11168 J. Agric. Food Chem. 2009, 57, 11168–11173
DOI:10.1021/jf903499y

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Papyracillic Acid, a Phytotoxic 1,6-Dioxaspiro[4,4]nonene Produced by *Ascochyta agropyrina* Var. *nana*, a Potential Mycoherbicide for *Elytrigia repens* Biocontrol

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A strain of *Ascochyta agropyrina* var. *nana* was isolated from *Elytrigia repens* (quack grass), a noxious perennial weed widespread through the cold regions of the northen and southern hemispheres. Papyracillic acid was isolated for the first time from the fungal solid culture and identified using spectroscopic methods, including X-ray diffractometric and CD analysis for the assignment of the relative and absolute stereochemistries. Some key derivatives were prepared and used in a structure−activity relationship study. Tested by leaf disk-puncture assay, papyracillic acid at the concentration of 1 mg/mL was shown to be phytotoxic both for the host plant and a number of nonhost plants of the fungus. Papyracillic acid was active against bacteria (*Xanthomonas campestris* and *Bacillus subtilis*) and the fungus *Candida tropicalis* at 6 μg/disk. Derivatives of papyracillic acid were significantly less active than original toxin. However, the monoacetyl derivative of the toxin did not possess antimicrobial activity but remained highly phytotoxic to quack grass. Hence, papyracillic acid and its analogues have potential as nonselective herbicides of natural origin. Some structure−activity relationship observations for papyracillic acid and its derivatives were also made.

KEYWORDS: *Ascochyta agropyrina* var. *nana*; weeds; *Elytrigia repens*; phytotoxins; 1,6-dioxaspiro-[4,4]nonenes; papyracillic acid; bioherbicides

INTRODUCTION

Elytrigia repens L. Desv. ex Nevski (quack grass) is a perennial noxious weed widespread through temperate regions of the northern and southern hemispheres. This adaptable grass spreads by seed and rhizomes and produces phytotoxic metabolites suppressing the growth of other plants. The only control measure is the application of preemergence herbicides or spot treatment with postemergence herbicides (*1*, *2*). Such restrictions induce the search for natural compounds with herbicidal activity against quack grass.

Microbial phytotoxins have been evaluated for the development of new agrochemicals against weeds for a long time (*3*−*5*). It is well-known that many plant pathogens, especially necrotrophic and hemibiotrophic fungi, are capable of producing phytotoxins responsible for the development of disease symptoms (*6*). Therefore, appropriate pathogens can be a source of such herbicidal metabolites for the control of target weeds. For instance, phytotoxic metabolites were isolated from culture filtrate or mycelia of some mycoherbicidal fungi belonging to the genera *Alternaria*, *Ascochyta*, *Drechslera*, *Ophiobolus*, *Phoma*, and many others (*7*,*8*).

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Recently, the fungus identified as *Ascochyta agropyrina* (Fairman) Trotter var. *nana* Punith. was isolated from diseased leaves of the perennial weed of quack grass. A preliminary study showed that a strain of this fungus has potential for the production of phytotoxic and antimicrobial metabolites when grown in liquid and solid culture.

Fungi belonging to the genus *Ascochyta* are causal agents of diseases of wild plants and crops. They infect above-ground parts of plants, expressing as necrotic spots and lesions (*9*). The ability of many of these pathogens to produce phytotoxic metabolites has been ascertained, and their involvement in symptom appearance has been discussed (*10*−*12*). Different potent phytotoxins were isolated from *Ascochyta* species pathogenic to some weeds. Pyrenolide A and ascochytin were isolated from *A. hyalospora*, a pathogen of lamb's-quarters (*Chenopodium album*) (*13*). Three novel non-proteigenic toxic amino acids have been purified and identified from the liquid culture of *A. caulina* and proposed as natural herbicides for control of the same weed (*14*−*16*). Recently, ascosonchine, a new enol tautomer of 4-pyridylpyruvic acid, was isolated as the main phytotoxin produced by *A. sonchi* and proposed as a safe natural herbicide against *Sonchus arvensis* (*17*).

 Published on Web 11/05/2009

The primary aim of this work was to purify and characterize the phytotoxic metabolites produced by *A. agropyrina* var. *nana*, with emphasis on testing them against quack grass, a problem weed in various crops.

This paper will describe the results of the chemical and biological characterization of the main phytotoxic metabolite, identified as papyracillic acid (PA), produced for the first time *in vitro* by a strain of *A. agropyrina* var. *nana*. Papyracillic acid has been already reported as a fungal metabolite as well as an antimicrobial, nematicidal, and cytotoxic product (*18*), whereas its phytotoxicity is a very interesting novelty. Furthermore, phytotoxic and antimicrobial activities of its derivatives (**2**−**7**) will be also discussed.

