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Activity of the pterophyllins 2 and 4 against postharvest fruit pathogenic fungi. Comparison with a synthetic analog and related intermediates

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ABSTRACT

The antifungal activity of pterophyllin 2, pterophyllin 4, a 5-desmethyl analog of the latter and some of their synthetic intermediates, against three postharvest phytopathogenic fungi, was evaluated. The target fungi were Rhizopus stolonifer, Botrytis cinerea and *Monilinia fructicola*, which affect fruits worldwide, causing important economic losses. The tests were carried out with imazalil and carbendazim as positive controls. Minimum inhibitory concentrations and minimum fungicidal concentrations were determined, and the morphology of the colonies was examined microscopically. In liquid medium, it was found that pterophyllin 4 exhibited selective fungicidal activity toward *M. fructicola*, whereas its congener pterophyllin 2 proved to be less potent and not selective and the 5-desmethyl analog of pterophyllin 4 displayed a different activity profile. Morphological changes were observed in the colonies exposed to pterophyllin 4. The results highlighted the importance of small structural features for the antifungal behavior and also suggested that, in Nature, the pterophyllins may act as plant defenses against pathogens.

1. Introduction

Plant diseases caused by phytopathogenic fungi are responsible for important economic losses, which arise mainly from crop yield reduction, but are also a result of diminished quality and safety of the products. Sometimes, they also represent a risk for human and animal health, due to food contamination [1].

Rhizopus stolonifer (Ehrenb.: Fr.) Vuill, *Botrytis cinerea* (Pers.: Fr.) and *Monilinia fructicola* (G. Wint.) Honey are widely known phytopathogens and three of the main pathogenic fungi of concern to Argentine exports of fruits and also to their producers.

R. stolonifer is one of the most common and fastest-growing phytopathogenic species, especially under mild moisture conditions, being considered as one of the most devastating threats [2a]. It attacks a wide variety of hosts, causing the black mold rot; its quick penetration and colonization abilities result in fast spreading from infected to healthy fruit, at any stage between processing and consumers' houses.

B. cinerea is a ubiquitous and destructive plant pathogen, responsible for the botrytis bunch rot or gray mold, which causes damage on a large number of economically important agricultural and horticultural crops. It is an important disease which produces heavy losses to table and wine grapes [2b]. This pathogen is currently being controlled with pre- and postharvest fungicides [2c].

On the other hand, *M. fructicola* is the causal agent of the brown rot, a serious disease that affects the quality of peaches from the blossom period up to the harvest and storage stages. Its broad dissemination results in heavy production losses. For the Chinese market, the damage has been estimated as high as over 20% [2d].

Physical methods (X-rays and radio frequency, cold/hot water) [3], some inorganic salts [4], and synthetic biocides including sanitizing products [5] are among the main alternative strategies employed to ameliorate the threat posed by phytopathogens. These approaches are also being complemented by emerging, non-conventional resources, such as the use of natural antimicrobials [6] as biochemical control agents, and antagonist microorganisms [7] as biological control means.

All of them have some drawbacks, which range from damage to the sensory quality of the fruits, including their firmness [8a,b], to limited or variable efficacy [8c]. Further, the biological control agent *Saccharomyces cerevisiae* has been reported as the causal agent of some clinical infections [9], especially dangerous in immunocompromised patients [10].

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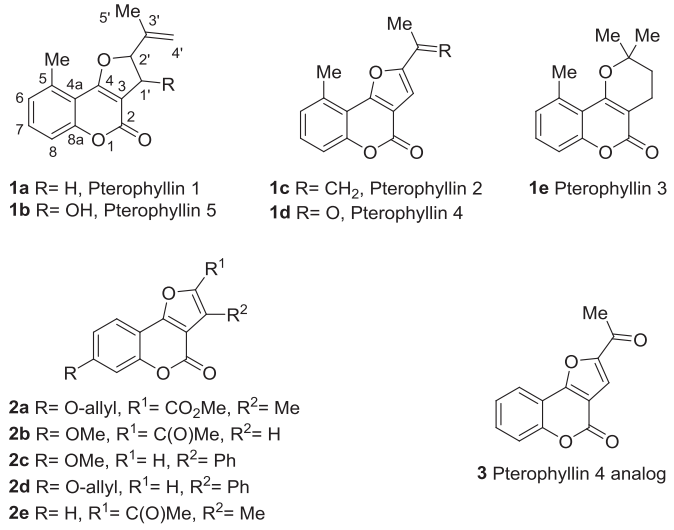


Fig. 1. Chemical structures of the pterophyllins 1–5 (**1a–e**), osthole derivatives (**2a–d**) and a pterophyllin 4 analog (**3**).

Since regulations on the use of new and existing fungicides are becoming more and more stringent, it urges to identify and develop new chemical entities with antifungal properties. However, increasing consumer awareness [11a,b], coupled to concerns regarding food quality and safety, demand from the novel fungicides an increasing eco-friendly character, as well as lack of cross-resistance with existing products [11c].

Naturally-occurring non-toxic chemicals have emerged as promising alternatives to the synthetic fungicides, as they may furnish effective protection against postharvest deterioration without exhibiting unwanted effects. Different natural products [12], semisynthetic compounds [13a,b], and plant derivatives [13c], including extracts, formulations based on chitosan [14], essential oils [15a,b], polyphenolics [15c,d], and carnauba wax [15e] have been tested as part of this strategy.

The pterophyllins 1–5 (**1a–e**) are 5-methyl substituted coumarin derivatives (Fig. 1), isolated in tiny amounts from *Ekebergia pterophylla* (C.D.C.) Hofmeyr (Meliaceae), a small evergreen tree known as Rock Ash, which grows on the Natal Group Sandstone outcrops, in South Africa [16]. Except for pterophyllin 3 (**1e**), they share a furo[3,2-*c*] coumarin core. Pterophyllin 2 (**1c**) was isolated from the methanolic extract of the bark (4.1 mg, 0.0016%), whereas pterophyllin 4 (**1d**) was found in the chloroformic extract of the wood (4.6 mg, 0.00077%).

