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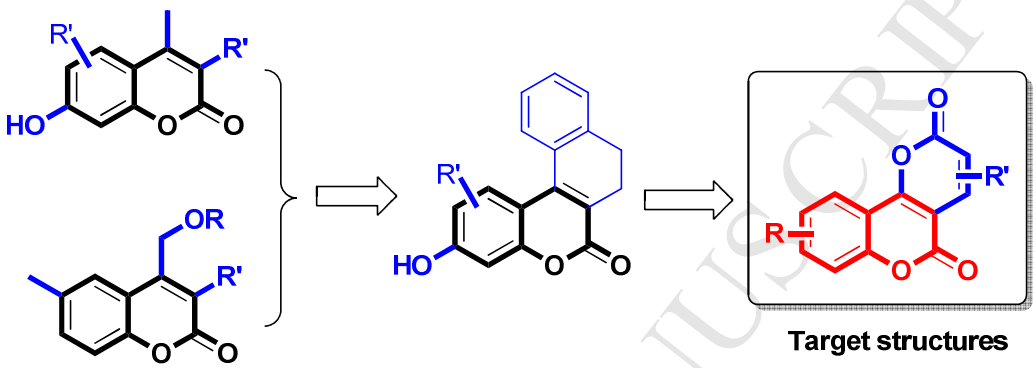

A series of novel fused coumarin derivatives were designed and synthesized through an optimized microwave-assisted protocol. Antifungal activity screening against five phytopathogenic fungi led to the identification of compound **5d** as the most promising candidate for further study.

Microwave-Assisted Synthesis and Antifungal Activity of Novel Coumarin Derivatives: Pyrano[3,2-*c*]chromene-2,5-diones

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Abstract:

A series of novel fused coumarin analogues pyrano[3,2-*c*]chromene-2,5-diones have been synthesized through an optimized microwave-assisted protocol. All target compounds were tested and evaluated for their antifungal activity against *Botrytis cinerea*, *Colletotrichum copsica*, *Alternaria solani*, *Gibberella zeae* and *Rhizoctorzia solani*. The bioassay results indicated that some of the compounds exhibited potent antifungal activities at concentration less than 50 ppm. For the compounds **5d**, **6c** and **7b**, EC_{50} values against *Botrytis cinerea* were as low as 0.141, 0.082 and 0.091 μM, respectively, which represents better antifungal activity than that of the commonly used fungicide Azoxystrobin. Compounds **5d** (57%) and **6c** (55%) also exhibited more effective control than Azoxystrobin (44%) against *Colletotrichum capsica*.

KEY WORDS: coumarin derivatives; microwave-assisted; synthesis; antifungal activity

1. Introduction

Coumarin, also known as benzopyran-2-one, is a fragrant organic compound which is found naturally in some plants. These include the Tonka bean, which is known by the French as coumarou, and is one of the sources from which coumarin was first isolated as a natural product by

A. Vogel in 1820[1, 2]. Since coumarin was first synthesized by the English chemist William Henry Perkin in 1868[3], the application of coumarin derivatives as bioactive molecules against different kinds of diseases has gained great interest from medicinal chemists. Coumarin derivatives demonstrate a wide spectrum of biological activities such as anticancer, anticoagulant, anti-inflammatory, and are usually associated with low toxicity[4-9]. The diversity of bioactivities among coumarin compounds is so huge that coumarin is described as one of the "privileged scaffolds", a term first introduced by Evans to define structures that can bind more than one receptor[10].

As the structural core, coumarin is used regularly as a scaffold in medicinal and agricultural chemistry. This is highlighted by **Warfarin** and **Acenocoumarol** (shown in **Figure 1**), which are anticoagulant agents that function as vitamin K antagonists, and are normally used in the prevention of thrombosis and thromboembolism[11, 12]. Owing to the vast number of coumarin-containing molecules in the literature, this paper is primarily focused on antifungal agents, and serves as a mini-review of currently published coumarin antifungal agents, as limited reports have been published in this area.

As shown in **Figure 1, Osthole** is a natural *O*-methylated coumarin found in many plants and has a broad scope of antifungal activities against *Rhizoctonia solani* and other phytopathogenic fungi[13]. It has also been widely used as a fungicide in China for a long history[14]. The recently reported compound **Cou-NO_2** (7-hydroxy-6-nitro-2*H*-1-benzopyran-2-one), a biosynthetic coumarin derivative, has been reported for its antifungal activity, and exhibits bioactivity against *Aspergillus* mycelial growth and spore germination[15]. **Hemiarin** (7-methoxycoumarin, or ayapanin) and **Scopoletin** are inducible antifungal compounds, and can be considered as defense tools for plants against pathogenic fungi. **Xanthotoxin**, a common methoxylated furanocoumarin, is reported to exhibit activity in darkness against *Candida albicans* and *Cryptococcus laurentii*[16]. **Mammeisin**, a coumarin derivative isolated from *Kielmeyera elata*, is reported to present a minimum inhibitory concentration (MIC) value similar to ketoconazole and display a better result than fluconazole against *Candida tropicalis*, which is the most common cause of human fungal infections worldwide[17]. **Coumoxystrobin** (shown in **Figure 1**) is a coumarin derivative containing (*E*)-methyl 3-methoxy-2-phenylacrylate with a broad spectrum of antifungal

activity[18]. **Excavarin-A**, a new γ-lactone coumarin, shows antifungal activity against fifteen fungal strains that are pathogenic against plants and humans. It is also stronger than a standard antimicrobial nystatin, against some clinically important pathogens, such as *A. fumigatus*, *C. tropicalis* and *M. circinelloides*. Additionally, it is more potent than the standard fungicide bavistin against some plant pathogenic fungi, such as *R. solani* and *F. oxysporum*[19].