MATERIALS AND METHODS

General Experimental Procedures. Optical rotation was measured in $CHCl_3$, unless otherwise noted, on a Jasco (Tokyo, Japan) polarimeter; IR spectra were recorded as deposit glass film on a Perkin-Elmer (Norwalk, CT) spectrometer, and UV spectra were measured in MeCN, unless otherwise noted, on a Perkin-Elmer spectrophotometer. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 600 or 300 MHz and at 75 MHz, respectively, in $CDCl_3$, on Bruker (Kalsruhe, Germany) spectrometers. The same solvent was used as internal standard. Carbon multiplicities were determined by distortionless enhancement by polarization transfer (DEPT) spectra (*19*). DEPT, correlation spectroscopy (COSY)-45, heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC), and nuclear Overhauser enhancement spectroscopy (NOESY) experiments (*19*) were performed using standard Bruker microprograms. Electrospray ionization (ESI) mass spectra (MS) were recorded on a Waters Micromass (Milford, MA) spectrometer. The X-ray analysis of papyracillic acid was carried out on a colorless crystal obtained from EtOAc/*n*-hexane (1:5) solution (slow evaporation). Data were acquired on a Bruker-Nonius Kappa CD diffractometer (graphite-monochromated Mo Kα radiation). CD spectra were recorded on a Jasco spectropolarimeter. For the solid state CD measurement of papyracillic acid, the disk was prepared by mixing 128 mg of KCl (optical grade, heated at 100−120 °C for 1−2 h) and 1.6 mg of **1** and minced in an agate mortar. Then the thin powder was pressed at 10 tons with a press under vacuum to get a transparent disk. To check the microscopic anisotropy of the KCl disk, more spectra were recored by rotating the disk with 90° intervals, which were slightly different. Analytical and preparative thin layer chromatographies (TLC) were performed on silica gel (Kieselgel 60, F_{254}, 0.25 and 0.5 mm, respectively, Merck, Darmstadt, Germany) plates. The spots were visualized by exposure to UV radiation (253 nm) or by spraying first with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in EtOH, followed by heating at 110 °C for 10 min. Column chromatography was performed on a silica gel column (Merck, Kieselgel 60, 0.063−0.200 mm).

Fungal Strain, Culture Medium, and Growth Conditions. The fungus was isolated from naturally infected leaves of *E. repens*. The microorganism was identified as *A. agropyrina* (Fairman) Trotter var. *nana* Punith., previously described on litter of *Agropyron* and *Koeleria* species (*20*). A monoconidial isolate (A-10) was deposited in the culture collection of the All-Russian Research Institute of Plant Protection, Pushkin, Saint Petersburg, Russia. The isolate was maintained in sterile tubes containing potato−dextrose agar. Solid cultures were obtained on autoclaved pearl barley: 15 flasks (1 L) with 100 g of pearl barley and 60 mL of water. The fungus was grown on the solid substrate at 24 °C for 10 days and stirred every day to prevent clumping.

Extraction and Purification of Papyracillic Acid (1). The fungal culture was dried by an air stream in a laminar box. The dried material (1 kg) was extracted with 1 L of acetone/water (1% NaCl) (1:1, v/v). The mixture was vacuum-filtered through cheesecloth. The solid was re-extracted with the same mixture two more times under the same conditions, and then the combined liquid phase evaporated under reduced pressure to eliminate acetone. The resulting aqueous phase was extracted (3 × 3 L) with EtOAc. The combined organic extracts were dried (Na_2SO_4) and evaporated under reduced pressure to yield a brown solid residue (2.7 g). The residue tested at concentrations of 5 mg/mL as described below was found to be phytotoxic against *E. repens*, and it was then submitted to bioassay-guided fractionation through column chromatography on silica gel, eluted with the $CHCl_3$/*i*-PrOH (9:1, v/v). Ten homogeneous fraction groups were collected and screened for their phytotoxic activity. The residues (265.1 and 1293.0 mg, respectively) of fourth and fifth fractions, containing the main metabolite, as checked by TLC (silica gel, same solvent), were independently crystallized from an EtOAc/*n*-hexane mixture (1:5), yielding white needles, identified as below reported as the main isomer of papyracillic acid (**1**, R_f 0.57, 905.9 mg/kg).

Papyracillic Acid. Papyracillic acid had $[\alpha]^{25}_D$, IR, UV, and ^1H and ^{13}C NMR spectra similar to those previously reported (*21*); ESI MS (+), *m*/*z* 475 $[2M + Na]^+$, 265 $[M + K]^+$, 249 $[M + Na]^+$, 209 $[M + H - H_2O]^+$.

