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Ascosonchine, the enol tautomer of 4-pyridylpyruvic acid with herbicidal activity produced by *Ascochyta sonchi*

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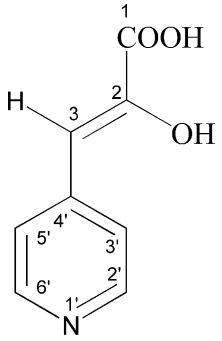
Abstract

A new phytotoxic enol tautomer of 4-pyridylpyruvic acid, named ascosonchine, was isolated from the culture filtrate of *Ascochyta sonchi*. Such a leaf pathogen is a potential biocontrol agent of *Sonchus arvensis*, a perennial herbaceous weed occurring throughout the temperate regions of the world. Ascosonchine, characterised as (*Z*)-2-hydroxy-3-(4-pyridyl)-2-propenoic acid by spectroscopic methods, showed selective herbicidal properties, that are not associated with antibacterial, antifungal or zootoxic activities.

Keywords: Ascosonchine; *Sonchus arvensis*; *Ascochyta sonchi*; Mycoherbicide; Phytotoxins; 4-Pyridylpyruvic acid; Weed biocontrol

1. Introduction

Phytopathogenic fungi belonging to the genus *Ascochyta* are responsible for several diseases, that cause necrotic lesions on leaves and stems (Melnik, 1971). Some *Ascochyta* spp. have also been proposed as mycoherbicides for the biological control of noxious weeds, i.e.: *A. caulina* against *Chenopodium album* (Netland et al., 2001), or *A. cypericola* against *Cyperus rotundus* (Upadhyay et al., 1991). The ability of many of these pathogens to produce phytotoxins has been ascertained and their involvement in symptoms appearance has been discussed (Evidente et al., 1993a,b; Strange, 1997). Recently, three novel toxins have been purified and identified from the liquid culture of *A. caulina* and proposed as natural herbicides to be utilized in addition to or as an alternative to the use of the pathogen (Evidente et al., 1998, 2000; Vurro et al., 2001). *Ascochyta sonchi* (Sacc.) Grove is a natural pathogen isolated from necrotic leaves of sowthistle (*Sonchus arvensis* L.), a herbaceous weed occurring through the temperate regions of the world, that is being evaluated as a possible biocontrol agent (Berestelski, personal communication). Therefore, the production of toxic metabolites by this promising pathogen is of interest. This paper describes the isolation, structural elucidation and biological characterisation of the main toxic metabolite produced in liquid culture, named ascosonchine (**1**), whose structure was determined by extensive use of spectroscopic methods (IR, NMR, MS techniques).



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2. Results and discussion

The culture filtrate of *A. sonchi,* showing high phytotoxicity on leaves of the host plant, was examined to ascertain the chemical nature of the phytotoxic metabolites. Preliminary experiments *in vitro* revealed that the fungus produces hydrophilic phytotoxins, because the compounds remained in the aqueous phase after exhaustive extraction of the culture filtrates with organic solvents having increased polarity (*n*-hexane $<CHCl_3<$ EtOAc $<$ BuOH). The phytotoxic metabolites had molecular weights lower than 3500, as deduced from dialysis experiments. A TLC analysis of the culture filtrates on silica gel plates (eluent A) also showed the presence of basic metabolites, probably bearing NH_2-groups, and amino acids and/or peptides. Purification of the crude culture filtrate by cationic exchange chromatography resulted in two fractions. The residue obtained by lyophilization of the basic eluate yielded a material containing the main metabolites in almost pure form (see Experimental) as shown by TLC analysis on silica gel (eluent A and B). The residue, a yellowish highly water-soluble powder having phytotoxic activity, was further purified by a medium pressure silica gel column. Eleven groups of homogeneous fractions were collected. The residue obtained from the third fraction group, containing a mixture of less polar metabolites, proved to be phytotoxic on leaves. Similar effects occur with the final eluate (eleventh fraction), however, their activity was significantly lower than those shown, in the same bioassay, by both the culture filtrate and the mixture of the metabolites obtained from the basic eluate of the cationic-exchange chromatography. Therefore, probably this latter contains other bioactive metabolites having a peptide nature. The residue of the third active fraction was purified by preparative silica gel TLC (eluent C) yielding a homogenous amorphous solid metabolite (**1**), which was named ascosonchine.