The presence of the coumarin moiety is known to confer resistance in plant tissues against microbial attack. Since the antifungal activity of coumarin and its derivatives are mainly determined by the functional groups and positions of substituents on the benzopyran ring, various structural modifications to coumarin and their biological activities have been reported. For example, alkylcoumarins (3-, 4- and 6-alkyl) are reported to display antifungal activities[20, 21], and the derivatives of 4-methylcoumarin (Compound **A** in **Scheme 1**) especially showed good antifungal activities against different kinds of plant pathogens. 7-hydroxycoumarin (Umbelliferone) and its derivatives (Compound **B** in **Scheme 1**) have also been reported to possess antifungal activities against spore germination and hyphal growth of many fungi[22]. 4-Phenoxymethylcoumarins and their in vitro growth-inhibiting activities against various plant pathogenic fungi have been presented in a series of studies on coumarin based pesticides, and 4-(4-tert-butylphenoxy)methyl analogues (Compound **C** in **Scheme 1**) revealed the highest toxicity towards a majority of the tested fungi[23].

Only Bharat B. Gupta and coworkers have previously described the synthesis and antifungal activity of some fused coumarin compounds, such as 2-ethyl-7,8-dihydro-3-hydroxynaphtho[l,2-*c*]chromen-6-one (Compound **D** in **Scheme 1**), which possessed effective control with EC_{50} values ranging from 0.2 to 2.5 μg/mL against five tested phytopathogenic fungi in culture[24]. Other than this, no further structural modification and antifungal activity of fused coumarin analogues have been reported so far. The demand to improve antifungal activity necessity the discovery of novel fused coumarin analogues. Herein we report our work focused on a series of fused coumarin analogues: pyrano[3,2-*c*]chromene-2,5-diones.

2. Materials and methods

2.1 Chemicals and Instruments

All chemicals including 2'-Hydroxyacetophenone (**3a)** were purchased from commercial sources (e.g., Adamas, Crystal Chemicals) and used without further purification unless otherwise stated. The melting points of the ester derivatives of coumarin derivatives were determined on an X-4 apparatus (uncorrected), which was purchased from Shanghai Tech. ^{1}H NMR and ^{13}C NMR spectra were obtained using a Bruker Avance 400 MHz spectrometer in $CDCl_3$ solution with TMS as an internal standard. HR-MS (ESI) spectra were carried out with a Thermo Exactive spectrometer, and X-rays were measured at 296 K on a Bruker SMART APEX2 CCD area detector diffractometer. Most reaction yields were not optimized.

General procedure for the preparation of compounds 2a, 3b-d (Scheme 2)

2,4-dihydroxyacetophenone **2a** was synthesized through the reported method[25].

To a solution of 50 mL acetone in 250 mL one-neck flask, was added KI (0.1g) and K_2CO_3 (0.1g), followed by 13 mmol of 2,4-dihydroxyacetophenone **2a**. Once **2a** was completely dissolved, 13 mmol of methyl iodide (or allyl bromide, 1-chlorobutane) was added to the reaction solution to afford **3b**, **3c**, **3d** respectively, and the mixture was refluxed with stirring for 3 hours. After the mixture cooled to room temperature, the precipitate was filtered off and washed with 5 mL of acetone. The combined acetone solutions were concentrated in vacuum to obtain a residue, and the resulting residue was purified by column chromatography to give a white solid below 20□.Yields for **3b**, **3c**, **3d** are 85%, 75%, 82%, respectively.

General procedure for the preparation of compounds 4b-d (Scheme 2)

To a solution of anhydrous toluene (80 mL) in a 250 mL two-neck flask under N_2, was added 1.56g of NaH (26 mmol, 60%), followed by 13 mmol of 2,4-dihydroxyacetophenone **3a**. After stirring for 10 mins, 6 mL of diethyl carbonate in anhydrous toluene (20 mL) was added dropwise during 30 mins, and the mixture was refluxed with stirring for 3 hours. The reaction mixture was cooled to room temperature, and then poured into water, then extracted with diethyl ether for three times until the ether layer was colorless. The aqueous layer was acidified with 100 mL of 2 N hydrochloric acid, and the resulting precipitate was filtered off and recrystallized from ethanol to give a white solid. Yields for **4b**, **4c**, and **4d** were 85%, 86%, and 82%, respectively.

Microwave-assisted synthetic procedure for the preparation of compounds 5a-8d (Scheme 2)

In our initial study, the reaction of 4-hydroxycoumarin (**4a**) with ethyl 2-methylacetoacetate in the presence of toluene was chosen as a model to optimize the reaction conditions, and the experimental results were summarized in **Table 1**.

Compounds Data

4-Methyl-pyrano[3,2-*c*]chromene-2,5-dione (5a): a white solid; mp: 257.6-262.5□; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (s, 1H), 7.71 (s, 1H), 7.44 (s, 2H), 6.27 (s, 1H), 2.69 (s, 3H); ^{13}C NMR (101 MHz, DMSO) δ 135.09, 125.63, 123.99, 117.14, 114.29, 40.51, 40.40, 40.19, 39.91, 39.78, 39.59, 39.46, 22.31; IR (KBr) ν (cm^{-1}) 1711, 1623, 1446, 1272; HR-MS (ESI): m/z calcd for $C_{13}H_9O_4$ ($[M + H]^+$) 229.05009, found 229.04944.

3-Fluoro-4-methyl-pyrano[3,2-*c*]chromene-2,5-dione (5b): a white solid; mp: 247.3 -247.5□;□^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 (s, 1H), 7.69 (s, 1H), 7.41 (s, 2H), 2.64 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.03, 157.69, 152.61, 145.45, 142.95, 134.91, 134.34, 125.30, 123.69, 116.96, 112.73, 103.25, 12.84; IR (KBr) ν (cm^{-1}) 1695, 1616, 1446, 1268; HR-MS (ESI): m/z calcd for $C_{13}H_9FO_4$ ($[M + H]^+$) 247.04066, found 247.03992.

3,4-Dimethyl-pyrano[3,2-c]chromene-2,5-dione (5c): a white solid; mp: 223.5-223.5□; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (s, 1H), 7.69 (s, 1H), 7.41 (s, 2H), 2.69 (s, 3H), 2.26 (s,3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.52, 159.36, 158.65, 152.73, 149.46, 133.86, 124.90, 123.64, 122.41, 116.67, 113.20, 104.18, 18.18, 13.23; IR (KBr) ν (cm^{-1}) 1727, 1660, 1446, 1191; HR-MS (ESI): m/z calcd for $C_{14}H_{10}O_4$ ($[M + H]^+$) 243.06573, found 243.06502.