Papyracillic Acid Methyl Ester (2). To the main isomer of papyracillic acid (**1**, 15.0 mg), dissolved in MeOH (0.5 mL), was added an ethereal solution of diazomethane. The reaction was carried out overnight at room temperature in the dark. The reaction was stopped by evaporation under a N_2 stream. The residue (20.0 mg) was purified by preparative TLC on silica gel [(eluent $CHCl_3$/*i*-PrOH (95:5, v/v)], yielding papyracillic acid methyl ester as an oil (**2**, R_f 0.68, 2.4 mg). Derivative **2** had $[\alpha]^{25}_D$, IR, UV, and ^1H NMR very similar to those previously reported (*21*); ESI MS (+) spectrum, *m*/*z* 263 $[M + Na]^+$.

Papyracillic Acid Methyl Acetal (3). To the main isomer of papyracillic acid (10.0 mg) dissoveld in MeOH (100 μL) was added CF_3COOH (100 μL). The reaction was carried out at room temperature and under stirring overnight. The reaction was stopped by evaporation under a N_2 stream. The residue (10.6 mg) was purified by two preparative TLC steps on silica gel [(eluents $CHCl_3$/*i*-PrOH (9:1, v/v) and *n*-hexane/EtOAc (6:4, v/v), respectively], yielding papyracillic acid methyl acetal as an amorphous solid (**3**, R_f 0.68, 2.4 mg). Derivative **3** had $[\alpha]^{25}_D$, IR, UV, and ^1H NMR spectra very similar to those previously reported (*21*); ESI MS (+) spectrum, *m*/*z* 503 $[2M + Na]^+$, 279 $[M + K]^+$, 263 $[M + Na]^+$, 209 $[M - OMe]^+$.

Acetylation of Papyracillic Acid. To the main isomer of papyracillic acid (**1**, 20.0 mg) dissolved in acetic anhydride (940 μL) was added NaOAc (40.0 mg). The reaction was carried out at 80 °C overnight. The reaction was stopped by the addition of MeOH and evaporation by a N_2 stream. The residue (18.0 mg) was purified by preparative TLC on silica gel [eluent *n*-hexane/EtOAc (1:1, v/v)], yielding three different derivatives, all as homogeneous oils (**4**, R_f 0.76, 14.4 mg; **5**, R_f 0.60, 3.4.mg; and **6**, R_f 0.45, 1.5 mg). Derivative **4** (a 1:1 epimeric mixture) had $[\alpha]^{25}_D$, IR, UV, and ^1H NMR spectra very similar to those previously reported (*22*); ESI MS (+), *m*/*z* 291 $[M + Na]^+$, 249 $[M + Na - CH_2CO]^+$, 231 $[M + Na - AcOH]^+$. Derivative **5** had IR, UV, and ^1H NMR spectra very similar to those previously reported (*21*); ESI MS (+), *m*/*z* 291 $[M + Na]^+$, 249 $[M + Na - CH_2CO]^+$, 231 $[M + Na - AcOH]^+$. Derivative **6** had $[\alpha]^{25}_D$ −5.0° (*c* 0.05); IR ν_{max} 1772, 1742, 1714, 1640, 1603 cm^{-1}; UV λ_{max} nm (log ε) 268 (4.09); ^1H NMR spectrum, see **Table 1**; ESI MS (+) spectrum, *m*/*z* 291 $[M + Na]^+$, 249 $[M + Na - CH_2CO]^+$, 231 $[M + Na - AcOH]^+$.

Hydrogenation of Papyracillic Acid. Papyracillic acid (50 mg) dissolved in MeOH (2.5 mL) was added, under stirring, to a H_2 presaturated suspension of 10%Pd/C in MeOH (2.5 mL). The reaction was carried out at room temperature and atmospheric pressure. After 1 h, the catalyst was removed by funnel filtration, and the clear solution was evaporated under vacuum, yielding an oily residue (48 mg). The latter was purified by preparative TLC on silica gel [eluent $CHCl_3$/*i*-PrOH (95:5, v/v)], yielding the main derivative as a homogeneous oil (**7**, R_f 0.75, 30 mg). The dihydroderivative of papyracillic acid had $[\alpha]^{25}_D$ −24.0° (*c* 0.42); IR ν_{max} 3425, 1755, 1637 cm^{-1}; UV λ_{max} nm (log ε) 222 (3.69); ^1H and ^{13}C NMR spectra, see **Table 1**; ESI MS (+), *m*/*z* 267 $[M + K]^+$, 251 $[M + Na]^+$, 229 $[M + H]^+$, 211 $[M + H - H_2O]^+$; ESI MS (−), *m*/*z* 227 $[M - H]^-$.

