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Screening study for potential lead compounds for natural product-based fungicides: I. Synthesis and *in vitro* evaluation of coumarins against *Botrytis cinerea*†

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Abstract: An efficient, one-pot synthesis of angular and linear dihydropyranocoumarins, along with C-6 and C-8 prenylated coumarins is reported. These compounds, together with single- and furanocoumarins, were tested for their potential antifungal activity against the phytopathogen *Botrytis cinerea* Pers ex Fr. The results show that furanocoumarins may be able to control the fungus *B cinerea*.

Keywords: crop-protection agents; antifungal activity; *Botrytis cinerea*; coumarins

1 INTRODUCTION

Botrytis cinerea Pers ex Fr, by far the most important of the *Botrytis* species, is also an interesting fungal pathogen because it can live both pathogenically and saprophytically.[1]

Since the mid-1990s, new compounds with excellent activity against *B cinerea* have been commercialized. However, strategies to control the fungus with classic fungicides may produce side-effects, notably environmental contamination and the development of multi-resistant fungal strains. Some of these fungicides, such as the dicarboximide procymidone, are persistent enough to be detected after several weeks in vegetables[2] and soil,[3] and even after vinification.[4]

Application of synthetic fungicides for the control of fungal diseases in major agricultural crops is a standard tool of farm production in Europe. Although modern fungicides have reached a high level of efficacy coupled with increasingly reduced toxicity, some problems associated with environmental impacts and toxicological effects remain. Consequently, there is a great deal of interest in developing novel, non-persistent antifungal agents, especially those with activity against particularly damaging fungi such as *B cinerea*.

Recent progress in the identification of the pathogenicity factors of this harmful fungus has opened significant options for major innovations in the control of plant disease.[5] Thus, the putative role of the phytotoxins excreted by *B cinerea* in the infection mechanism has been reported,[6,7] and a new alternative to synthetic fungicides has been obtained by using compounds structurally analogous to the biosynthetic intermediate of the naturally expressed toxins of the fungus.[8,9] In this context, detoxification studies of some these antifungal compounds have revealed that compounds with C6–C5 fused rings in the basic skeleton and different substitution patterns could lead to the design of more active compounds.[10]

Coumarins, including furanocoumarins, are bioactive compounds which exhibit a wide range of biological properties against fungi, viruses and bacteria,[11] and they have a basic skeleton which could be considered analogous to the intermediates of the biosynthesis of both melanin[12] and botrydial.[13]

In the present paper we report, in addition to the synthesis of dihydropyranocoumarins, the antifungal activity of coumarins against *B cinerea* in relation to their chemical structure.

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2 EXPERIMENTAL

2.1 General experimental procedures

Melting points were measured with a Reichert–Jung Kofler block and are uncorrected. Optical rotations were determined with a Perkin–Elmer 241 polarimeter. IR spectra were recorded on a Mattson Genesis spectrophotometer, series FTIR. ^{1}H and ^{13}C NMR spectra were recorded on Varian Gemini 200 MHz (^{1}H at 199.975 MHz, ^{13}C at 50.289 MHz) and Varian Unity 400 MHz (^{1}H at 399.952 MHz, ^{13}C at 100.570 MHz) spectrometers. Chemical shifts are quoted relative to tetramethylsilane (TMS) in deuterochloroform. Mass spectra were recorded on GC-MS Thermoquest, model Voyager, and VG-Autospec spectrometers. HPLC was performed with a Hitachi/Merck L-6270 apparatus equipped with an UV-vis detector (L 4250) and a differential refractometer detector (RI-71). TLC was performed on Merck Kiesegel 60 F_{254}, 0.25 mm thick. Silica gel (Merck) was used for column chromatography. Purification by means of HPLC was carried out with a Silica gel column (LiChrospher Si-60, 10 μm, 1 cm wide and 25 cm long or 5 μm, 0.4 cm wide, and 25 cm long).