To date, the bioactivity profile of the pterophyllins remains unknown. It has been shown that some furo[3,2-*c*] coumarin derivatives of osthole (7-methoxy-8-prenyl coumarin), such as **2a–e**, are active against phytopathogenic fungi [17]. Since **2e** can be regarded as an analog of pterophyllin 4 (**1d**), we conjectured that the pterophyllins may display antifungal activity. The small amounts of the natural products found in Nature and the difficulty of preparing analogs from them suggested chemical synthesis as a convenient strategy for their study.

We have recently reported the total syntheses of pterophyllin 2 (**1c**) and pterophyllin 4 (**1d**) [18a]. Therefore, herein we wish to report the study of the antifungal activity of **1c**, **1d**, and **3** (a 5-desmethyl analog of **1d**) against *R. stolonifer*, *B. cinerea* and *M. fructicola*, three relevant phytopathogenic fungi, that infect fruits mainly during the postharvest stage. For the sake of comparison, some heterocyclic synthetic intermediates toward the pterophyllins [18a] have also been included.

2. Results and discussion

2.1. Chemistry

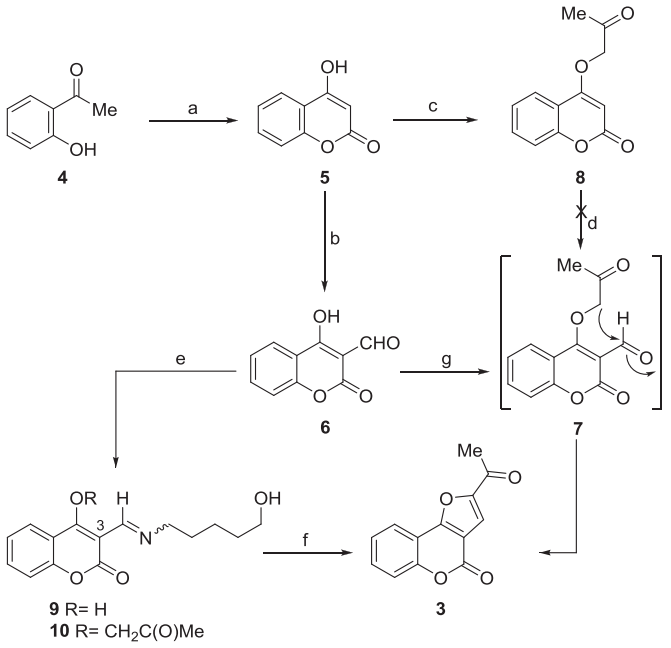
The access to the proposed analog **3** was achieved through a strategy involving the scarcely precedented [18a] Casnati-Skattebøl C-3 selective formylation of a 4-hydroxycoumarin and a one-pot acetonylation-cyclodehydration sequence, as its main features.

To that end, the economic and readily available 2-hydroxy acetophenone (**4**) was submitted to reaction with diethyl carbonate in THF; when K^{t}BuO was employed as basic promoter, the reaction gave 82% yield of the expected 4-hydroxy coumarin intermediate **5** [19a]. In turn, the latter was exposed to anhydrous paraformaldehyde in THF at 50 °C, in the presence of the $MgCl_2$-Et_3N reagent system [18b,c], cleanly affording 91% yield of the 3-formyl derivative **6** [19b]. The use of carefully dried reagents and anhydrous solvent in the Casnati-Skattebøl reaction was critical for the attainment of good yields. It was observed that the transformation failed, or its performance was sensitively lower when commercial paraformaldehyde (> 5% H_2O) was employed or when the $MgCl_2$ was not scrupulously dry.

Attempts at *O*-acetonylation of **6** under different base-promoted conditions (K_2CO_3, $MgCO_3$) failed to afford the expected intermediate **7**, and resulted in degradation of the starting material. Therefore, in search of an alternative path, the *O*-acetonylation of **5** was performed instead, with chloroacetone and K_2CO_3 in absolute EtOH. The reaction furnished 52% of the acetonyl ether **8**; [20] however, the latter missed to deliver the 3-formyl derivative **7**, when the Vilsmeier-Haack conditions ($POCl_3$, DMF) were employed.

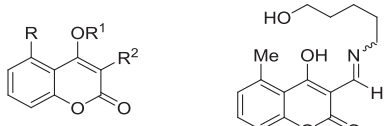
Suspecting that the high reactivity of the formyl moiety of **6** was responsible for the observed results, the aldehyde was submitted to imination with 5-aminopentanol under azeotropic conditions [21], to afford **9** in 75% yield. This was followed by the one-pot K_2CO_3-assisted *O*-acetonylation of the so formed imine **9** in DMF and the subsequent cyclization to **3** through the acetonyl ether **10**. Fortunately, the latter stages resulted in direct access to the tricycle **3**, albeit in a meager 28% yield from **9** (4 steps, 16% overall yield from **4**) (Scheme 1).

In order to simplify and improve the performance of the synthetic sequence, the use of different bases was explored. Luckily, stirring a warm dichloromethane mixture of **6** and chloroacetone with activated basic alumina (Brockmann I) smoothly furnished the final product **3** in 88% yield, presumably through the intermediacy of the acetonyl ether



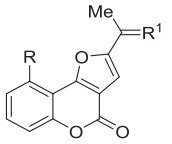
Scheme 1. *Reagents and conditions*: a) 1. K^{t}BuO, THF, r.t., 10 min.; 2. Et_2CO_3, reflux, 12 h (82%); b) $MgCl_2$ (anh.), Et_3N, $(CH_2O)_n$, THF, 50 °C, 1 h (91%); c) $ClCH_2COCH_3$, K_2CO_3, EtOH, 45 °C, overnight (52%); d) $POCl_3$, DMF, 0 °C; e) $H_2N(CH_2)_4CH_2OH$, PhMe, 90 °C, 1 h (75%); f) $ClCH_2COCH_3$, K_2CO_3, DMF, 75 °C, overnight (28%); g) $ClCH_2COCH_3$, Al_2O_3, CH_2Cl_2, 40 °C, 72 h (**3**, 88%).