Crystal Structure Determination of the Main Isomer of Papyracillic Acid (1). Single crystals of **1** (colorless prism, 0.30 × 0.10 × 0.03 mm^3) suitable for X-ray analysis were obtained by recrystallization from 1:5 EtOAc/*n*-hexane solution (slow evaporation). Data collection was performed at ambient temperature on a Bruker-Nonius KappaCCD diffractometer (graphite monochromated Mo Kα radiation, φ and ω scans to fill asymmetric unit). Structure was solved and refined by standard procedures using the SHELX97 software package. The absolute configuration was assigned by solid state CD data.

Table 1. ^{1}H and ^{13}C NMR Data of the New Monacetyl and Dihydro Derivatives of Papyracillic Acid (**6** and **7**)[a]

	6		7		
position	δH	J (Hz)	δ[b,c]	δH	J (Hz)
1			169.4, 169.4, 169.3, 169.3 s		
2	5.22 s		90.7, 90.5, 90.1, 90.0 d	5.11, 5.09, 5.08, 5.04 s	
3			176.7, 176.1, 176.1, 175.6 s		
4			109.3, 107.7, 107.7, 107.7 s		
5			46.9, 45.1, 44.9, 43.9 d	2.59, 2.29, 2.27, 1.97 dq	12.3, 6.6
6	4.22 q	6.9	48.9, 43.5, 41.1, 40.1 d	3.11, 2.74, 2.39, 1.87 dq	12.3, 6.4
7			110.6, 110.6, 109.6, 109.5 s		
8	2.18 s		27.7, 27.1, 23.8, 22.5 q	1.56, 1.53, 1.52, 1.50 s	
9	4.95 d	12.0	12.7, 11.4, 11.0, 10.6 q	1.07, 1.03, 0.99, 0.97 d	6.6
	4.85 d	12.0			
10	1.26 d	6.9	10.1, 9.7, 9.3, 8.1 q	0.94, 0.93, 0.88, 0.85 d	6.4
OMe	4.0 s		59.7, 59.5, 59.4, 59.3 q	3.91, 3.90, 3.89, 3.87 s	
OAc	1.97 s				

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

Leaf Disk-Puncture Assay. Organic extract from solid culture of *A. agropyrina* var. *nana*, the chromatographic fractions and pure compounds PA and its derivatives **2**−**6**, and the dihydro derivative of PA were assayed by leaf disk-puncture (*24*) bioassay on quack grass (*E. repens*), and Canada thistle (*Cirsium arvense*). Papyracillic acid was tested additionally on a number of other nonhost plants: Asian dock (*Rumex confertus*), dandelion (*Taraxacum officinalis*), barley (*Hordeum vulgare*), timothy grass (*Phleum pretense*), fat hen (*Chenopodium album*), double-cinnamon rose (*Rosa cinnamomea*), perennial sowthistle (*Sonchus arvensis*), hemp (*Cannabis sativa*), and red clover (*Trifolium pretense*).

Antimicrobial Assay. The antifungal activity of PA, its derivatives **2**−**6**, and the dihydro derivative of PA was assayed on *Candida tropicalis*, and their antibacterial activity was tested on *Xanthomonas campestris* and *Bacillus subtilis* at the concentration of 50 μg per disk according to the method previously described (*25*).

Statistical Analysis. All of the bioassays were performed twice with at least three replicates. When appropriate, means were compared using Fisher's least significance difference (LSD).

RESULTS AND DISCUSSION

The solid culture of *A. agropyrina* var. *nana* (1 kg) was exhaustively extracted as reported under Materials and Methods. The organic extract, showing high phytotoxic activity on host and nonhost plants, was purified by column chromatography as described under Materials and Methods, affording the main metabolite as a crystalline solid (905.9 mg/kg), which was recrystallized and identified as papyracillic acid (**1**, **Figure 1**).

Fortunately, our crystalline metabolite, **1**, corresponds to the main isomer and permits single-crystal X-ray. The results of crystal structure analysis were the same previously reported (*23*), when it was isolated together with its methyl acetal (**3**, **Figure 1**), named papyracillic acid B, palmarumycins, and microsphaeropsins, belonging to two different classes of naturally occurring compounds, from the *Microsphaeropsis* sp. The fungus, internal strain 7291, was isolated from a branch of the tree *Larix decidus*, growing in Herjeting, Denmark, and its culture extract was found to have good antifungal and antibacterial activities (*22*). As expected, also the spectroscopic and physical properties of the main metabolite isolated from *A. agropyrina* var. *nana* were identical to those previously reported for papyracillic acid (*21*,*23*) as well as its absolute configuration determined in solid state CD spectra (*23*). Papyracillic acid was also previously isolated together with lachnumon and mycorrhizin A, chlorinated metabolites belonging to different classes of naturally occurring compounds, from the ascomycete *Lachum papyraceum*, an efficient producer of nematicidal and antimicrobial metabolites (*21*). Successively, the same authors also extensively studied the