3-Ethyl-4-methyl-pyrano[3,2-c]chromene-2,5-dione (5d): a white solid; mp: 143.4-144.3□; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (d, *J* = 7.9 Hz, 1H), 7.69 (t, *J* = 7.8 Hz, 1H), 7.42 (dd, *J* = 13.8, 8.0 Hz, 2H), 2.73 (dd, *J* = 15.2, 7.7 Hz, 2H), 2.70 (s, 3H), 1.21 (q, *J* = 7.7 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 159.54, 159.13, 158.71, 152.75, 149.08, 133.86, 128.15, 124.88, 123.67, 116.66 , 113.24, 104.31, 20.76, 17.67, 12.52; IR (KBr) ν (cm^{-1}) 1715, 1620, 1537, 1449; HR-MS (ESI): m/z calcd for $C_{15}H_{12}O_4$ ($[M + H]^+$) 257.08139, found 257.08069.

4-Trifluoromethyl-pyrano[3,2-c]chromene-2,5-dione (5e): a white solid; mp: 200.5-203.0□; ^{1}H NMR (400 MHz, CDCl3) δ 8.13 (dd, *J* = 8.0, 1.4 Hz, 1H), 7.80-7.72 (m, 1H), 7.49-7.41 (m, 2H),

6.88 (d, *J* = 0.7 Hz, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.80, 156.30, 154.66, 153.70, 142.35, 141.93, 135.75, 125.38, 124.15, 117.16, 115.97 (q, *J* = 7.3 Hz, 1C), 112.37, 99.63; IR (KBr) ν (cm^{-1}) 1724, 1607, 1530, 1458, 1389, 1099, 833; HR-MS (ESI): m/z calcd for $C_{13}H_5F_3O_4$ ([M + H]$^+$) 283.02182, found 283.02127.

8-Methoxy-4-methyl-pyrano[3,2-c]chromene-2,5-dione (6a): a white solid; mp: 242.0-242.1□; ^{1}H NMR (400 MHz,$CDCl_3$)δ 8.02(s, 1H),6.99(s, 1H),6.87(s, 1H),6.17(s, 1H),3.97(s, 3H),2.66(s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.13, 162.71, 158.69, 158.18, 156.45, 155.36, 125.40, 113.70, 112.72, 106.30, 101.51, 100.55, 56.04, 22.67; IR (KBr) ν (cm^{-1}) 1709, 1611, 1541, 1281; HR-MS (ESI): m/z calcd for $C_{14}H_{10}O_5$ ([M + H]$^+$) 259.06065, found 259.05988.

8-Methoxy-3,4-dimethyl-pyrano[3,2-c]chromene-2,5-dione (6b): a white solid; mp: 212.3-212.9□; ^{1}H NMR(400 MHz,$CDCl_3$) δ 8.01(s, 1H), 6.97 (s, 1H), 6.85 (s, 1H), 3.95 (s, 3H), 2.67 (s, 3H), 2.23 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.54, 159.80, 159.70, 159.01, 154.74, 149.83, 124.85, 120.69, 113.44, 106.33, 101.83, 100.34, 55.98, 18.14, 13.06; IR (KBr) ν (cm^{-1}) 1702, 1609, 1550, 1275; HR-MS (ESI): m/z calcd for $C_{15}H_{12}O_5$ ([M + H]$^+$) 273.07630, found 273.07553.

3-Ethyl-8-methoxy-4-methyl-pyrano[3,2-c]chromene-2,5-dione (6c): a white solid; mp: 143.1-143.3□; ^{1}H NMR (400 MHz,$CDCl_3$) δ 8.01 (dd, *J*= 18.7,8.9 Hz,1H), 7.00-6.94 (m,1H), 6.84 (dd, *J*= 12.5, 2.2 Hz, 1H), 3.95 (s,3H), 2.71 (dd, *J*= 14.6,7.1 Hz, 2H), 2.68 (s, 3H), 1.18 (t, *J*= 7.5 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.56, 160.10, 159.36, 159.12, 154.80, 149.47, 126.48, 124.92, 113.40, 106.41, 101.97, 100.35, 55.97, 20.63, 17.64, 12.60; IR (KBr) ν (cm^{-1}) 1715, 1607, 1397, 1275, 1022; HR-MS (ESI): m/z calcd for $C_{16}H_{13}O_5$ ([M + H]$^+$) 287.09195, found 287.09115.

8-Methoxy-4-trifluoromethyl-pyrano[3,2-c]chromene-2,5-dione (6d): a white solid; mp: 225.6-225.8□; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (dd, *J* = 21.3, 8.4 Hz, 1H), 7.09-6.94 (m, 1H), 6.94-6.74 (m, 2H), 3.98 (d, *J* = 8.6 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.08, 164.17, 156.66, 156.05, 155.09,143.37, 142.29, 125.49, 115.09, 114.38-113.99 (m, 1C), 105.54, 100.57, 97.02, 56.21, IR (KBr) ν (cm^{-1}) 1765, 1731, 1609, 1376, 1150; HR-MS (ESI): m/z calcd for $C_{14}H_7F_3O_5$ ([M + H]$^+$) 313.03238, found 313.03183.

8-Allyloxy-4-methyl-pyrano[3,2-c]chromene-2,5-dione (7a): a white solid; mp: 178.1-178.3□; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (s, 1H), 7.01 (s, 1H), 6.88 (s, 1H), 6.17 (s, 1H), 6.09 (s, 1H), 5.46 (s, 2H), 4.70 (s, 2H), 2.66 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.02, 162.62, 158.63,

158.12, 156.41, 155.21, 131.70, 125.36, 118.89, 114.14, 112.72, 106.34, 101.51, 101.35, 69.53, 22.65; IR (KBr) ν (cm^{-1}) 1711, 1609, 1544, 1489, 1279, 1089; HR-MS (ESI): m/z calcd for $C_{16}H_{11}O_5$ ($[M + H]^+$) 285.07630, found 285.07554.