Ascosonchine has a molecular formula of $C_8H_7NO_3$, corresponding to 6 degrees of unsaturations, as deduced from the molecular weight of 165.04259, measured by HR EI mass spectrometry. It has not an α-amino acidic nature, considering the atypical yellow-pale colour observed when its TLC chromatogram is sprayed with ninhydrin. The investigation of its ^{1}H NMR spectrum recorded in CD_3OD (Table 1) showed two doublets ($J = 8.6$ Hz) at δ 8.01 and 6.82, attributable to the two pairs of the *ortho*- (H-2′ and H-6′) and *meta*- (H-3′ and H-5′) positioned aromatic protons of a suitable monosubstituted pyridine. Furthermore, a singlet, typical of enol proton (H-3), was observed at δ 8.00 in part overlapping the doublet of H-2′ and H-6′ (Pretsch et al., 1989). The presence of these two partial structures, the pyridyl and the enol moieties, was supported by the examination of the ^{1}H NMR, recorded in DMSO-d_6 and the ^{13}C NMR, COSY and HSQC spectra (Braun et al., 1998) recorded in both solvents (Table 1). In fact, the ^{1}H NMR spectrum, recorded in DMSO-d_6 (Table 1) showed a signal pattern very similar to that in CD_3OD, being the proton H-3 resolved better with respect to the doublet of H-2′ and H-6′. In addition, it showed the significant presence of a broad singlet at the typical chemical shift value of δ 12.4, due to the exchangeable proton of the hydroxy group of both the enol and the carboxyl groups (Pretsch et al., 1989). The ^{13}C NMR spectrum (in DMSO-d_6) (Table 1) showed the presence of the two pairs of the protonated and the quaternary carbons of a suitable 4-substituted pyridyl residue at δ 132.2 (C-2′ and C-6′), 126.6 (C-4′) and 115.2 (C-3′ and C-5′). The enolic hydroxylated and protonated carbons were present at the typical chemical shift values of δ 163.6 and 147.4 (C-2 and C-3), as well as that of the carboxyl group at δ 186.0 (C-1) (Breitmaier and Voelter, 1987).

On the basis of these results and of the correlation observed in the COSY and HSQC spectra, the chemical shifts of all the protons and the corresponding carbons were assigned in both solvent (CD_3OD and DMSO-d_6) as reported in Table 1, and the structure of the enol tautomer of the 4-pyridylpyruvic acid was attributed to ascosonchine.

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

	CD_3OD				DMSO-d_6			
C	δ[b]	δH	*J* (Hz)	HMBC	δ[b]	δH	*J* (Hz)	HMBC
1	189.2 *s*			8.00	186.0 *s*			8.00
2	165.5 *s*				163.6 *s*			
3	149.2 *d*	8.00 *s*		8.01, 6.82	147.4 *d*	8.00 *s*		7.91, 6.81
2′,6′	134.2 *d*	8.01 *d*	8.8		132.2 *d*	7.91 *d*	8.8	
4′	129.5 *s*			8.00, 6.82	126.6 *s*			8.00, 6.81
3′,5′	116.5 *d*	6.82 *d*	8.8		115.2 *d*	6.81 *d*	8.8	
COOH, OH						12.4 *br s*		

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

The structure is in agreement with the typical absorptions observed in IR spectrum for the hydroxy, the olefinic, the carboxyl and pyridyl groups, which are typical of the enolised form of an arylpyruvic acid and its derivatives in which the enol OH group is probably intramolecularly hydrogen-bonded with the carboxyl group (Josien et al., 1957; Nakanishi and Solomon, 1977; Cassidei et al. 1980; Lee et al., 1998). The enol form, and therefore the structure assigned to **1**, was also supported by the absorption maxima observed in the UV spectrum, which are consistent with an enol double bond conjugated with both a carboxyl and a pyridyl residue (Scott, 1964; Cassidei et al. 1980), and with the $^{1}H,^{13}C$ long-range correlations observed in the HMBC spectrum (Table 1) (Braun et al., 1998).

This structure was further supported by the HR EIMS spectrum which showed significant fragmentation peaks at *m*/*z* 138, 137, 121 and 94, besides the molecular ion at *m*/*z* 165.04259. They were generated from the parent ion by alternative and/or consecutive losses of HCN, CO and CO_2. The other significant peak observed at *m*/*z* 93 $[C_6H_7N]^+$ was probably produced by the molecular ion, as a result of a benzylic cleavage, in agreement with the fragmentation mechanisms described for alkylpyridines and phenylpyruvic acid (PPA) derivatives (Porter, 1985; Cassidei et al., 1980). The ES MS spectrum recorded in positive modality, showed clustered sodium and the pseudomolecular $[M+H]^+$ ions at *m*/*z* 188 and 166, respectively. As expected for the presence of a carboxyl group, the ES MS spectrum recorded in negative modality exhibited a very intense pseudomolecular ion $[M-H]^-$ at *m*/*z* 164, which generated the ions at *m*/*z* 146 and 120 by the alternative loss of H_2O or CO_2.