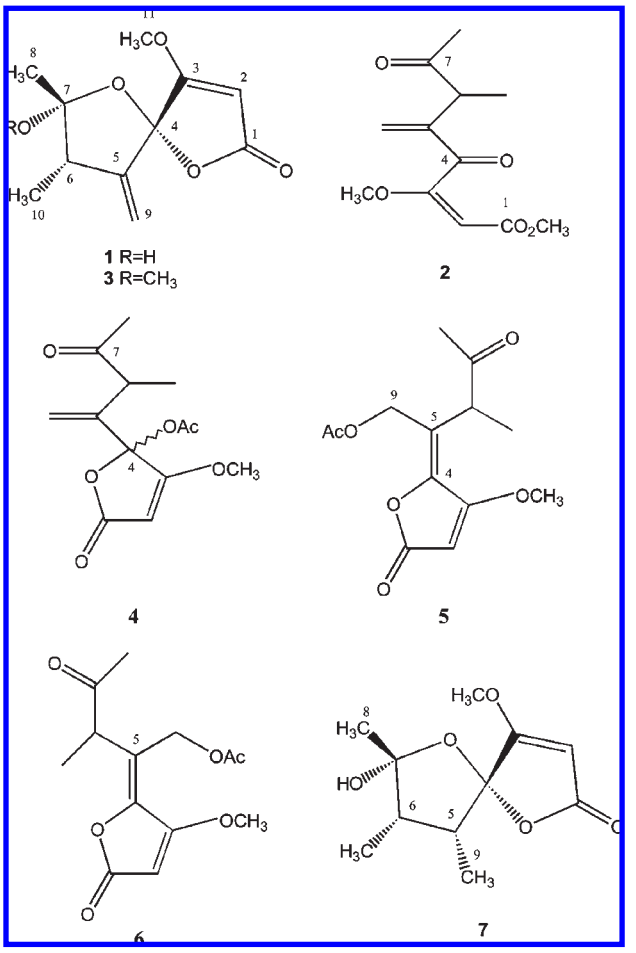

Figure 1. Structures of the main isomer of papyracillic acid (**1**) and its derivatives (**2**−**7**).

reactivity of papyracillic acid, essentially by reaction with cysteine and cysteine methyl ester (*22*).

The main isomer of papyracillic acid **1** was used for the preparation of seven derivatives to carry out a structure−activity relationship study aimed to find a derivative with increased phytotoxicity and specificity.

Papyracillic acid (**1**) was converted to its methyl ester and methyl acetal (**2** and **3**, **Figure 1**). The $[\alpha]_D$ and IR, UV, and ^{1}H NMR spectra of **2** and **3** were practically identical to those previously reported (*21*). The acetylation of the main isomer of

papyracillic acid (**1**), carried out in the usual conditions with pyridine and acetic anhydride, as previously reported (*22*), gave a complex dark mixture, which was very difficult to purify. When the reaction was carried out with acetic anhydride and sodium acetate, some acetyl derivatives were obtained. The major product, as reported (*22*), was found to be an unseparable epimeric mixture of the two monoacetyl derivatives **4** (**Figure 1**) in a ratio of ca. 1:1 as deduced by its ^{1}H NMR spectrum, whereas another one was the monoacetyl derivative **5**. Their $[\alpha]_D$ and IR, UV, and ^{1}H NMR spectra were practically identical to those previously reported (*22*). The ESI MS spectrum of **5** showed the same ions observed in the spectrum of **4**. A new monoacetyl derivative (**6**) was also prepared, which proved to be the *E*-diastereomer of **5** on the basis of its physical and spectroscopic properties. In fact, IR and UV spectra were very similar to those of **5**, but its ^{1}H NMR spectrum (**Table 1**) was significantly different. In particular, the doublet of H-9A (J = 12.0 Hz) and the quartet (J = 6.9 Hz) of H-6 appeared upfield and downfield shifted ($\Delta\delta$ 0.15 and 0.29, respectively) at δ 4.95 and 4.22, respectively. The diastereomeric structure assigned to **6** compared to **5** was further supported by data from their NOESY spectra. As expected, in both a coupling between the olefinic proton H-2 and the OMe group at C-3 was observed. Furthermore, a coupling was observed in **6** between the latter group and the methyl of the acetoxy group at C-9, whereas in **5** the MeO coupled with the secondary methyl group Me-10. The ESI MS spectrum showed the sodium clustered ion $[M + Na]^+$ at m/z 291 and the significant fragmentation ions $[M + Na - CH_2CO]^+$ and $[M + Na - AcOH]^+$ at m/z 249 and 231, respectively. Finally, catalytic hydrogenation of the main isomer of papyracillic acid (**1**) gave the expected dihydro derivative, the main isomer of which is **7** (**Figure 1**). The structure of **7** was supported by the data obtained by IR and UV spectra and essentially by NMR (COSY, HSQC, HMBC, and NOESY) experiments, which also allowed assignment of the chemical shift to all protons and corresponding carbons as reported in **Table 1**. Compared to **1**, the main differences observed in the ^{1}H and ^{13}C NMR spectra were the signals of a further secondary methylated (Me−CH-5) carbon appearing as a double quartet (J = 12.3 and 6.6 Hz) and a doublet (J = 6.6 Hz) at δ 1.97 (H-5) and 1.07 (Me-9) and at δ 46.9 (C-5) and 11.4 (Me-9), respectively. Furthermore, the same spectra showed a significant upfield shift of the signal of the other secondary methylated carbon (Me-CH-6), resonating as a double quartet (J = 12.3 and 6.4) and a doublet (J = 6.4 Hz) at 2.39 (H-6) and 0.94 (Me-10) and at δ 43.5 (C-6) and 10.1 (Me-10), respectively. As already observed for papyracillic acid, the ^{1}H and ^{13}C NMR spectra of its dihydro derivative, when recorded in the same solvent but with an increased acidity and/or for a longer time, showed systems consistent with the presence of a mixture of four isomers in the ratio of ca. 1:1:2:4; the ^{1}H and ^{13}C NMR data are reported in **Table 1**. Its ESI MS spectrum, recorded in positive mode, showed the potassium $[M + K]^+$ and sodium $[M + Na]^+$ clustered, the pseudomolecular $[M + H]^+$ ions at m/z 267, 251, and 229, respectively, and the significant fragmentation ion $[M + H - H_2O]^+$ at m/z 211. The same spectrum recorded in negative modality showed the pseudomolecular ion $[M - H]^-$ at m/z 227. The relative configuration of the main isomer of the dihydro derivative (**7**) was deduced by the couplings observed in the NOESY spectrum. In particular, besides the expected effect between H-2 and the methoxy group, a clear coupling was observed between H-6 and both H-5 and Me-8, consistent with their *cis*-configurations. These results were in full agreement with an inspection of a Dreiding model of **7**. Therefore, considering that the absolute stereochemistry at C-4, C-6, and C-7 in **7** was the same as **1**, the absolute stereochemistry of **7** was assigned as depicted in **Figure 1**.