8-Allyloxy-3,4-dimethyl-pyrano[3,2-c]chromene-2,5-dione (7b): a white solid; mp: 192.7-193.3□; ^{1}H NMR (400 MHz, DMSO) δ 7.79 (s, 1H), 7.15 (s, 1H), 6.32 (s, 1H), 6.10 (s, 1H), 5.39 (d, *J* = 40.7 Hz, 2H), 4.78 (s, 2H), 3.33 (s, 3H), 2.21 (s, 3H); ^{13}C NMR (101 MHz, CDCl3) δ 163.44, 159.77, 159.02, 154.63, 149.81, 131.80, 124.86, 120.72, 118.80, 113.92, 106.41, 101.86, 101.17, 69.45, 18.15, 13.07. IR (KBr) ν (cm^{-1}) 1718, 1605, 1541, 1489, 1291, 1105; HR-MS (ESI): m/z calcd for $C_{17}H_{14}O_5$ ($[M + H]^+$) 299.09195, found 299.09109.

8-Allyloxy-3-ethyl-4-methyl-pyrano[3,2-c]chromene-2,5-dione (7c): a white solid; mp: 143.1-143.3□; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (d, *J* = 8.9 Hz, 1H), 7.00-6.88 (m, 1H), 6.82 (d, *J* = 2.3 Hz, 1H), 6.05 (ddd, *J* = 22.5, 10.6, 5.3 Hz, 1H), 5.41 (ddd, *J* = 13.9, 11.6, 1.1 Hz, 2H), 4.63 (d, *J* = 5.3 Hz, 2H), 2.67 (dd, *J* = 14.9, 7.4 Hz, 2H), 2.63 (s, 3H), 1.14 (t, *J* = 7.5 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.46, 160.04, 159.31, 159.09, 154.67, 149.43, 131.81, 126.52, 124.90, 118.79, 113.92, 106.47, 101.98, 101.17, 69.46, 20.63, 17.63, 12.59; IR (KBr) ν (cm^{-1}) 1704, 1616, 1544, 1503, 1277, 1205; HR-MS (ESI): m/z calcd for $C_{18}H_{16}O_5$ ($[M + H]^+$) 313.10760, found 313.10669.

8-Butoxy-4-methyl-pyrano[3,2-c]chromene-2,5-dione (8a): a white solid; mp: 183.0-183.9□，^{1}HNMR (400 MHz, $CDCl_3$) δ 8.03 (d, *J* = 8.9 Hz, 1H), 6.99 (dd, *J* = 8.9, 2.2 Hz, 1H), 6.85 (d, *J* = 2.2 Hz, 1H), 6.17 (d, *J* = 1.0 Hz, 1H), 4.11 (t, *J* = 6.5 Hz, 2H), 2.66 (d, *J* = 0.9 Hz, 3H), 1.91 – 1.79 (m, 2H), 1.55 (dt, *J* = 14.7, 7.4 Hz, 2H), 1.04 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.74, 162.73, 158.73, 158.21, 156.48, 155.35, 125.30, 114.07, 112.59, 106.02, 101.36, 100.93, 68.73, 30.90, 22.67, 19.14, 13.77; IR (KBr) ν (cm^{-1}) HR-MS (ESI): m/z calcd for $C_{17}H_{16}O_5$ ($[M + H]^+$) 301.10760, found 301.10675.

8-Butoxy-3,4-dimethyl-pyrano[3,2-c]chromene-2,5-dione (8b): a white solid; mp: 172.5-172.7℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97 (d, *J* = 8.9 Hz, 1H), 6.93 (dd, *J* = 8.9, 2.3 Hz, 1H), 6.79 (d, *J* = 2.2 Hz, 1H), 4.05 (t, *J* = 6.5 Hz, 2H), 2.63 (s, 3H), 2.19 (s, 3H), 1.88 – 1.73 (m, 2H), 1.52 (dq, *J* = 14.7, 7.4 Hz, 2H), 1.00 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.17, 159.98, 159.77, 159.13, 154.78, 149.89, 124.80, 120.56, 113.84, 106.11, 101.73, 100.76, 68.62, 30.92, 19.15, 18.15, 13.77, 13.05; HR-MS (ESI): m/z calcd for $C_{18}H_{18}O_5$ ($[M + H]^+$) 315.12325, found 315.12239.

8-Butoxy-3-ethyl-4-methyl-pyrano[3,2-c]chromene-2,5-dione (8c): a white solid; mp: 144,3 -144.7□; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97 (dd, *J* = 8.9, 1.3 Hz, 1H), 6.92 (dd, *J* = 8.9, 2.1 Hz, 1H), 6.79 (s, 1H), 4.05 (t, *J* = 6.4 Hz, 2H), 2.67 (dd, *J* = 13.2, 5.7 Hz, 2H), 2.63 (s, 3H), 1.88 – 1.76 (m, 2H), 1.51 (dt, *J* = 14.9, 7.4 Hz, 2H), 1.26 (s, 1H), 1.14 (t, *J* = 7.5 Hz, 3H), 1.00 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.17, 160.15, 159.38, 159.18, 154.80, 149.51, 126.35, 124.83, 113.83, 106.15, 101.83, 100.74, 68.61, 30.93, 20.61, 19.15, 17.64, 13.77, 12.60; HR-MS (ESI): m/z calcd for $C_{19}H_{20}O_5$ ($[M + H]^+$)329.13890, found 329.13809.

7,8,9,10-Tetrahydro-5,12-dioxa-chrysene-6,11-dione (9a): a white solid; mp: 211.2-212.3□; ^{1}H NMR (400MHz,$CDCl_3$) δ 8.09 (s,1H), 7.65 (s,1H), 7.37 (s,2H), 3.14 (s,2H), 2.59 (s,2H), 1.80 (s,4H); ^{13}C NMR (101 MHz, CDCl3) δ 159.29, 159.17, 158.40, 152.73, 150.76, 133.77, 124.87, 123.79, 123.56, 116.67, 113.27, 103.83, 28.55, 24.50, 21.53, 20.84; IR(KBr) ν (cm^{-1}) 1720, 1620, 1605, 1541, 1189; HR-MS (ESI): m/z calcd for $C_{16}H_{12}O_4$ ($[M + H]^+$) 269.08139, found 269.08072.