Table 1
MIC and MFC values of the tested compounds against postharvest fruit pathogenic fungi.[a]



5 R= R^1= R^2= H
8 R= R^2= H, R^1= $CH_2C(O)Me$
11 R= Me, R^1= R^2= H
12 R= Me, R^1= H, R^2= CHO

13



3 R= H, R^1= O
1c R= Me, R^1= CH_2
1d R= Me, R^1= O

Comp. no[b]	MIC (μg/mL)			MFC (μg/mL)		
	Rs	*Bc*	*Mf*	*Rs*	*Bc*	*Mf*
1c	250	250	250	250	250	250
1d	250	I	62.5	I	I	62.5
3	250	250	250	I	250	I
5	I	I	I	I	I	I
8	I	I	I	I	I	I
11	I	I	I	I	I	I
12	250	125	125	I	125	125
13	250	250	125	I	I	125
Imz	31.25	0.12	0.49	62.5	0.25	0.97
Cbz	7.8	0.49	0.12	7.8	0.49	0.12

[a] MIC: Minimum Inhibitory Concentration; MFC: Minimum Fungicidal Concentration; *Rs*: *Rhizopus stolonifer*; *Bc*: *Botrytis cinerea*; *Mf*: *Monilinia fructicola*. Imazalil (Imz) and carbendazim (Cbz) were used as positive controls. I: inactive (MIC > 250 μg/mL).
[b] For the synthesis of **1c**, **1d**, and **11–13**, see Ref. 18a.

derivative **7** [19c], which was detected by TLC but could not be isolated. This approach enabled the efficient acquisition of **3** in three steps and 66% overall yield from ketone **4**.

All the compounds were characterized by their melting points and spectroscopic means. Notably, the ^{1}H NMR spectrum of **9** was unusually complex. Its analysis and the examination of NOE spectra revealed that the imine was obtained as an inseparable 7:3 (*anti:syn*) mixture of isomers; further, each isomer was observed as a mixture of two rotamers, as a consequence of the hindered rotation of the whole aldimino moiety around the C−3−C single bond.

2.2. Antifungal activity. Bioassay studies

Table 1 summarizes the Minimum Inhibitory Concentration (MIC) values and the Minimum Fungicidal Concentration (MFC) data of the tested compounds against the fruit pathogenic fungi *R. stolonifer*, *B. cinerea* and *M. fructicola*.

The MIC values were determined by the broth microdilution technique, according to the procedures reported by CLSI [22], whereas the MFC values were obtained by sub-culturing a sample of media from MIC tubes showing no growth, onto drug free agar plates. Carbendazim and imazalil, two imidazole-type fungicides commonly employed on several crops, were used as positive controls.

The observed results revealed that, except for **5**, **8** and **11**, the tested compounds were active against at least two of the fruit pathogenic fungi. Further, all compounds were at best fungistatic against *R. stolonifer* (MIC = 250 μg/mL) and only pterophyllin 2 (**1c**) proved to be fungicide against this fungus, with MFC = 250 μg/mL. It is worth mentioning that this phytopathogen is fast growing and one of the most difficult to control, as inferred from the MIC and MFC data of the commercial antifungal drug imazalil (31.25 and 62.5 μg/mL, respectively).

Regarding *B. cinerea*, the most active compound was aldehyde **12**, which behaved as fungistatic and fungicide at 125 μg/mL. On the other hand, compounds **3** and **1c** showed moderate activities, with MIC and MFC values of 250 μg/mL; in addition, **13** was not fungicide, being barely fungistatic, and **1d** resulted inactive (MIC > 250 μg/mL) against this pathogen.

Finally, *M. fructicola* proved to be the most susceptible microorganism, since except for the fully inactive heterocycles, the remaining compounds under evaluation inhibited its growth. Compound **3** resulted only fungistatic (MIC = 250 μg/mL); however, the rest of the compounds were also fungicides with MFC values between 62.5 and 250 μg/mL. Interestingly, this important stone fruit pathogen was particularly sensitive to **1d** with MIC and MFC values of 62.5 μg/mL (~258 μM). Compared with the commercial agents, pterophyllin 4 (**1d**) was 1.8 and 2.7 orders of magnitude less potent than Imz and Cbz, respectively.

The antifungal activity of pterophyllin 4 against the three fungi was also assessed in agar plates containing the heterocycle, by verifying the inhibition of the radial growth of their mycelia (Fig. 2). The solid medium assay was performed with **1d** at a final concentration of 250 μg/mL; at this level, the compound inhibited the hyphal growth of all tested phytopathogenic fungi.

The heterocycle inhibited the mycelial growth compared to the control (Fig. 2A and E) of *R. stolonifer* (antifungal index = 70% after 38 h). Notably however, after 43 h, the growth control (Fig. 2F) began to exhibit its characteristic hairy look, and essentially covering the whole plate. In comparison, the treated sample (Fig. 2B) coated approximately 15% of plate surface.

On the other hand, despite pterophyllin 4 (**1d**) was considered to be inactive toward *B. cinerea* in the liquid medium test (MIC > 250 μg/mL), it displayed a noticeable reduction of the mycelial growth (antifungal index = 66%) compared with the corresponding control (Fig. 2C and G). Further, the control plate exhibited a compact core and a fluffy periphery, whereas the treated colony showed a more homogeneous growth.

As previously observed in the liquid medium, under the same conditions, *M. fructicola* demonstrated to be the most sensitive species (antifungal index = 82%). The untreated colony (Fig. 2H) displayed its characteristic pattern of greyish concentric rings, which was rather absent in the treated sample (Fig. 2D).

The morphology of the colonies in the Petri plates was examined employing optical microscopy. Representative photographs exhibiting hyphae malformations of the three phytopathogens in response to their exposure to pterophyllin 4 (**1d**) and the corresponding controls, are