Coumarins **2–4** (Fig 1) were obtained from commercially available umbelliferone (**1**) following procedures described previously.[14,15] The furanocoumarins **16** and **19** (Fig 2) were obtained by reacting 8- and 6-iodoumbelliferone (**8**,**9**), respectively, with ethylpropiolate and copper(I) oxide in *N*,*N*-dimethylformamide, as reported.[16] Alkaline hydrolysis and subsequent decarboxylation with copper powder in quinoline yielded angelicine (**16**) (78%) and psoralene (**19**) (81%). Furanopinnarin (**17**),[11] xanthotoxin (**18**),[17] chalepensin (**20**),[17] pyranocoumarin (**13**)[11,18] and the coumarins **14**[19] and **15**[11,18] were generously provided by Professor FR Luis, who isolated them from natural sources or obtained them using synthetic methods.

2.2 Synthesis

2.2.1 General procedure for reactions of coumarins with 3,3-dimethylallyl bromide and tri-n-butyltin hydride (THB)

The coumarin and 3,3-dimethylallyl bromide (0.5 ml) were dissolved in dry benzene. The mixture was stirred and refluxed, after which tri-*n*-butyltin hydride (THB; 0.5 ml) and a catalytic amount of azobisisobutyronitrile were added. After 24 h the reaction mixture was cooled and filtered on silica gel and washed with hexane until the THB was eliminated. Ethyl acetate was then passed through the column to yield the reaction products.

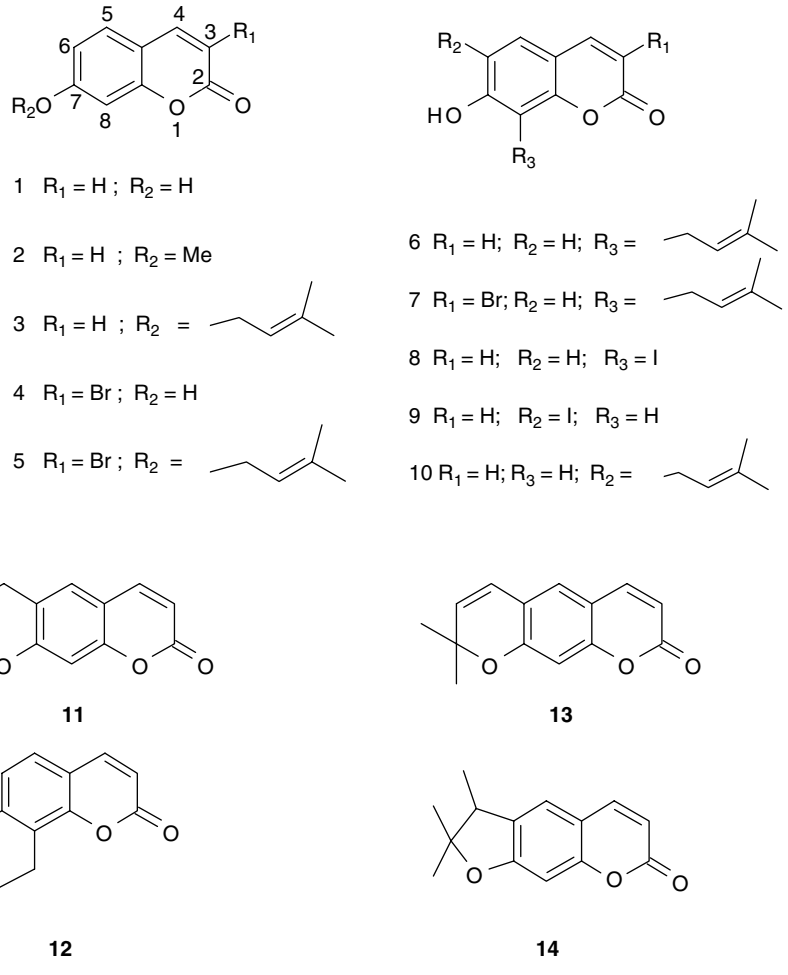

Figure 1. Structures of test compounds **1–14**.