2-Methoxy-7,8,9,10-tetrahydro-5,12-dioxa-chrysene-6,11-dione (9b): a white solid, mp: 238.2-240.8□; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (s, 1H), 6.95 (s, 1H), 6.82 (s, 1H), 3.91 (s, 3H), 3.12 (s, 2H), 2.56 (s, 2H), 1.78 (s, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.48, 159.88, 159.42, 158.81, 154.77, 151.13, 124.81, 122.11, 113.41, 106.44, 101.49, 100.39, 55.96, 28.55, 24.37, 21.57, 20.93; IR (KBr) ν (cm^{-1}) 1702, 1605, 1528, 1275; HR-MS (ESI): m/z calcd for $C_{17}H_{14}O_5$ ($[M + H]^+$) 299.09195, found 299.09109.

2-Allyloxy-7,8,9,10-tetrahydro-5,12-dioxa-chrysene-6,11-dione (9c): a white solid, mp: 206.9 -207.3□; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (d, *J* = 8.9 Hz, 1H), 7.07-6.93 (m, 1H), 6.86 (d, *J* = 2.2 Hz, 1H), 6.09 (ddd, *J* = 22.4, 10.6, 5.3 Hz, 1H), 5.45 (dd, *J* = 36.7, 13.9 Hz, 2H), 4.68 (d, *J* = 5.3 Hz, 2H), 3.16 (s, 2H), 2.60 (s, 2H), 1.87 – 1.71 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.40, 159.85, 159.40, 158.81, 154.67, 151.11, 131.83, 124.82, 122.15, 118.78, 113.89, 106.53, 101.53, 101.23, 69.45, 28.54, 24.37, 21.57, 20.92; IR(KBr)ν (cm^{-1}) 1702, 1611, 1550, 1277, 1110; HR-MS (ESI): m/z calcd for $C_{19}H_{16}O_5$ ($[M + H]^+$) 325.10760, found 325.10675.

2-Butoxy-7,8,9,10-tetrahydro-5,12-dioxa-chrysene-6,11-dione (9d): a white solid; mp: 146.7-147.1℃; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97 (d, *J* = 8.9 Hz, 1H), 6.92 (dd, *J* = 8.9, 2.3 Hz, 1H), 6.79 (d, *J* = 2.2 Hz, 1H), 4.05 (t, *J* = 6.5 Hz, 2H), 3.11 (s, 2H), 2.56 (s, 2H), 1.89-1.69 (m, 6H), 1.60-1.42 (m, 2H), 1.00 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 162.71, 159.17, 158.84, 158.19, 155.43, 141.00, 130.49, 122.90, 113.44, 109.11, 105.24, 101.85, 68.45, 61.27, 30.98 ,

29.70, 19.17, 14.24, 13.78, 10.17; HR-MS (ESI): m/z calcd for $C_{20}H_{20}O_5$ ($[M + H]^+$) 341.13890, found 341.13835.

2.2. X-ray Diffraction Analysis

White crystals of compound 5b (0.130 × 0.130 × 0.050 mm^3) were mounted on a quartz fiber with protection oil. Cell dimensions and intensities were measured at 296 K on a Bruker SMART APEX2 CCD area detector diffractometer with graphite mono-chromated MoKα radiation (λ= 0.71073 Å); θ_{max} = 25.010; 1578 independent reflections (R_{int} = 0.0334). The structure was solved by direct methods using SHELXS-97; all other calculations were performed with Bruker SAINT System and Bruker SMART programs. Full-matrix least-squares refinement based on F^2 using the weight of ω= 1/[σ2(Fo2) + (0.0659P)2 + 0.0500P] gave final values of R = 0.0334, ωR = 0.1054, in the title compound, the moiety formula is $C_{13}H_7FO_4$.

White crystals of compound 6b (0.520 × 0.480 × 0.210 mm^3) were mounted on a quartz fiber with protection oil. Cell dimensions and intensities were measured at 296 K on a Bruker SMART APEX2 CCD area detector diffractometer with graphite monochromated Mo Kα radiation (λ= 0.71073 Å); θ_{max} = 27.559;1838 independent reflections (R_{int} = 0.0487). Data were corrected for Lorentz and polarization effects and for absorption (T_{min} = 0.942; T_{max} = 0.975). The structure was solved by direct methods using SHELXS-97; all other calculations were performed with Bruker SAINT System and Bruker SMART programs. Full-matrix least-squares refinement based on F^2 using the weight of ω= 1/[σ2(Fo2) + (0.0633P)2 + 0.4557P] gave final values of R = 0.0868, ωR = 0.1424, max/min residual electron density = 0.205 and -0.171 e.$Å^{-3}$. Hydrogen atoms were observed and refined with a fixed value of their isotropic displacement parameter. In the title compound, the moiety formula is $C_{15}H_{12}O_5$.

The crystallographic data have been deposited with the Cambridge Crystallographic Data Centre (CCDC) as supplementary publication number: CCDC 1047356 (5b), CCDC 1419461 (6b). Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB12 1EZ, UK (Fax +44 1223 336033 or E-mail: deposit@ccdc.cam.ac.uk).

2.3. Biological Assays

The antifungal activities of all the synthesized compounds were carried out at a concentration of 50 ppm using a mycelia growth inhibitory rate method, including Azoxystrobin used as a positive control. The tested fungi were provided by the Laboratory of Plant Disease Control, Nanjing Agricultural University, the fungal strains and experimental methods used in the biological assays are the same as those reported in the literature[26]. After retrieval from the storage tube, the strains were incubated in PDA at 25 □ for a week to get new mycelia for the antifungal assay. All the tested compounds were first dissolved in 5 mL N,N-dimethylformamide to generate a 100 ppm stock solution. The needed test solutions were prepared by diluting the above solution. The antifungal results of all the compounds against *Botrytis cinerea*, *Collecterichu mcapsica*, *Alternaria solani*, *Gibberella zeae*, and *Rhizoctorzia solani* are listed in **Table 3**.