The spontaneous tautomerism of a keto group could produce both *Z*- and *E*-enol tautomers. The stereochemistry of the double bond present in ascosonchine was deduced by spectroscopic IR and NMR experiments. In fact, the IR absorption bands observed for the hydroxy, double bond and carboxyl groups in ascosonchine are in agreement with those reported for the *Z*-enol of phenylpyruvic acid derivatives, in which the enol OH group is intramolecularly hydrogen-bonded with the carboxyl group (Cassidei et al., 1980; Lee et al., 1998). This *Z*-configuration was further supported by the $^{1}H,^{13}C$ coupling constants recorded in the undecoupled ^{13}C NMR spectrum of **1** (Table 2). In particular, the value of the vicinal $^{1}H\text{-}C=C\text{-}^{13}COOH$ $[^{3}JC_1H_3 = 3.7$ Hz], is typical for a *cis* arrangement of the coupled nuclei in fragments $^{1}C\text{-}C=C\text{-}^{13}C$ with similar sums of electronegative substituents (Stobbe and Kenyon, 1971; Vögeli and von Philipsborn, 1975; Sciacovelli et al., 1976). This result was in agreement with the stable green-blue colour yielded when **1**, dissolved in DMSO, reacted with $FeCl_3$, as already observed only for *Z*-enol tautomer of the *p*-hydroxy PPA treated in the same conditions (Cassidei et al., 1980). Presumably, the stability of the *Z*-enol form is due to the conjugation between phenyl ring, olefinic and carboxyl groups (Cassidei et al., 1998), as also shown by the results of the quantum mechanical calculations (Lee et al, 1998) and the X-ray crystal structure (Halet et al., 1984; Le Bihan et al., 1988) for the methyl and ethyl esters of PPA, respectively.

Therefore, ascosonchine can be formulated as (*Z*)-2-hydroxy-3-(4-pyridyl)-2-propenoic acid (**1**).

Ascosonchine (**1**), produced by *A. sonchi*, is isolated for the first time as naturally occurring compound and phytotoxic metabolite. It belongs to the group of α-ketoacids and in particular to that of the heteroarylpyruvic acids. In several cases, as also for **1**, they exist exclusively under the enolic form (Sciacovelli et al., 1976; Dalla et al, 1997). The α-ketoacids, such as PPA, are metabolic products that are biologically important (Sukarai, 1956; Meister, 1965; Casey and Dobb, 1992). PPA is an intermediate in the shikimic acid pathway for the biosynthesis of the crucial amino acids, L-phenylalanine and L-tyrosine in plants and bacteria (Ganem, 1978; Haslam, 1993).

Tested with the leaf puncture assay on the host plant, the toxin (**1**) already after 2 days produced necrotic circular lesions resembling those caused by the pathogen. The diameter of the necrotic area appeared very wide (up to 0.5 cm) when 15 or 3 μg/droplets (around 6 and 1.2 10^{-3} M, respectively) were applied to the leaf surface, and was still quite evident at a concentration five times lower.

Assayed on several weedy and cultivated plants, both monocots and dicots, at 15 μg/droplet, ascosonchine showed interesting selective properties. In fact, as shown in Table 3, it was completely ineffective on all the solanaceous species assayed (tomato, eggplant, red pepper, potato), was slightly (1–2 mm) active or almost inactive on leguminous (bean and chickpea) and cucurbitaceous (melon and zucchino) plants, but caused severe (up to 1 cm) necrosis on many other species, such as *Euphorbia*, *Salvia*, *Valerianella*, or *Triticum*. Even if further assessments are needed, this semi-selective toxin could have practical applications as a herbicidal compound. It is interesting to note that the toxin is still very active when used at a quite low concentration.