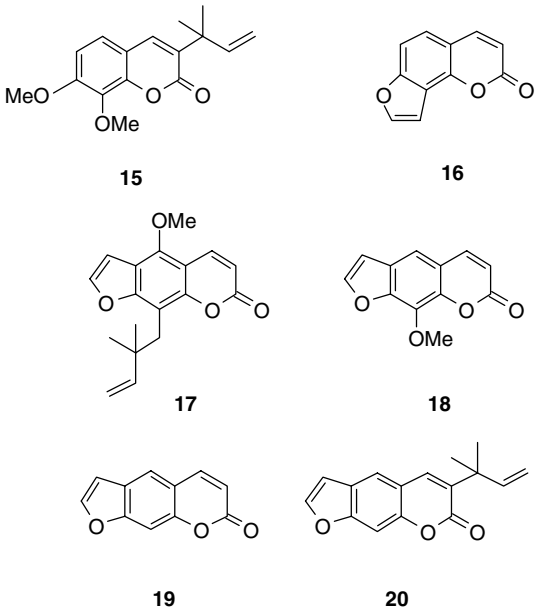

Figure 2. Structures of test compounds **15–20**.

*2.2.2 Prenylation of 3-bromoumbelliferone (**4**)*

3-Bromoumbelliferone (**4**; 48 mg) and 3,3-dimethylallyl bromide (0.5 ml) were dissolved in dry benzene (5 ml). The mixture was stirred and refluxed following the general procedure described in Section 2.2.1 and then subjected to chromatography to yield compounds **5** (15.5%) and **7** (40%) (Table 1):

3-Bromo-7-(3,3-dimethylallyloxy)coumarin (**5**): colourless oil; IR (potassium bromide) ν_{max} 1725, 1600, 1493, 1270, 1113 cm^{-1}; HREIMS calcd for $C_{14}O_3H_{13}Br$ M^+ 308.0048, found 308.0051.

3-Bromo-7-hydroxy-8-(3,3-dimethylallyl)coumarin (**7**): colourless oil; IR (potassium bromide) ν_{max} 3350, 1700, 1600, 1490, 1125 cm^{-1}; EIMS *m/z* (%), M^+, $(M^+ + 2)$ 308, 310 (3,1); 240 (35); 69 (100); HREIMS calcd for $C_{14}O_3H_{13}Br$ M^+ 308.0048, found 308.0042.

Table 1. ^{1}H NMR signals of coumarins **5**, **7**, 3′,4′-dihydroxanthyletin (**11**) and 3′,4′-dihydroseselin (**12**) (deuterochloroform, 200 MHz)

	^{1}H signals (δ) for compound			
Resonance atom	**5**	**7**	**11**	**12**
H-3	—	—	6.07 d	6.07 d
H-4	7.94 s	7.99 s	7.58 d	7.47 d
H-5	7.26 d	7.10 d	7.07 d	7.04 s
H-6	6.80 dd	6.73 d	6.68 d	—
H-8	6.75 d		—	6.6 s
H-1′	4.50 d	3.54	—	—
H-2′	5.39 t	5.16	—	—
H-3′			1.79 t	1.73 t
H-4′	—	—	2.82 t	2.70 t
CH_3	1.74 s	1.79	1.37 s	1.25 s
CH_3	1.70 s	1.69	1.37 s	1.25 s

Selected coupling constants (Hz): **5** ($J_{H5-H6} = 8.6$; $J_{H6-H8} = 2.4$; $J_{H1'-H2'} = 6.7$); **7** ($J_{H5-H6} = 8.6$; $J_{H1'-H2'} = 6.7$); **11** ($J_{H3-H4} = 9.4$; $J_{H5-H6} = 8.6$; $J_{H3'-H4'} = 6.8$); **12** ($J_{H3-H4} = 9.4$; $J_{H3'-H4'} = 6.8$).

*2.2.3 Prenylation of umbelliferone (**3**)*

Umbelliferone (**1**) dissolved in dry benzene was treated with 3,3-dimethylallyl bromide and THB following the general procedure described in Section 2.2.2. After column chromatography on silica gel, 3′,4′-dihydroxanthyletin (**11**; 24%) and 3′,4′-dihydroseselin (**12**; 35%) were obtained (Table 1):

3′,4′-Dihydroxanthyletin (**11**): colourless oil; IR (potassium bromide) ν_{max} 1717, 1595, 1484 cm^{-1}; EIMS *m/z* (%) 230 $[M]^+$ (96), 215 (22), 187 (28), 175 (100), 146 (28); HREIMS calcd for $C_{14}O_3H_{14}$ 230.0943, found 230.0947.