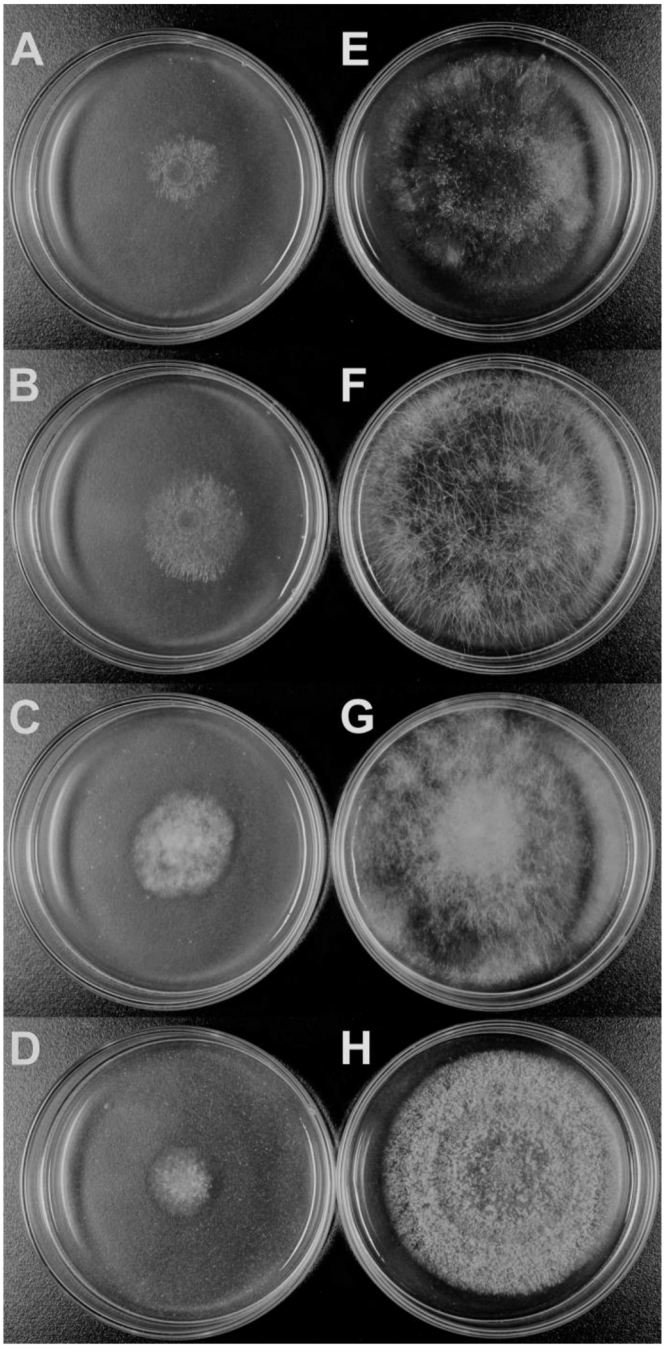


Fig. 2. Representative pictures of the inhibition of mycelial growth in *R. stolonifer* (A, B), *B. cinerea* (C) and *M. fructicola* (D) when treated with pterophyllin 4 (**1d**, final concentration 250 μg/mL) for 38, 43, 96 and 96 h, respectively. Plates E-H at the right are the corresponding growth controls.

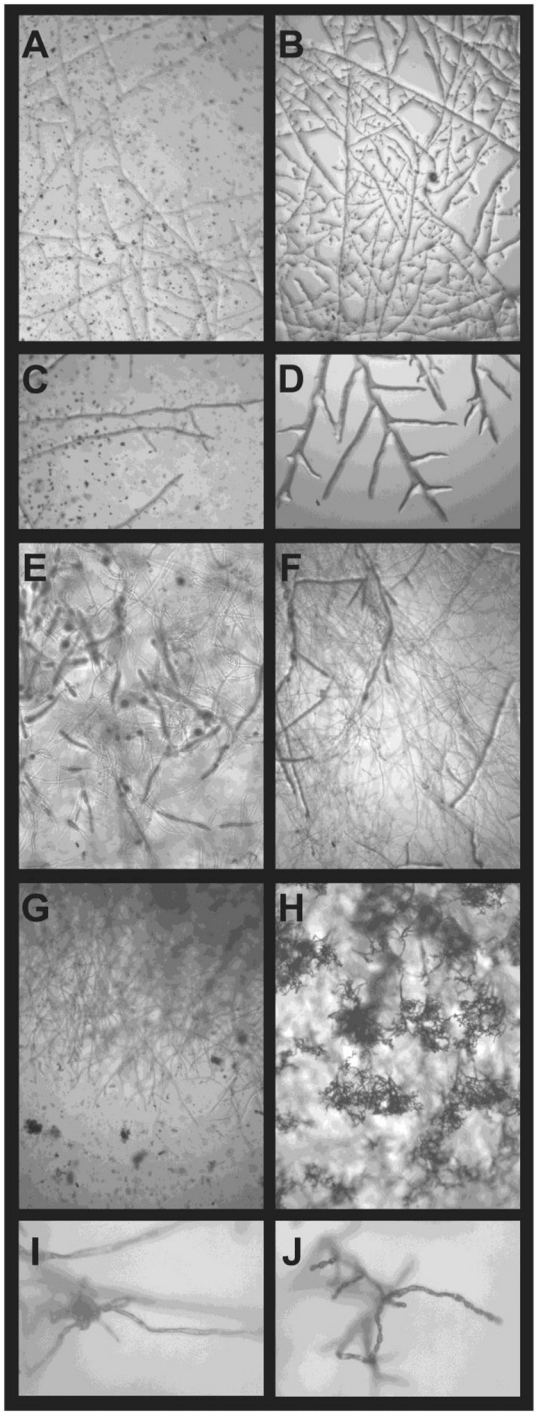


Fig. 3. Hyphal growth of the fungi at 40 × (light microscopy). Left: in presence of pterophyllin 4 (**1d**, 250 μg/mL). Right: Corresponding controls. *R. stolonifer* (A, B); *B. cinerea* (E, F) and *M. fructicola* (G, H). Details at 100 × of *R. stolonifer* (C, D) and *M. fructicola* (I, J). The photographs of the colonies were taken after 43, 96 and 96 h of incubation, respectively.

shown in Fig. 3.

The Fig. 3A and B show the images of mycelia obtained from the outer part of the colonies of *R. stolonifer*, which displayed some differences. In the control plate (Fig. 3B), the hyphae were lush and growing vigorously, as stemmed from the presence of a good number of young hyphae of smaller diameter. However, in the colony grown for 43 h on the solid medium containing pterophyllin 4 (**1d**), the hyphae were more scattered (Fig. 3A).

A further magnification of the image revealed that the number of hyphae and their degree of branching were notably reduced in the treated sample (Fig. 3C) compared with the control (Fig. 3D). This observation explains the noticeably diminished hyphal density visually detected in the plates of the treated colonies (Fig. 2A and B), with regard to the corresponding controls (Fig. 2E and F).