Table 2
$^{1}H,^{13}C$ coupling constants measured for ascosonchine (**1**) in the undecoupled ^{13}C NMR spectrum (CD_3OD)

C	δ	*J* (Hz)
1	189.2	$^{3}J(C_1H_3) = 3.7$
2	165.5	$^{4}J(C_2H_{3',5'}) = 9.4$
3	149.2	$^{1}J(C_3H_3) = 169.1$
2′,6′	134.2	$^{1}J(C_{2',6'}H_{2',6'}) = 161.3$, $^{4}J(C_{2',6'}H_3) = 7.6$,
4′	129.5	$^{2}J(C_{4'}H_3) = 2.1$, $^{3}J(C_4H_{2',6'}) = 7.7$
3′,5′	116.5	$^{1}J(C_{3',5'}H_{3',5'}) = 160.9$, $^{3}J(C_{3',5'}H_3) = 4.6$

Table 3
Effect of ascosonchine in the leaf-puncture assay

Common name	Scientific name	Family	Effect on leaves[a]
Alligatorweed	*Alternanthera philoxeroides*	Amaranthaceae	++
Artichoke	*Cynara scolymus*	Compositae	–
Bean	*Phaseolus vulgaris*	Leguminosae	–
Bindweed	*Convolvulus arvensis*	Convolvulaceae	–
Chickpea	*Cicer arietinum*	Leguminosae	+
Eggplant	*Solanum melongena*	Solanaceae	–
Four-o'clock	*Mirabilis jalapa*	Nyctaginaceae	+
Foxtail millet	*Setaria italica*	Poaceae	++
Lamb's lettuce	*Valerianella locusta*	Valerianaceae	+++
Melon	*Cucumis melo*	Cucurbitaceae	–
Red pepper	*Capsicum annum*	Solanaceae	–
Potato	*Solanum tuberosum*	Solanaceae	–
Common sage	*Salvia officinalis*	Labiatae	++++
Sowthistle	*Sonchus arvensis*	Asteraceae	+++
Spinach	*Spinacia oleracea*	Chenopodiaceae	+
Sun spurge	*Euphorbia helioscopia*	Euphorbiaceae	++++
Tomato	*Lycopersicon esculentum*	Solanaceae	–
Wheat	*Triticum durum*	Poaceae	+++
Zucchino	*Cucurbita pepo*	Cucurbitaceae	–

[a] Toxicity determined with the following scale: – = no symptoms; + = necrosis with diameter around 1–2 mm; ++ = necrosis 2–3 mm; +++ = necrosis 3–5 mm; ++++ = wider necrosis.

In the antibiosis assay on *Geotrichum candidum*, ascosonchine assayed at concentrations up to 50 μg/disk proved to be completely inactive. The same negative result was observed when the toxin was tested on *Pseudomonas syringae* and *Lactobacillus plantarum* (a Gram- and a Gram+ bacterium, respectively). No effect was observed in the brine shrimp assay, tested at concentrations up to 10^{-4} M. Even if futher nontargeting tests of ascosonchine are required, the results from assays revealed only phytotoxic activity, whereas ascosonchine was completely ineffective on fungi, bacteria and arthropods. This could be very important from a practical point of view, and would confirm the hypothesis that more environmentally friendly and safe herbicides could be obtained by plant pathogenic fungi (Evidente and Motta, 2001).

Assayed on host leaves at the same concentration used for ascosonchine, the mixture obtained by the basic eluate of the cationic exchange chromatography (containing around 1% of ascosonchine) showed a good toxicity. Considering the production of the mixture of metabolites to be much easier than the purification of ascosonchine, and also their higher relative toxicity compared to ascosonchine, the idea of a practical application of this mixture as a natural herbicide seems to be a feasible option.

3. Experimental

3.1. General

IR and UV spectra were determined as neat and in MeOH solution, respectively, on a Perkin-Elmer Spectrum ONE FT-IR Spectrometer and a Shimadzu UV-1601 UV-Visible spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded at 400 and at 125, 100 and 75 MHz, respectively, in CD_3OD and/or DMSO-d_6, on Bruker spectrometers. The same solvent was used as internal standard. Carbon multiplicities were determined by DEPT spectra. DEPT, COSY-45, HSQC and HMBC experiments (Braun et al., 1998) were performed using Bruker microprograms. EI MS and HR EIMS were taken at 70 eV on a Fisons Trio-2000 and a Fisons ProSpec spectrometer, respectively. Electrospray 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) 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) BuOH–AcOH–H_2O (4:1:1.6); (B) *iso*-PrOH–H_2O (8:2), (C) $CHCl_3$–*iso*-PrOH (9:1); (D) EtOH–H_2O (1:1).