3′,4′-Dihydroseselin (**12**): colourless oil; IR (potassium bromide) ν_{max} 1720, 1600, 1500 cm^{-1}; EIMS *m/z* (%) 230 $[M]^+$ (95), 215 (28), 187 (22), 175 (100); HREIMS calcd for $C_{14}O_3H_{14}$ 230.0943, found 230.0939.

*2.2.4 Treatment of 8-iodoumbelliferone (**8**) with 3,3-dimethylallyl bromide*

Compound **8** (100 mg) was dissolved in benzene + tetrahydrofuran (1 + 1 by volume; 10 ml) and 3,3-dimethylallyl bromide (0.2 ml) was then added. Following the general procedure described in Section 2.2.1, tri-*n*-butyltin hydride (0.5 ml) and a catalytic amount of azobisisobutyronitrile were added and the reaction recovered to yield a mixture of compounds **3** (30%), **6** (38%) and **10** (18%).

2.3 Microorganism and antifungal assays

The culture of *B cinerea* employed in this work, *B cinerea* 2100, was obtained from the Centro Español de Cultivos Tipos (CECT), Facultad de Biologia, Universidad de Valencia, Spain, where a culture of this strain is deposited. Bioassays were performed by measuring inhibition of radial growth on an agar medium in a Petri dish. The test compound was dissolved in acetone to give final compound concentrations ranging from 25 to 200 mg $litre^{-1}$. Solutions of the test compound were added to a glucose–malt–peptone–agar medium (61 g $litre^{-1}$, pH 6.5–7.0). The final acetone concentration was identical in both control and treated cultures. The medium was poured into sterile plastic Petri dishes (13.5 cm diameter) and a 1-cm diameter mycelial disc of *B cinerea* cut from an actively growing culture was placed in the centre of the agar plate. Inhibition of radial growth was measured for 8 days. Growth inhibition was calculated as the percentage of inhibition of radial growth relative to the control (Table 2). The assays were carried out in triplicate and the results are shown as mean values of three replicates of colony diameters (±SD) (Fig 3A and B). The commercial fungicide dichlofluanid was used throughout as a standard for comparison. The IC_{50} value was determined by the linear regression of the probit of the test fungus percentage inhibition and the log of the studied compound concentrations.