Interestingly, as shown in Fig. 4, the microscopic observation of samples stained with lactophenol blue, revealed the frequent presence of typical *Rhizopus* sporangia in the control sample (Fig. 4B); however, these were rather absent in the treated colony (Fig. 4A).

In addition, Fig. 3E and F show the images of treated and growth control colonies of *B. cinerea*, respectively. The control exhibited thin hyphae with a smooth surface, forming a typical net-shaped structure

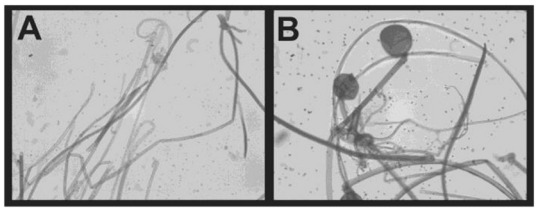


Fig. 4. Microscopic examination of the hyphal growth of *R. stolonifer*, stained with Lactophenol Blue. A) In the presence of pterophyllin 4 (**1d**, 250 μg/mL); B) Control.

(Fig. 3F). However, the image of the fungus exposed to pterophyllin 4 (**1d**) displayed more curly and swollen hyphae (Fig. 3E), suggesting that growth inhibition involves some deformation of the intimate structure of the fungal hyphae.

On the other hand, the exposed colony of *M. fructicola* microscopically displayed a radial growth of the hyphae (Fig. 3G), whereas the untreated counterpart showed groups of curled hyphae (Fig. 3H). Further, the image magnification of both samples revealed the presence of numerous typical lemon shaped conidia in the untreated specimen, produced in beads like strings (Fig. 3J). In sharp contrast, they were scarce in the exposed colony (Fig. 3I). No significant changes in the diameter of the hyphae were observed.

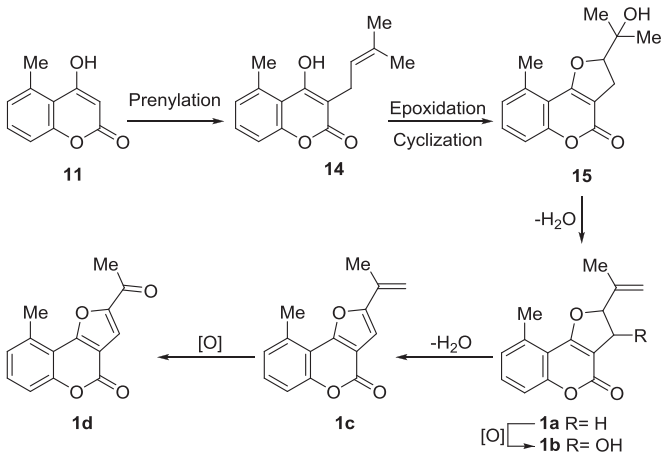
A simple evaluation of structure-activity relationships revealed that pterophyllin 4 (**1d**) is a selective fungicidal agent against *M. fructicola* (MFC = 62.5 μg/mL), not exhibiting fungicidal behavior against the other fungi at test concentrations over 250 μg/mL. However, the analog **3** which lacks the 5-methyl group, is barely fungicide only against *B. cinerea*. Interestingly, some structurally related open furocoumarins, were also found to be especially effective to control this fungus [23].

On the other hand, despite that structural differences between pterophyllin 2 (**1c**) and pterophyllin 4 (**1d**) are rather minor, their activity profiles, especially at the MFC level, are different. While the isopropenyl derivative **1c** is a low potency fungicide toward the three fungal strains (MFC = 250 μg/mL), the methyl ketone **1d** is a more potent and highly selective agent toward *M. fructicola*. In addition, the synthetic intermediates toward **1d** (**11** − **13**) displayed a rather different activity profile.

Overall, these results suggest that in order to yield more potent compounds, pterophyllin 4 (**1d**) could be a useful starting point, which still requires further development. In addition, and perhaps more revealing, they provide a hint that in Nature, pterophyllin 2 (**1c**) and pterophyllin 4 (**1d**) probably play plant defensive roles against phytopathogenic fungi [24].

It can be assumed that the pterophyllins are biogenetically related (Scheme 2). Pterophyllin 4 (**1d**) may result from the C-3 prenylation of 5-methyl-4-hydroxycoumarin (**11**) [25], which would afford **14** and a subsequent cyclization through the intermediacy of an epoxide, to give an intermediate like **15** [25a]. Sequential dehydration of the tertiary alcohol moiety in **15** to pterophyllin 1 (**1a**), followed by further allylic oxidation to pterophyllin 5 (**1b**) and final aromatization driven dehydration, may result in pterophyllin 2 (**1c**) [26].

In turn, pterophyllin 2 (**1c**) may undergo an oxidative fission of the exocyclic double bond, being converted into pterophyllin 4 (**1d**). Under these assumptions, it is interesting to somehow note the progressive increase in selectivity and potency with metabolite maturation (**11** → **1c** → **1d**) [27].



Scheme 2. Possible biogenetical path toward the natural products **1c** and **1d** from 4-hydroxy coumarin **11**.

3. Experimental

3.1. General information

All the reactions were performed under anhydrous argon atmospheres, employing oven-dried glassware and freshly distilled anhydrous solvents. CH_2Cl_2 was dried by means of a 4-hour reflux over P_2O_5 followed by atmospheric pressure distillation. Anhydrous THF was obtained from an M. Braun solvent purification and dispenser system. Anhydrous Et_3N was prepared by reflux and distillation of the PA product from CaH_2. Anhydrous DMF was prepared by heating the product over dry BaO and distillation of the solvent under reduced pressure. Anhydrous toluene was accessed by reflux over sodium and distillation from the sodium-benzophenone ketyl. Absolute EtOH was prepared by treatment of the PA reagent with clean magnesium turnings and a crystal of iodine, followed by reflux until complete consumption of the metal was achieved. The solvent was distilled from the so formed magnesium ethoxide. Anhydrous acetone was obtained by distillation of the PA product from $KMnO_4$; the distillate was stored over powdered pre-dried K_2CO_3. The anhydrous solvents were transferred *via* cannula and stored in dry Young ampoules. All other reagents were used as received.

