50 μg per disk. The zootoxic activity was tested on brine shrimps (*Artemia salina* L.) at 10^{-3}, 10^{-4} and 10^{-5} M, as previously described (Bottalico et al., 1990).

3.6. Calculations

A model of drazepinone was designed and refined with the SYBYL 6.3 package (*SYBYL*; Tripos Inc.: St. Louis, MO, 1996). The Tripos force field was used for all the calculations, with a charge distribution obtained with the Gasteiger–Hückel method. The structures were obtained by simulated annealing with molecular dynamics. Each conformation underwent a 10-ps heating from 300 to 1000 K, followed by 50 ps at 1000 K and then a 50-ps cooling from 1000 to 200 K. Each structure was then energy minimised with a final gradient norm criterion of 10^{-3} kcal mol^{-1} $Å^{-1}$. For each set of molecules, 100 conformers were obtained and the analyses were performed on the 10 most stable structures, satisfying the desired structural requirements.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2005.02.008.

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