3. Results and discussion

3.1 Synthetic Chemistry

Most synthetic procedures are costly, time consuming and not environmentally friendly, usually giving relatively poor yields. The application of microwave irradiation in synthetic chemistry has become increasingly popular in modern combinatorial and medicinal chemistry. Compared with traditional heating for organic reactions, the microwave irradiation used in this paper is an very efficient and green synthetic approach which dramatically reduces reaction time (reduced from days and hours to minutes and seconds) and improves the reaction yields[27]. As shown in **Table 1**, compound **5c** could be prepared in a moderate yield with toluene as the best solvent (Entry 3, 5, 6), using 640W microwave irradiation (Entry 6, 7, 8), and DMAP as the catalyst (Entry 4, 6). The yields increased consistently with time, from 34% (10 min) to a maximum of 69% (15min) under these conditions, however, when extending the reaction time longer than 20 min, the yield did not increase significantly (shown in Entry 5, 6 and 9). Therefore, we can conclude that the optimal conditions include using toluene as the solvent in the presence of DMAP under microwave irradiation (640 W) for 15 mins. This optimized method was used to synthesize the designed coumarin derivatives. Furthermore, compared with conventional heating (Entry 13-14), the reaction times for the target compounds can be reduced from about 6 hours to 15 mins at minimum (Entry 6), and the isolated yields can be improved from 30% to 76% at maximum (Compound **7a**).

With this efficient method, a focused library of 19 compounds including 3-(trifluoro)methyl (**5a**-**5e**), 8-alkoxy (**6a**-**6d**, **8a**-**8c**), 8-allyloxy (**7a**-**7c**) pyrano[3,2-*c*]chromene-2,5-diones and 3,4-cyclohexane pyrano[3,2-*c*]chromene-2,5-diones (**9a**-**9d**) have been designed and synthesized. All of the target molecules listed in **Table 2** were characterized on the basis of physical and spectral data. In addition, compounds **5b** and **6b** were further confirmed by X-ray diffraction crystallography (**Figure 2**).

3.2 Antifungal Activity and the Structure-Activity Relationships (SAR)

The results of the biological testing against seven phytopathogenic fungi are given in **Table 3**. For the purposes of analysis of structure-activity relationships, the antifungal activities of all target compounds were compared to the positive control Azoxystrobin, a marketed broad spectrum fungicide developed by Syngenta.

Data presented in **Table 3** reveal that the synthesized coumarin derivatives exhibit differential activities against five plant pathogenic fungi at 50 ppm. All of the compounds, excluding **6a** and **8a**, showed better antifungal activity against *Botrytis cinerea* than Azoxystrobin (34%). Compounds **5d** (64%), **5e** (60%), **6b** (60%), **7b** (64%) and **9b** (63%) demonstrate a two-fold increase in potency against Botrytis cinerea compared to Azoxystrobin. Compounds **5d** (57%) and **6c** (55%) exhibited better activity than Azoxystrobin (44%) against *Colletotrichum capsica*. However, these coumarin derivatives showed weak antifungal activity against *Alternaria solani*, though **5d** (22%) and **9a** (19%) showed improved antifungal activity compared to Azoxystrobin (16%). The bioscreening data also showed that all of these target compounds did not show effective control against *Gibberella zeae* and *Rhizoctorzia solani*, though compound **6c** (64%) showed activity equivalent to the positive control Azoxystrobin (69%) against *Rhizoctorzia solani*.

As **5d**, **6c** and **7b** showed effective control against *Botrytis cinerea* and *Colletotrichum capsica*, we further tested the EC_{50} values of these compounds together with the positive control. As shown in **Table 4**, we noticed that the EC_{50} values of compounds **6c** and **7b** were as low as 0.082 and 0.091 μM, respectively, which proves they are much more effective than Azoxystrobin (0.158 μM). Compound **5d** also exhibited much better activity than the control against *Collecterichum capsica*. The EC_{50} value was almost half that of Azoxystrobin, which was as low as

0.115 μM.

Although the antifungal activity of most of the fused coumarin derivatives has been proven to be very poor, making it difficult to extract a clear structure-activity relationship analysis, some broad conclusions can still be drawn. These compounds were noticeably more active against *Botrytis cinerea* and *Colletotrichum capsici*, but lacked potency against *Alternaria solani*, *Alternaria mali* and *Rhizoctonia solani*, as illustrated by the absence of activity against these three kinds of fungi. Compounds **5d**, **5e** and **9a** showed equivalent activity with the positive control Azoxystrobin against *Alternaria mali*. Secondly, the spectrum of antifungal activity is generally improved if R_2 and R_3 are Me, Et or CF_3, highlighted by **5d** and **6c**, both of which displayed a broad spectrum of activity and are particularly effective against *Botrytis cinerea*.

4. Conclusions

In summary, we have efficiently designed and synthesized a novel series of fused coumarin analogues through an optimized microwave-assisted protocol. The reaction times for the target compounds can be reduced from about 6 hours to 15 mins at minimum, and the isolated yields can be improved from 30% to 76% at maximum. Biological testing data showed that some of the fused coumarin analogues exhibited good antifungal activity against *Botrytis cinerea* and *Collecterichum capsica*. Compounds **5d**, **6c** and **7b** were identified as the most active ones, and EC_{50} values shows against *Botrytis cinerea* were as low as 0.141, 0.082 and 0.091 μM, respectively, which improved antifungal activity compared to Azoxystrobin. Compound **5d (**EC_{50} = 0.115 μM) also exhibited much better activity than the control **(**EC_{50} = 0.222 μM) against *Collecterichum capsica*. Compound **5d** was identified as the most active and therefore the most promising candidate for further study. Further structural optimization of fused coumarin analogues is well underway, with the aim of preparing analogues with improved antifungal activity.