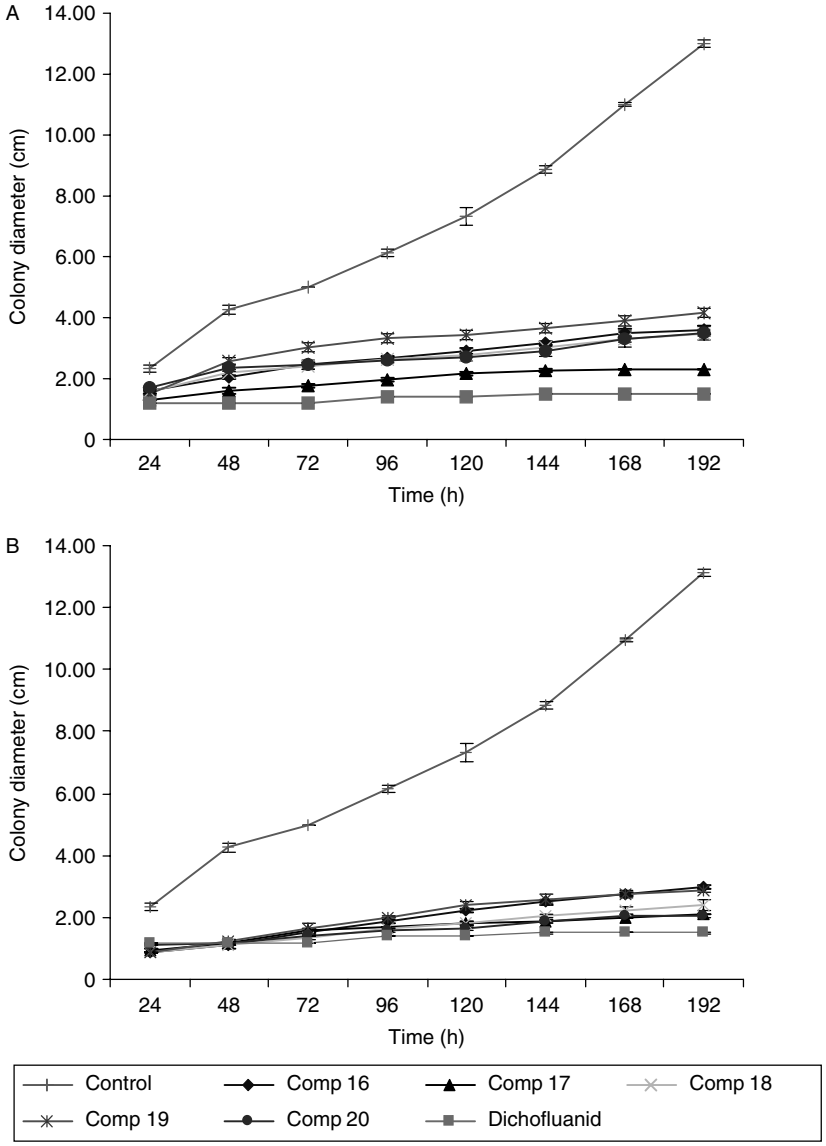

Figure 3. Antifungal effect of compounds **16**–**20** and dichlofluanid on *in vitro* growth of *Botrytis cinerea* at (A) 100 mg litre^{-1}, (B) 200 mg litre^{-1}. Results are shown as mean values of three replicates of colony diameters; bar = ±SD.

Table 2. Percentage inhibition of *Botrytis cinerea* mycelium radial growth and IC_{50} values of compounds **15**–**20**

Compound	Inhibition (%) 25	50	100 (mg litre^{-1})	150	200	IC_{50} (mg litre^{-1})
15	19.1	19.1	19.1	28.1	70.8	157.5
16	1.12	1.12	64.0	69.7	71.91	98
17	31.5	76.4	74.16	77.5	78.7	45.2
18	1.12	31.46	66.29	67.42	76.40	85.3
19	34.8	44.9	58.4	64.04	70.79	62.8
20	43.82	61.81	67.42	70.8	80	33.3

The IC_{50} was the average of three replications (Table 2).

3 RESULTS AND DISCUSSION

As mentioned above, the structural framework of coumarins endows them with promising potential as antifungal agents. When the 3-bromo derivative (**4**), obtained by treatment of umbelliferone (**1**) with trimethylphenyl perbromide,[20] was treated with 3,3-dimethylallyl bromine and THB, with a catalytic amount of azobisisobutyronitrile, compounds **5** (15.5%) and **7** (40%) (Table 1) were obtained via a clear radical reaction mechanism.

The synthetic route generally used to obtain prenylated coumarins in C-8 and C-6, compounds **6** and **10**, respectively, usually involves either Claisen rearrangements from C-7[21,22] or direct prenylation with alkaline metals.[11,18] Although both of these methods have been reported extensively, their application is limited due to their low yields.[18] These results prompted us to study an alternative methodology which leads to direct C-6 or C-8 prenylation of coumarins with acceptable yields.

With this goal in mind, umbelliferone (**1**) was treated with THB and azobisisobutyronitrile to afford 3′,4′-dihydroxanthyletin (**11**; 24%) and

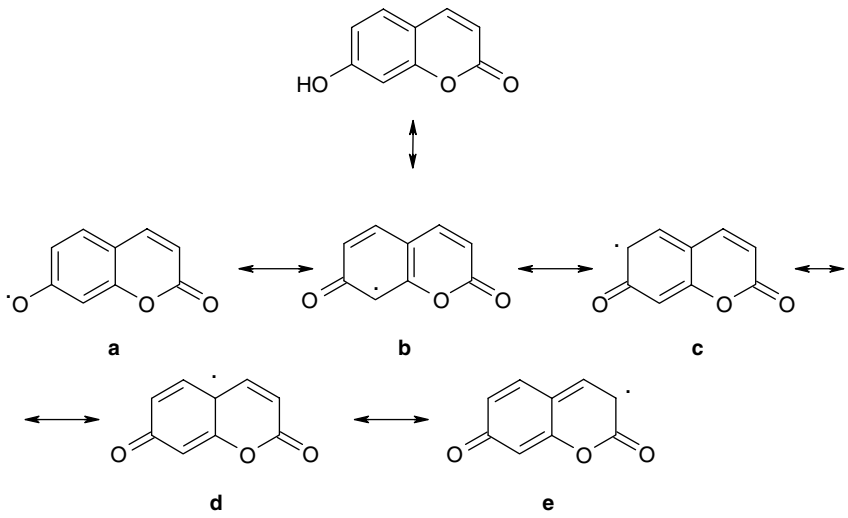

Figure 4. Resonance forms of 7-hydroxybenzpyrone.