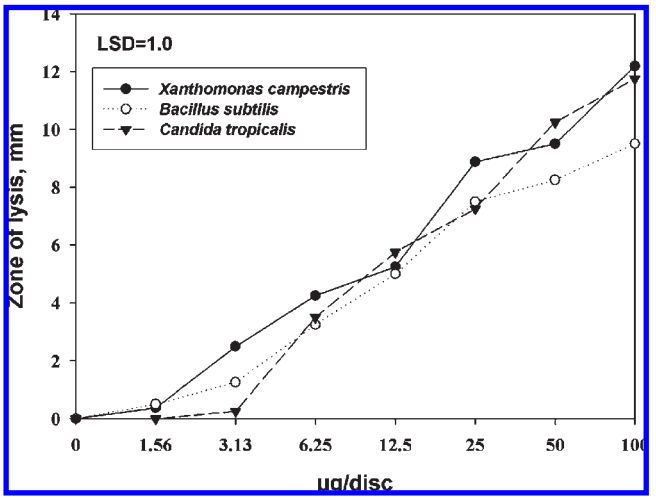

Figure 2. Effect of concentration of papyracillic acid on its antibiotic activity.

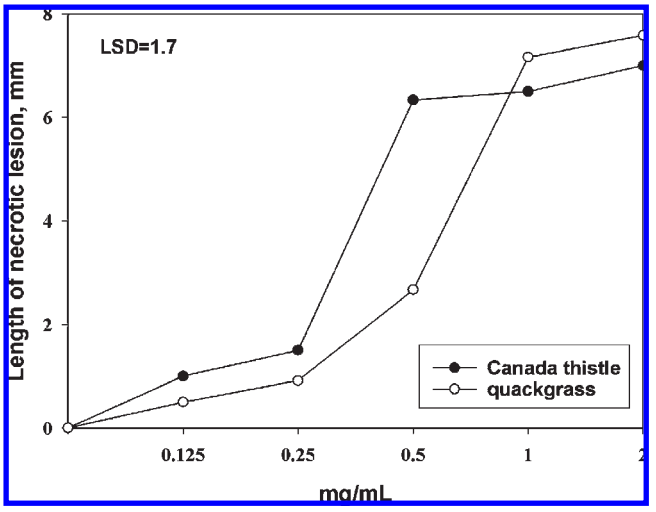

Figure 3. Effect of concentration of papyracillic acid on its phytotoxic activity (2 dpa).