Acknowledgement

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Figure 1. Structures of coumarin-containing drugs and antifungal agents

Figure 2. X-ray structures of compounds 5b and 6b

Scheme 1. The design of coumarin-containing antifungal compounds

Scheme 2. Synthetic route for target compounds **5a-9d**

Table 1. Reaction Condition Optimization for compound **5c**

Table 2. The structures and yields of compounds **5a-9d**

Table 3. Antifungal activity of coumarin analogues (inhibitory rate, %)

Table 4. EC_{50} determination of compounds **5d**, **6c** and **7b**

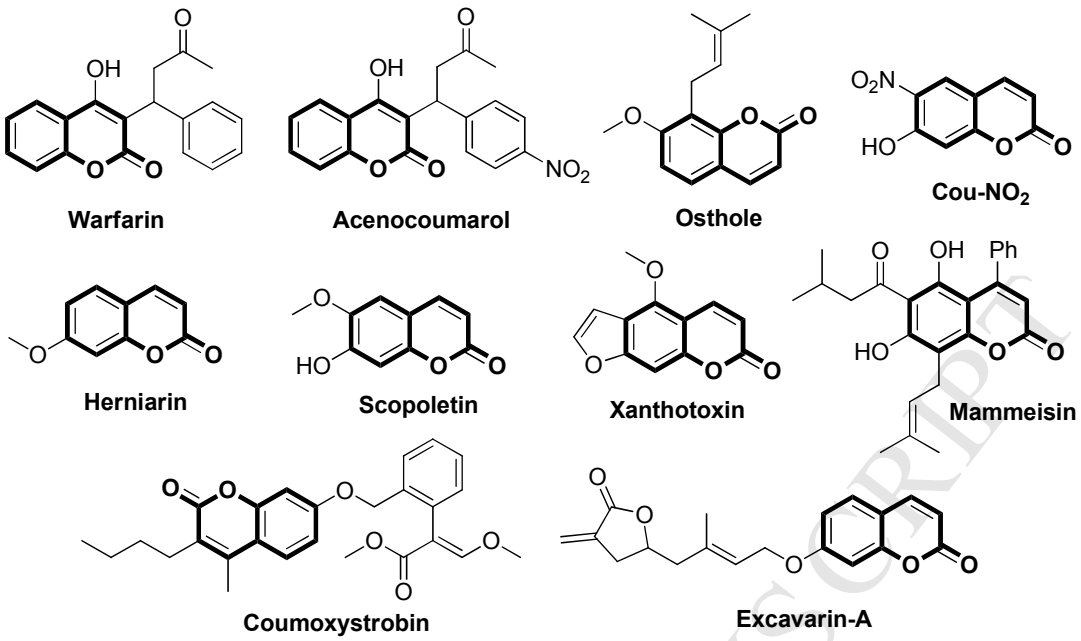
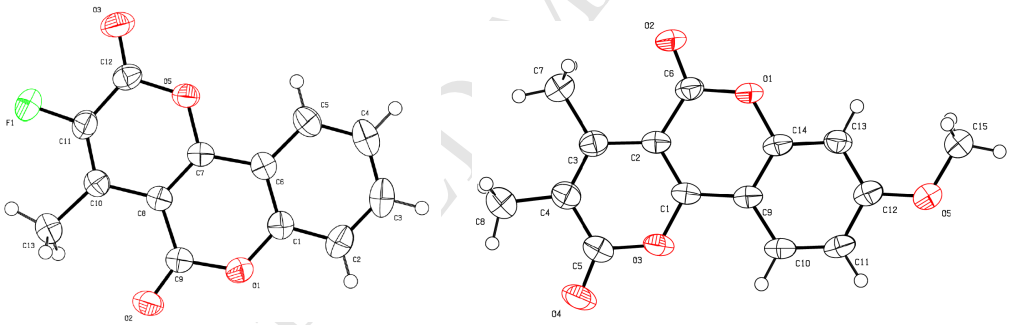
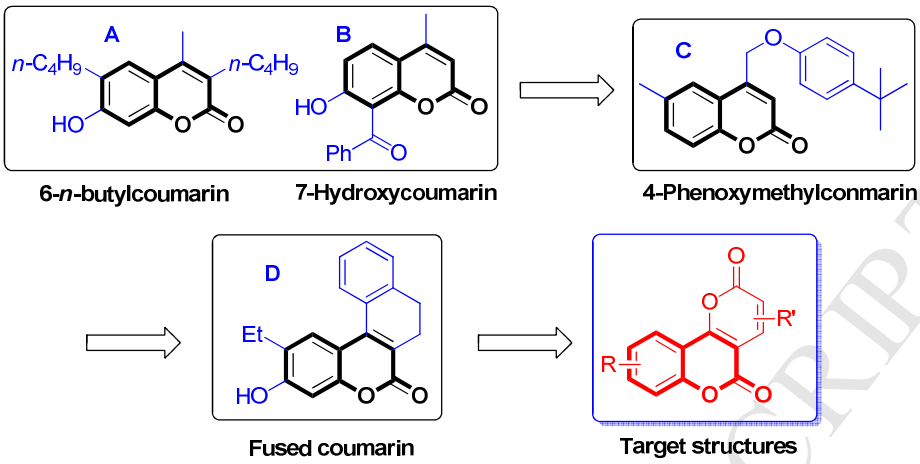