Brockmann I basic alumina was activated in an oven, by heating the commercial product for 30 min. at 250 °C. Anhydrous paraformaldehyde was prepared by placing the commercial product (~90% grade; 5–10% H_2O) in a flask fitted with an acetone/liquid nitrogen cold trap, and heating the solid at 60 °C under reduced pressure (< 5 mm Hg), in order to remove the hydration water.

The reactions were monitored by TLC (silica gel 60 GF_{254}) run in different hexane-EtOAc solvent mixtures. The chromatographic spots were detected by exposure to UV light at 254 nm, and by spraying with 1% methanolic $FeCl_3$, or ethanolic *p*-anisaldehyde/sulfuric acid reagent, followed by careful heating to improve selectivity.

In the conventional work-up procedure, the reaction mixture was diluted with saturated brine and the products were extracted several times with EtOAc; the combined organic extracts were then washed once with brine, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by chromatography.

The flash column chromatographic separations were performed employing silica gel 60 H (particle size 63–200 μm). The elution was carried out with mixtures of hexane-EtOAc, under positive pressure of N_2 and employing gradient of solvent polarity techniques.

3.2. Equipment

The melting points were measured on an Ernst Leitz Wetzlar model 350 hot-stage microscope and are informed uncorrected. The FT-IR spectra were recorded on a Shimadzu Prestige 21 spectrophotometer, as solid dispersions in KBr disks.

The nuclear magnetic resonance spectra were acquired in $CDCl_3$, on a FT-NMR Bruker Avance 300 spectrometer, at 300.13 (^{1}H) and 75.48 (^{13}C) MHz. The chemical shifts are consigned in parts per million in the δ scale. TMS was used as the internal standard (resonances of $CHCl_3$ in $CDCl_3$: δ 7.26 and 77.0 for ^{1}H and ^{13}C NMR, respectively). The

magnitudes of the coupling constants (*J*) are given in Hertz. In special cases, NOE and 2D-NMR experiments (COSY, HSQC, TOCSY and HMBC) were also employed.

The high-resolution mass spectra were obtained from UMYMFOR (Buenos Aires), with a Bruker MicroTOF-Q II instrument. The detection of the ions was performed by electrospray ionization (ESI) in positive ion mode.

The optical microscopy studies were carried out with the aid of a Correct optical microscope, fitted with 10 ×, 40 × and 100 × objectives and equipped with a 5.0 Megapixels Beion CMOS digital camera [resolution (H × V) = 2592 × 1944].

3.3. Chemical synthesis

3.3.1. Chloroacetone (warning: strong lachrymator agent)

A stirred solution of H_2SO_4 (45 μL) in anhydrous acetone (200 mL) was cooled in an ice-water bath and treated portion-wise with finely powdered trichloroisocyanuric acid (85% w/w, 28 g, 105 mmol). After all the solids were dissolved, the mixture was warmed to room temperature and stirring continued for 3 h. Then, the liquid was decanted from the solid cyanuric acid by-product, powdered $CaCO_3$ (100 mg) was added, and the mixture was fractionally distilled under atmospheric pressure, collecting the liquid boiling in the range 117–119 °C. The distillate was stored in a brown bottle, at − 20 °C, over powdered $CaCO_3$.

3.3.2. 4-Hydroxy-2H-chromen-2-one (5)

A solution of 2-hydroxyacetophenone (**4**, 500 mg, 3.7 mmol) in THF (10 mL) was added to a stirred suspension of K^{t}BuO (1.24 g, 11 mmol) in THF (15 mL). After stirring for 10 min. at room temperature, the mixture was treated dropwise with Et_2CO_3 (1.4 mL, 11 mmol) and the reaction was further stirred overnight under reflux. Then, the solvent was evaporated under reduced pressure and the residue was treated with 1 M HCl (20 mL). The reaction products were extracted with EtOAc (4 × 20 mL) and the combined organic extracts were washed with brine (1 × 10 mL), dried ($MgSO_4$) and concentrated under reduced pressure. Chromatography of the residue gave **5** (488 mg, 82%), as an orange solid, mp: 210–211 °C; Lit.: 211–213 °C [19a]. IR (KBr, ῡ): 2918, 2717, 2577, 1701, 1611, 1545, 1312, 1277, 949, 833 and 746 cm^{-1}. ^{1}H NMR *δ*: 7.81 (dd, 1H, *J* = 7.8 and 1.6, H-5), 7.63 (ddd, 1H, *J* = 8.6, 7.2 and 1.6, H-7), 7.32–7.38 (m, 1H, H-6), 7.31 (dd, 1H, *J* = 7.2 and 1.1, H-8) and 5.58 (s, 1H, H-3). ^{13}C NMR *δ*: 166.1 (C-4), 162.3 (C-2), 154.0 (C-8a), 133.2 (C-7), 124.4 (C-6), 123.7 (C-5), 116.8 (C-8), 116.3 (C-4a) and 91.4 (C-3).

3.3.3. 4-Hydroxy-2-oxo-2H-chromene-3-carbaldehyde (6)

Anhydrous $MgCl_2$ (520 mg, 5.6 mmol) was added at once to a stirred solution of **5** (450 mg, 2.8 mmol) and anhydrous Et_3N (1.2 mL) in dry THF (18 mL). After stirring for 10 min, anhydrous paraformaldehyde (250 mg, 8.4 mmol) was added to the resulting suspension, and the reaction was heated at 65 °C for 1 h. The mixture was then cooled to room temperature, diluted with 1 M HCl (20 mL), and the products were extracted with EtOAc (3 × 35 mL). The combined organic extracts were washed with brine (1 × 20 mL), dried over $MgSO_4$ and concentrated under reduced pressure to afford a brownish solid, which was recrystallized from EtOAc to furnish **6** (480 mg, 91%), as a light brown solid, mp: 133–134 °C (dec.); Lit.: 135–136 °C [19b]. IR (KBr, ῡ): 3421, 2922, 1715, 1543, 1489, 1375, 1296, 1153, 958, 895 and 760 cm^{-1}; ^{1}H NMR *δ*: 10.04 (s, 1H, CHO), 8.05 (dd, 1H, *J* = 7.9 and 1.6, H-5), 7.73 (ddd, 1H, *J* = 8.5, 7.4 and 1.6, H-7), 7.38 (ddd, 1H, *J* = 7.9, 7.4 and 1.1, H-6) and 7.35 (d, 1H, *J* = 8.5, H-8).