When tested at a range of concentrations from 1.5 to 100 μg/disk, PA showed activity against the Gram-positive and Gram-negative bacteria *Bacillus subtilis* and *Xanthomonas campestris*, respectively, and the fungus *Candida tropicalis* at the concentration of 6.25 μg/disk (**Figure 2**). High antimicrobial, nematicidal, and cytotoxic activity of PA (at 5−10 μg/mL) was reported previously (*18*, *21*). The antibacterial activity was reported against *Bacillus brevis*, *B. subtilis*, *Micrococcus luteus* (Gram-positive) and *Enterobacter dissolvens* (Gram-negative), whereas the antifungal activity was assayed against *Nematospora coryli*, *Mucor miehie*, *Penicillium notatum*, and *Paecilomyces varioti* (*18*). Nematicidal activity was assayed against *Caenorhabditis elegans* and *Meloidogyne incognita* (*21*). Here we also report phytotoxic activity of PA that was observed on *E. repens* and *Cirsium arvense* when tested at a range of concentrations from 0.06 to 2 mg/mL (0.6−20 μg/disk). On these plants significant symptoms appeared at the concentration of 0.5 mg/mL (**Figure 3**). Among nine plant species, both dicots and monocots, eight plants were differentially sensitive to PA assayed at 1 mg/mL. The most sensitive plant was quack grass, whereas hemp was insensitive to PA (**Figure 4**).

Among seven derivatives (including the epimeric mixture **4** and that of the four dihydro derivatives) of PA compounds **3**−**6** were phytotoxic for *E. repens* assayed at 1 mg/mL (**Figure 5**), but they

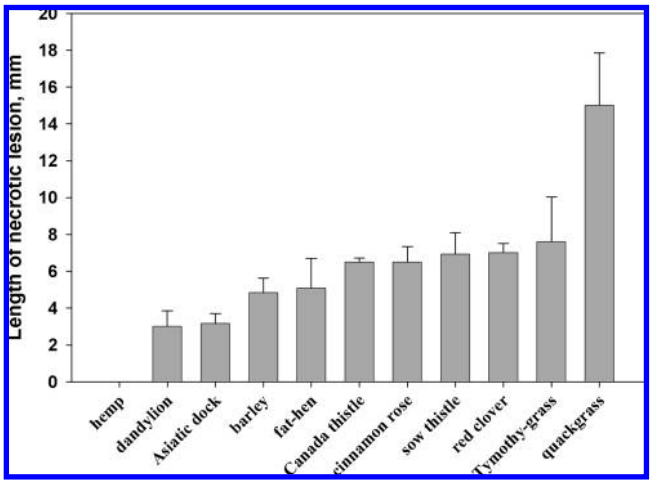

Figure 4. Sensitivity of different plant species to papyracyllic acid at the concentration of 1 mg/mL (3 dpa). Error bars indicate standard error (±).

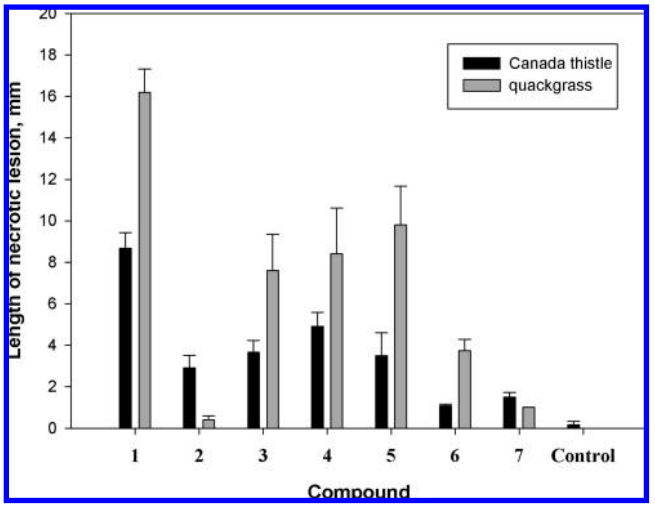

Figure 5. Phytotoxic activity of papyracillic acid and its derivatives (**2**−**7**) tested at the concentration 1 mg/mL (3 dpa). Error bars indicate standard error (±).

were significantly less active than PA. Canada thistle leaves were sensitive to compounds **2**−**5**, but at less than PA (**Figure 5**).

Antimicrobial activity of PA derivatives (**2**−**7**) was assayed against *B. subtilis*, *X. campestris*, and *C. tropicalis* at 50 μg/disk. Derivatives **3**, **4**, and **6** showed antibacterial activity. Only the derivative **3** inhibited growth of the fungus *C. tropicalis*. All of these derivatives **2**−**6** and the dihydro derivative of PA were less active than PA. Considering that compound **5** had phytotoxic activity but did not demonstrate antimicrobial activity, it can be evaluated as a prospective novel herbicide (**Figure 6**). However, further whole plant assays as well as safety and mode of action studies are need to confirm this conclusion. This work is in progress.