Figure 1. Structures of coumarin-containing drugs and antifungal agents

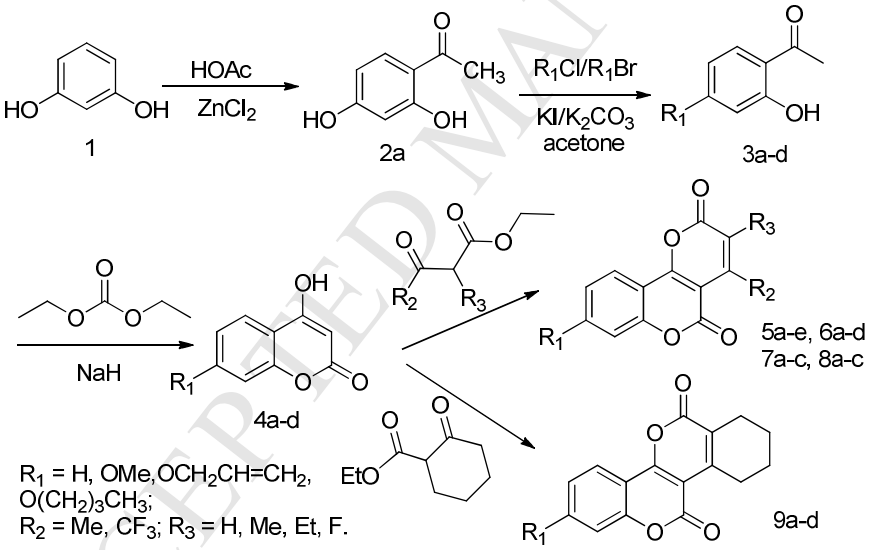


5b **6b**

Figure 2. X-ray structures of compounds 5b and 6b

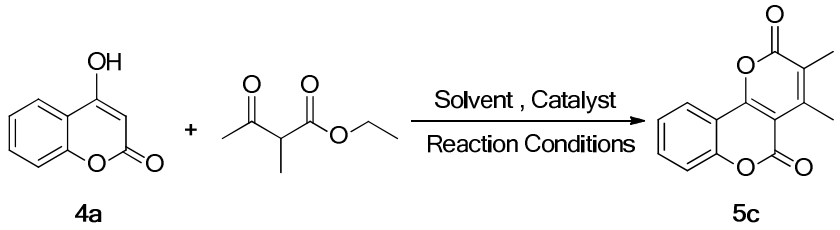


Scheme 1. The design of coumarin-containing antifungal compounds



Scheme 2. Synthetic route for target compounds **5a-9d**

Table 1. Reaction Condition Optimization for compound **5c**



4a **5c**

Entry	Solvent	Catalyst	MW	Temperature	Time	Yield [a]
1	DMF	NH_4OAc	640 W	r.t.	15 min	45%
2	DMF	NH_4OAc	640 W	r.t	20 min	40%
3	DMF	DMAP	640 W	r.t.	15 min	35%
4	Toluene	NH_4OAc	640 W	r.t	15 min	45%
5	Toluene	DMAP	640 W	r.t.	10 min	34%
6	**Toluene**	**DMAP**	**640 W**	**r.t.**	**15 min**	**69%**
7	Toluene	DMAP	560 W	r.t.	15 min	55%
8	Toluene	DMAP	720 W	r.t.	15 min	20%
9	Toluene	DMAP	640 W	r.t.	20 min	65%
10	Ethanol	NH_4OAc	640 W	r.t.	20 min	48%
11	Ethanol	DMAP	640 W	r.t.	20 min	54%
12	DMF	NH_4OAc	/ [b]	Reflux	6h	30%
13	DMF	DMAP	/ [b]	Reflux	6h	40%
14	Toluene	DMAP	/ [b]	Reflux	6h	45%

[a] Yields after recrystallization from ethanol. [b] Conventional heating, no microwave irradiation.

Table 2. The structures and yields of compounds **5a-9d**

Compd.	R_1	R_2	R_3	Yield (%) [a]	Compd.	R_1	R_2	R_3	Yield (%) [a]
5a	H	H	CH_3	72	7b	$OCH_2CH=CH_2$	CH_3	CH_3	66
5b	H	F	CH_3	13	7c	$OCH_2CH=CH_2$	Et	CH_3	69
5c	H	CH_3	CH_3	68	8a	$O(CH_2)_3CH_3$	H	CH_3	70
5d	H	Et	CH_3	64	8b	$O(CH_2)_3CH_3$	CH_3	CH_3	62
5e	H	H	CF_3	20	8c	$O(CH_2)_3CH_3$	Et	CH_3	64
6a	OCH_3	H	CH_3	75	9a	H	$(CH_2)_4$		74
6b	OCH_3	CH_3	CH_3	71	9b	OCH_3	$(CH_2)_4$		65
6c	OCH_3	Et	CH_3	69	9c	$OCH_2CH=CH_2$	$(CH_2)_4$		67
6d	OCH_3	H	CF_3	31	9d	$O(CH_2)_3CH_3$	$(CH_2)_4$		75
7a	$OCH_2CH=CH_2$	H	CH_3	76	[a] *Yields after recrystallization or column chromatography*				

Table 3. Antifungal activity of coumarin analogues (inhibitory rate, %)

Species [a]	BOT	COL	ALT	GIB	RHI
Compound Rate(ppm)	50	50	50	50	50
5a	47 [b]	21	5	15	21
5b	47	12	4	4	14
5c	47	15	5	3	16
5d	64	57	22	30	30
5e	60	10	16	6	14
6a	31	15	4	6	19
6b	60	15	13	16	53
6c	67	55	13	44	64
6d	50	22	5	38	44
7a	41	15	4	8	26
7b	64	40	4	57	28
7c	54	13	13	4	29
8a	23	36	7	23	26
8b	41	27	5	15	31
8c	36	34	10	18	21
9a	35	14	19	13	41
9b	63	15	11	6	21
9c	54	15	11	6	21
9d	40	21	5	15	21
Azoxystrobin	34	44	16	68	69

[a] BOT, Botrytis cinerea; COL, Collecterichum capsica; ALT, Alternaria solani; GIB, Gibberella zeae; RHI, Rhizoctorzia solani.

[b] All the data was the average of three replications.

Table 4. EC_{50} determination of compounds **5d**, **6c** and **7b**

Compound	Pathogen [a]	Toxic regression	R	EC_{50} (μM) [b]	95% confidence interval
5d	BOT	Y=4.2485+0.4824x	0.9988	0.141	0.131-0.151
6c	BOT	Y=3.8914+0.8086x	0.9959	0.082	0.074-0.091
7b	BOT	Y=3.1704+1.2718x	0.9986	0.091	0.085-0.097
Azoxystrobin	BOT	Y=3.0886+1.0598x	0.9997	0.158	0.145-0.171
5d	COL	Y=4.3614+0.4345x	0.9960	0.115	0.103-0.128
6c	COL	Y=3.4096+1.0175x	0.9995	0.128	0.122-0.136
7b	COL	Y=0.9851+2.2526x	0.9988	0.203	0.184-0.225
Azoxystrobin	COL	Y=3.9631+0.5312x	0.9985	0.222	0.194-0.254

[a] BOT, Botrytis cinerea; COL, Collecterichum capsica. [b] The EC_{50} value was the average of three replications.

Highlights

The microwave-assisted synthesis of novel fused coumarin derivatives was described.

Antifungal activity screening of the designed compounds against five fungi was screened.

Some compounds exhibited potent antifungal activity with **5d** as the most promising candidate for further study.

The structure-activity relationships of the fused coumarin derivatives were summarized.