3.3.4. 4-(2-Oxopropoxy)-2H-chromen-2-one (8)

Chloroacetone (7 μL, 0.09 mmol) was added dropwise at 0 °C to a stirred solution of **5** (16.5 mg, 0.09 mmol) and K_2CO_3 (14 mg, 0.095 mmol) in absolute EtOH (0.5 mL). The reaction was stirred overnight at 45 °C. The solvent was then evaporated, brine (5 mL) was added and the organic compounds were extracted with EtOAc (3 × 10 mL). The combined organic extracts were washed with brine (1 × 10 mL), dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Silica gel column chromatography (Hexane:EtOAc) gave **8** (10.2 mg, 52%) as a white solid, mp. 172–174 °C; Lit.: 173–175 °C [20c]. ^{1}H NMR *δ*: 7.91 (dd, 1H, *J* = 7.9 and 1.7, H-5), 7.59 (ddd, 1H, *J* = 8.3, 7.9 and 1.6, H-6), 7.34 (d, 1H, *J* = 8.3, H-8), 7.32 (t, 1H, *J* = 7.9, H-6), 5.54 (s, 1H, H-3), 4.74 (s, 2H, H-2′) and 2.35 (s, 3H, H-4′). ^{13}C NMR *δ*: 200.8 (C-3′), 164.5 (C-4), 162.3 (C-2), 153.4 (C-8a), 132.8 (C-7), 124.2 (C-6), 123.0 (C-5), 116.9 (C-8), 115.2 (C-4a), 91.3 (C-3), 72.6 (C-2′) and 26.6 (C-4′). HRMS *m/z* calcd for $C_{12}H_{10}NNaO_4$ 241.0471 $[M + Na]^+$; found: 241.0467.

3.3.5. 4-Hydroxy-3-{[(5-hydroxypentyl)imino]methyl}-2H-chro men -2-one (9)

A stirred solution of aldehyde **6** (20 mg, 0.10 mmol) in toluene (1 mL) was treated with 5-aminopentan-1-ol (11 mg, 0.11 mmol) and the mixture was heated at 90 °C for 1 h. The solvent was then evaporated, water (5 mL) was added and the organic compounds were extracted with EtOAc (3 × 10 mL). The combined organic extracts were washed with brine (1 × 10 mL), dried ($MgSO_4$) and concentrated under reduced pressure. Chromatography of the residue (silica gel, Hexane:EtOAc, 95:5, with 0.1% Et_3N) gave a 7:3 (*anti:syn*) isomeric mixture of imines **9** (22 mg, 75%), as a yellow oil. *Anti*-isomer - ^{1}H NMR *δ*: 8.40 (s, 0.5H, H-1′ rotamer-1), 8.35 (s, 0.5H, H-1′ rotamer-2), 8.00 (dd, 1H, *J* = 7.8 and 1.5, H-5), 7.50–7.59 (m, 1H, H-7), 7.24 (dt, 1H, *J* = 7.8 and 1.0, H-6), 7.21 (dd, 1H, *J* = 8.3 and 1.0, H-8), 3.66 (t, 2H, *J* = 6.2, H-7′), 3.53 (t, 2H, *J* = 6.8, H-3′), 1.68–1.85 (m, 2H, H-4′), 1.55–1.68 (m, 2H, H-6′) and 1.42–1.55 (m, 2H, H-5′). ^{13}C NMR *δ*: 181.4 (C-4), 164.0 (C-2), 162.3 (C-1′), 154.8 (C-8a), 134.3 (C-7), 125.6 (C-5), 124.0 (C-6), 120.6 (C-4a), 117.3 (C-8), 96.9 (C-3), 62.3 (C-7′), 51.0 (C-3′), 31.9 (C-6′), 29.9 (C-4′) and 22.8 (C-5′). *Syn*-isomer - ^{1}H NMR *δ*: 8.56 (s, 0.5H, H-1′ rotamer-1), 8.51 (s, 0.5H, H-1′ rotamer-2), 8.07 (dd, 1H, *J* = 7.8 and 1.6, H-5), 7.50–7.59 (m, 1H, H-7), 7.24 (dt, 1H, *J* = 7.8 and 1.0, H-6), 7.21 (dd, 1H, *J* = 8.3 and 1.0, H-8), 3.65 (t, 2H, *J* = 6.1, H-7′), 3.57 (t, 2H, *J* = H-3′), 1.68–1.85 (m, 2H, H-4′), 1.55–1.68 (m, 2H, H-6′) and 1.42–1.55 (m, 2H, H-5′). ^{13}C NMR *δ*: 178.6 (C-4), 165.3 (C-2), 160.8 (C-1′), 154.7 (C-8a), 134.3 (C-7), 126.4 (C-5), 124.2 (C-6), 120.8 (C-4a), 117.2 (C-8), 96.8 (C-3), 62.3 (C-7′), 50.8 (C-3′), 31.9 (C-6′), 29.7 (C-4′) and 22.7 (C-5′).

3.3.6. 2-Acetyl-4H-furo[3,2-c]chromen-4-one (3)