These results showed that the butenolide ring is an important feature imparting phytotoxicity. In fact, whereas the derivatives **3**−**6** and dihydro derivative of PA had this moiety unaltered, the PA methyl ester **2**, which showed the opening of the hemiacetalized 1,6-dioxospiran system, was practically inactive on the host and hemp plants and poorly active on Canada thistle. The inactivity of the dihydroderivative of PA, in which the butenolide ring is unaltered, also showed the importance for the phytotoxicity of the exocyclic methylene group at C-5. Furthermore, the heptasubstituted trihydrofuran seems to be unessential,

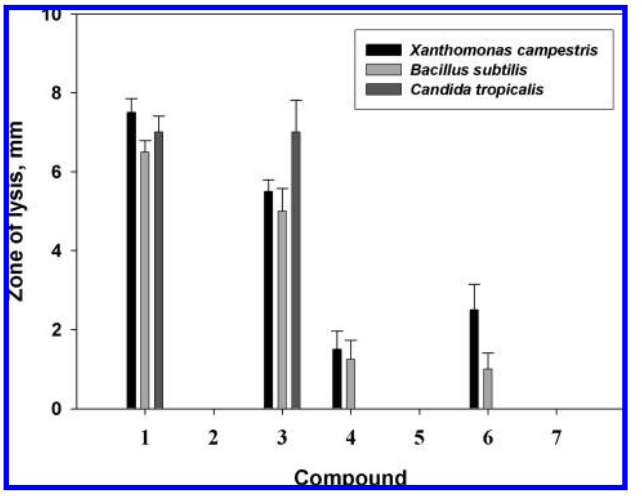

Figure 6. Antimicrobial activity of papyracillic acid and its derivatives (**2**−**7**) on Gram-positive and Gram-negative bacteria and a yeast fungus tested at the concentration 50 μg/disk. Error bars indicate standard error (±).

considering the activity of the three monoacetyl derivatives **4**−**6**, in which it is open and converted into differently C-4 -substituted butenolides. These latter derivatives also showed the absence of the exocyclic methylene group at C-5 but the presence of an exocyclic double bond belonging to the different side chain at C-4. Probably the different functionalities and stereochemistry of this latter justified the little difference observed in the phytotoxicity of **4**−**6**. The reduced activity of the PA acetal **3** also suggested a role in activity for the hemiacetalic hydroxy group at C-7.

These results were not surprising as for other butenolides, such as seiridins and 7′-hydroxyseridins produced by different *Seridium* species pathogenic to cypress (*27*−*29*); the integrity of the butenolide ring appeared to be fundamental to preserve both the phytotoxic and antimicrobial activities (*30*).

Papyracillic acid is an analogue of penicillic acid, a classical mycotoxin produced by various fungi including strains of the genera *Penicillium* and *Aspergillus*. Together with patulin, isopatulin, and ascladiol, penicillic acid constitutes a class of chemically relatively simple five-membered cyclic lactones, which, due to their toxicity and carcinogenicity, are considered to be potential health hazards to animals and man (*26*). Papyracillic acid was reported to have high antimicrobial and cytotoxic activity (~10 μg/mL), but no mutagenic activity (*23*). Our work represents the first isolation of papyracillic acid as a phytotoxin with potential herbicide activity and as a metabolite of *A. agropyrina* var. *nana*. Furthermore, this fungus appears to be a superproducer of this bioactive metabolite, considering the very high yield of papyracillic acid (>900 mg/kg or almost 33% of extract) obtained by solid culture, whereas the yields of PA from liquid culture of *L. papyraceum* were 10 mg/L (*21*) and 60 mg from 6.5 g of extract of *Microsphaeropsis* sp. (*23*). This result is very important in view of a potential practical application of papyracillic acid as a natural safe herbicide for the possibility of producing it by large-scale fermentation.

ACKNOWLEDGMENT

We thank the Servizio di Spettrometria di Massa del CNR, Pozzuoli, Italy. The NMR spectra were recorded in the laboratory of Istituto di Chimica Biomolecolare del CNR, Pozzuoli, Italy. We also thank the Centro Regionale di Competenza − Nuove Tecnologie per le Attività Produttive (CRdC-NTAP) of

Regione Campania and the Centro Interdipartimentale di Metodologie Chimico Fisiche (CIMCF) of the University of Naples Federico II for X ray facilities. The assistance of undergraduate students of Saint Petersburg State Agrarian University (Pavel Prokofyev and Alexandra Kurlenya) in preliminary investigations is acknowledged.

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Received for review July 23, 2009. Revised manuscript received October 22, 2009. Accepted October 22, 2009. This work was supported in part by a grant of the government of Saint Petersburg and Russian Fund of Basic Research 08-04-001354. Contribution DISSPAPA N. 199.