3.2. Fungal strain, culture medium and growth conditions

A strain of *A. sonchi* (Sacc.) Grove, isolated from diseased leaves of S. *arvensis* was supplied by Dr. Alexander Berestetski, All-Russian Research Institute of Plant Protection, St. Petersburg, Russia, and stored as a single spore culture in the Collection of the "Istituto di Scienze delle Produzioni Alimentari, CNR, Bari Italy (ITEM 6217). The fungus was maintained on potato-

dextrose agar medium. For the production of toxic metabolites a conidial suspension (1 ml containing approximately 10^6 conidia) was added to 1 l Roux bottles containing 200 ml of M-1 D medium (Pinkerton and Strobel, 1976). The cultures were incubated under static conditions at 25° C in the dark for 4 weeks, then filtered, assayed for phytotoxic activity and lyophilized for the successive purification steps.

3.3. Purification of ascosonchine (*1*)

The lyophilized residue (78.8 g, corresponding to 3.5 l of culture filtrate) was dissolved in 20 ml of 1M HCOOH and adsorbed onto a Dowex 50 [H^+] resin, packed in a chromatographic column (30×4 cm i.d.). The column was firstly washed with ultrapure water (1.5 l), in order to remove both the large amount of saccharose used as carbon source in the culture medium, as well as the other non-basic substances, and then eluted with 1M NH_4OH (500 ml). The basic substances were collected. Both the aqueous and ammonia eluates were analysed by silica gel TLC (eluents A and B). The presence of saccharose was ascertained only in the first eluate, whereas the second one contained a mixture of metabolites, including the toxins, as confirmed by its high phytotoxicity. The basic eluate was flushed with a stream of nitrogen in order to remove the ammonia and then lyophilized, yielding a solid residue (580 mg) containing an almost pure (99.3%) mixture of the phytotoxic and peptide metabolites. This latter was further purified by a medium pressure (15 bar) silica gel column (eluent B), affording 11 groups of homogenous fractions, of which the groups 3 and 11 showed phytotoxic activity. By TLC analysis (silica gel, eluent C) the group 3 (18 mg) showed to contain a mixture of less polar metabolites, which was further purified by preparative TLC yielding a solid homogeneous toxic metabolite (4 mg, 1.1 mg l^{-1}, R_f 0.43 and 0.75 by TLC on silica gel and reverse phase using eluents C and D, respectively) named ascosonchine (**1**).

3.4. Ascosonchine (*1*)

Compound **1** had: IR ν_{max} cm^{-1} 3359, 1661, 1600, 1585, 1514, 1453, 1247; UV λ_{max} (log ϵ) nm: 305 (3.61), 230 (3.66); ^1H and ^{13}C NMR: Table 1; HR EIMS (rel. int) *m/z*: 165.04259 ($C_8H_7NO_3$, calcd 165.04261, 100) $[M]^+$, 138 $[M–HCN]^+$ (76), 137 $[M-CO]^+$ (78), 121 $[M-CO_2]^+$ (99), 94 $[M-HCN-CO_2]^+$ (70), 93 $[C_6H_7N]^+$ (81). ES MS (+) *m/z*: 188 $[M+Na]^+$, 166 $[MH]^+$, 121 $[M-CO_2]^+$; ES MS (−) *m/z*: 164 $[M-H]^-$, 146 $[M-H-H_2O]^-$, 120 $[M-H-CO_2]^-$.

3.5. Bioassay methods

Culture filtrates, their chromatographic fractions including the mixture of metabolites obtained by the basic eluate of the cationic exchange column, and pure ascosonchine were assayed on host plants using the leaf-puncture assay. The toxin, as well as the fractions, were first dissolved in a small amount of methanol and then diluted to the desired final concentration with distilled water (final concentration of methanol: 1%). Droplets (15 μl) of assay solution were applied to punctured detached leaves stored in moistened chambers. Symptom appearance was observed 2 or 3 days after application. Ascosonchine was tested at concentrations of 1, 0.2 and 0.04 μg/μl on host leaves and at 1 μg/μl using the same assay, on several plants, as shown in Table 3. The extract from cationic exchange chromatography was tested at 1 μg/μl.

The antifungal activity of the toxins was checked on *Geotrichum candidum*, whereas the antibiotic activity was assayed on *Pseudomonas syringae* and on *Lactobacillus plantarum*, as already described (Bottalico et al., 1990), at concentrations up to 50 μg/disk. The zootoxic activity was tested on *Artemia salina* brine shrimps at concentrations up to 20 μg/ml of sea solution, as previously described (Bottalico et al., 1990).

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