3.3.6.1. Method A. Anhydrous K_2CO_3 (15 mg, 0.11 mmol) was added to a stirred solution of the imine **9** (28 mg, 0.1 mmol) in anhydrous DMF (1 mL), and the system was treated with chloroacetone (0.010 mL, 0.11 mmol). The system was warmed to 75 °C and left to react overnight. After assessing the complete consumption of the starting material by TLC, the reaction was diluted with brine (10 mL), and the products were extracted with EtOAc (4 × 20 mL). The combined organic extracts were washed with brine 1 × 5 mL, dried ($MgSO_4$) and concentrated under reduced pressure. Chromatography of the residue gave **3** (6.6 mg, 28%), as a colorless solid, mp: 210 °C (dec.); Lit.: 206 °C [19c]. IR (KBr, ῡ): 2922, 1749, 1684, 1624, 1541, 1450, 1296, 1177, 968 and 750 cm^{-1}. ^{1}H NMR *δ*: 8.03 (dd, 1H, *J* = 7.7 and 1.7, H-5), 7.68 (s, 1H, H-1′), 7.63 (ddd, 1H, *J* = 8.5, 7.4 and 1.7, H-7), 7.48 (dd, 1H, *J* = 8.5 and 0.9, H-8), 7.41 (dt, 1H, *J* = 7.7 and 0.9, H-6) and 2.61 (s, 3H, H-4′). ^{13}C NMR *δ*: 186.0 (C-3′), 159.5 (C-4), 157.3 (C-2), 153.8 (C-8a), 153.2 (C-2′), 132.6 (C-7), 125.0 (C-6), 122.0 (C-5), 117.7 (C-8), 115.2 (C-1′), 112.0 (C-4a), 111.6 (C-3) and 26.4 (C-4′). HRMS *m/z* calcd for $C_{13}H_8NNaO_4$ 251.0315 $[M + Na]^+$; found: 251.0307.

3.3.7. 2-Acetyl-4H-furo[3,2-c]chromen-4-one (3)

3.3.7.1. Method B. Activated basic alumina (Brockmann I, 54 mg, 0.53 mmol) was added to a solution of aldehyde **6** (20 mg, 0.10 mmol)

in anhydrous CH_2Cl_2 (1 mL) and the stirred suspension was treated with $ClCH_2COMe$ (0.021 mL, 0.26 mmol). The reaction was further stirred for 24 h at room temperature, when it was filtered off through Celite, and the solid was washed with CH_2Cl_2 (1 mL). The combined filtrates were concentrated under reduced pressure and the residue was chromatographed, furnishing **3** (21 mg, 88%), as a colorless oil. The spectral data of the product were in full agreement with those of the compound obtained employing *Method A*.

3.4. Antifungal studies

3.4.1. Microorganisms and media

For the evaluation of the antifungal activity, standardized strains from the Department of Microbiology of the Chemical Engineering Faculty, National University of Litoral (UNL, Santa Fe, Argentina), the Micology Reference Center (CEREMIC, Rosario, Argentina) and the National Institute of Agricultural Technology (INTA, San Pedro, Argentina) were used.

Strains of *Rhizopus stolonifer* LMFIQ-317, *Botrytis cinerea* CCC-100 and *Monilinia fructicola* INTA-SP345 were grown on Potato-Dextrose-Agar (PDA) medium using Petri plates for 48 h to 6 days at 15–20 °C (as needed for the growth of each one), and sub-cultured every 15 days to prevent pleomorphic transformations. Inocula of spore suspensions were obtained according to the CLSI reported procedures and adjusted to 1×10^4 Colony Forming Units (CFU)/mL [22].

3.4.2. Susceptibility tests

The Minimum Inhibitory Concentration (MIC) values were determined by using broth microdilution techniques according to the CLSI guidelines for filamentous fungi (document M 38 A2). MIC values were determined in RPMI-1640 (Sigma, St. Louis, MO, USA) medium buffered to pH 7.0 with MOPS (Remel, Lenexa, KS, USA). Microtiter trays were incubated at 15–20 °C in a moist and dark chamber, and MIC values were visually recorded at 24 h for *R. stolonifer* and at a time according to the growth control, for the remaining fungi.

For the assay, solutions of 12.5 mg/mL concentration of each compound were prepared in DMSO. 40 μL of these, were diluted in 960 μL of media RPMI to obtain stock solutions of 250 μg/mL that were serially diluted with RPMI from 250 to 7.8 μg/mL (final volume = 100 μL) in the corresponding wells of a microtiter plate. A volume of fungal suspension (100 μL) was added to each well, except for the sterility control, where sterile water was added to the well instead. The commercial antifungal agents imazalil and carbendazim were used as positive controls. The MIC endpoints were defined as the lowest concentration of compounds visually resulting in total inhibition of fungal growth compared to the growth in the control wells containing no antifungal agent.

The Minimum Fungicidal Concentration (MFC) was defined as the lowest concentration of compound that resulted in total inhibition of visible growth. The MFC values of the compounds against each fungal strain were determined after assessing the MIC values, by transferring sample aliquots (5 μL) from each clear well of the microtiter tray onto a 150-mm RPMI-1640 agar plate buffered with MOPS. The inoculated plates were incubated at 15–20 °C; the MFC values were recorded after 24 h for *R. stolonifer* and at later times, according to the corresponding growth control, for the rest of the fungi.

3.4.3. Mycelial growth inhibition

The antifungal activity of pterophyllin 4 (1d) was demonstrated *in vitro* by examining the inhibition of the mycelial radial growth of the three phytopathogenic fungi (*R. stolonifer*, *B. cinerea* and *M. fructicola*). A stock solution of 1d was prepared by dissolving the test compound in DMSO. This was diluted to a testing concentration of 250 μg/mL with Potato-Dextrose-Agar (PDA) medium at 50 °C and poured into sterilized Petri dishes (6 cm). After solidification, 10 μL of conidial suspensions adjusted to 1×10^4 CFU/mL of the active fungi were placed at the center of the Petri dishes.

The dishes were sealed and incubated at 15–20 °C for 1–4 days as appropriate for each of the fungi. The corresponding growth controls were prepared by admixing volumes of DMSO with PDA to the equivalent final concentrations. The growth of the fungal mycelia for the treated plates, was measured as the average of three orthogonal diameters when the control plates reached full growth. The percentage of reduction in colony diameter [antifungal index (%)] was calculated by the formula of Eq. (1),

$$\text{Antifungal index}\,(\%) = 100 \times (C - T)/C \quad (1)$$

where T and C are the mean diameters of the mycelia in the Test and corresponding Control samples.

4. Conclusion

In conclusion, our findings suggest that pterophyllin 4 (**1d**) exhibits antifungal activity, potentially useful for the elaboration of improved agents to control postharvest disease of fruits, especially those that may be caused by *M. fructicola*.

These results encourage prospection of natural products for their antifungal properties against fruits pathogens as new leads. They also reinforce the idea that, in the search for upcoming eco-friendly post-harvest pest control programs, natural products can be promising sources of inspiration for the development of new leads and more efficient compounds.

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