3′,4′-dihydroseselin (**12;** 35%) in a one-pot reaction (Table 1). Likewise, when 8-iodoumbelliferone (**8**)[16] underwent similar treatment, compounds **3** (30%), **6** (38%) and **10** (18%) were obtained. In order to examine the role of the hydroxyl group on C-7, 7-methoxyumbelliferone[14,15] and 7-acetoxyumbelliferone were both treated with THB and 3,3-dimethylallyl bromine. No reaction was observed. This fact indicates not only that the hydroxyl group is crucial for this reaction, but also that its participation occurs via an initial radical in the oxygen on C-7. The appearance of compounds **6** and **10** can be explained by assuming that the canonical resonance forms **b** and **c**, with the radical on C-8 and C-6 to conserve the pyrone ring, are especially stable (Fig 4). Curiously, the dihydropyranocoumarins **11** and **12** are obtained via a post-cyclization of the prenylic chain when halogen atoms are not present on the coumarin skeleton. This can be accounted for if we considerer that the halogen atom promotes the radical termination step.

The antifungal properties of the coumarin derivatives described herein were determined against the growth of *B cinerea* by means of the 'poisoned food' technique.[23,24] While coumarins **1–10** displayed no activity, pyrano- and dihydropyranocoumarins **11–13** showed some weak activity. The furanocoumarins **16–20** exhibited the maximum percentage growth inhibition, followed by the 3-(1,1-dimethylallyl) coumarin **15**, which showed significant activity from 200 mg litre^{-1}. In contrast, the dihydropyranocoumarins **14** was inactive. Figure 3 shows the antifungal effect displayed by compounds **16–20** at 100 and 200 mg litre^{-1}.

It is interesting that the single furanocoumarins **16** and **18** displayed activity in the centre-point inoculation disk assay from 100 mg litre^{-1}, producing 65% average inhibition for 7 days, while the psoralene (**19**) showed activity at 25 mg litre^{-1} for 4 days. The inhibition of the mycelial growth produced by **19** was 71% at 200 mg litre^{-1} and the IC_{50} was 62.8 mg litre^{-1}.

The prenylated 1,1-dimethylallyl-furanocoumarins **17** and **20** exhibited 79% and 80% inhibition, respectively, at 200 mg litre^{-1}, but already displayed activity at 25 mg litre^{-1}. The IC_{50} values for compounds **17** and **20** were 45.2 and 33.3 mg litre^{-1}, respectively. According to the data obtained, the most promising antifungal agents for control of *B cinerea* were furanopynnarin (**17**) and chalepensin (**20**), which showed equivalent effects to the fungicide dichlofluanid from 50 mg litre^{-1} (for compound **17**) and 100 mg litre^{-1} (for compound **20**). Figure 3 shows the results obtained when compounds **16–20** and dichlofluanid were compared under the same assay conditions.

The results of the activity displayed by the coumarin derivatives studied thus demonstrate that the presence of a furan ring is critical for the antifungal activity of these molecules. It is, however, also worth noting that the 1,1-dimethylallyl chain seems to play an important role in the inhibition mechanism by enhancing the antifungal activity of these compounds, while the presence of a 3,3-dimethylallyl chain does not seem to have any effect on their antifungal activity.

4 CONCLUSIONS

According to the results described in this paper, the most promising antifungal agents for the control of *B cinerea*, as judged on its consistency in inhibiting growth of the fungus, were in general the furanocoumarins (Fig 4) and specially the 1,1-dimethylallylfuranocoumarins **17** and **20**. Work is in progress to study the mode of action of these compounds in fungal metabolism.

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