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DESIGN, SYNTHESIS, AND ANTIFUNGAL ACTIVITY OF ALKYL GALLATES AGAINST PLANT PATHOGENIC FUNGI *IN VITRO* AND *IN VIVO*

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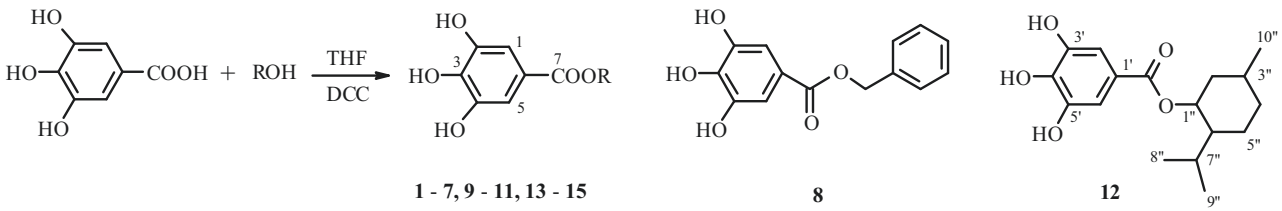
A series of alkyl gallates was synthesized by reacting gallic acid with the corresponding alcohols. Their structures were determined on the basis of spectroscopic data, including NMR and MS. The antifungal activities of these compounds against plant pathogenic fungi in vitro *and* in vivo *were assessed.*

Keywords: gallic acid, alkyl gallates, antifungal activity, inhibitor.

Gallic acid is a polyphenolic compound that is widely distributed across the plant kingdom, such as in leaves of beriberry and roots and bark of pomegranates, gall nuts, hops, and so on [1–3]. Gallic acid and many of its derivatives have a large number of applications in various fields of science and are well known for their natural and strong antioxidative, antimutagenic, anticarcinogenic, antiallergic, anti-inflammatory, antiviral, antibacterial, antifungal, antidiabetic, antityrosinase, and antiarteriosclerosis activities [2–25]. Although gallic acid and its derivatives have many biological activities, it is difficult to utilize these compounds as chemopreventive agents due to the small quantities present in plants. Therefore, interest in the design and synthesis of gallic acid compounds with enhanced chemopreventive action has intensified in recent years. On the other hand, a rare report that focuses on the antifungal activities of gallic acid compounds against plant pathogenic fungi has appeared to date [8, 11, 12]. Based on the above concept, a series of alkyl gallates (Scheme 1) was synthesized and tested for their antifungal activities against several plant pathogenic fungi *in vitro* and *in vivo*. The results showed that this series of compounds is significantly effective against the selected fungi except *Gaeumannomyces graminis*, and, in particular, compounds **9** and **10** exhibited the best activity *in vitro*. By way of contrast, compound **5** was seen to be the most active against *Erysiphe graminis in vivo*.



1 - 7, 9 - 11, 13 - 15 **8** **12**

1: R = CH_3; **2:** R = CH_2CH_3; **3:** R = $CH(CH_3)_2$; **4:** R = $CH_2CH(CH_3)_2$; **5:** R = $CH_2CH_2CH(CH_3)_2$
6: R = $CHCH_3(CH_2)_3CH_3$; **7:** R = $CH_2(CH_2)_5CH_3$; **9:** R = $CH_2(CH_2)_6CH_3$; **10:** R = $CH_2(CH_2)_7CH_3$
11: R = $CH_2(CH_2)_8CH_3$; **13:** R = $CH_2(CH_2)_{10}CH_3$; **14:** R = $CH_2(CH_2)_{14}CH_3$; **15:** R = $CH_2(CH_2)_{16}CH_3$

Scheme 1.

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TABLE1. Fungicidal Activities of Alkaly Gallates (**1–15**) against *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, and *Fusarium graminearum in vitro*

Compound (Number of carbon in R)	Inhibition of mycelial growth, % (± SD)[a]		
	Sclerotinia sclerotiorum	*Rhizoctonia solani*	*Fusarium graminearum*
Gallic acid (0)	10.3 (± 5.4) i	21.1 (± 3.9) h	23.5 (± 5.1) hg
1 (C_1)	20.9 (± 0.5) h	35.9 (± 3.0) efg	28.4 (± 3.5) fg
2 (C_2)	23.1 (± 0.7) gh	41.1 (± 6.0) edf	24.4 (± 0.7) hg
3 (C_3)	4.4 (± 1.6) j	33.4 (± 4.1) gf	6.1 (± 1.9) i
4 (C_4)	28.4 (± 4.6) gf	37.5 (± 3.9) ef	35.0 (± 3.7) de
5 (C_5)	42.3 (± 5.5) e	28.3 (± 0.9) gh	45.8 (± 6.2) c
6 (C_6)	32.9 (± 3.2) f	43.6 (± 5.2) de	36.4 (± 6.9) de
7 (C_7)	61.7 (± 2.9) c	72.2 (± 7.5) bc	54.6 (± 1.4) b
8 (C_7)	21.6 (± 3.2) h	4.2 (± 2.9) i	20.6 (± 0.8) h
9 (C_8)	74.4 (± 1.4) b	81.5 (± 7.9) a	52.1 (± 3.9) b
10 (C_9)	71.1 (± 5.4) b	76.7 (± 4.7) ab	73.7 (± 1.0) a
11 (C_{10})	72.6 (± 3.6) b	68.6 (± 1.2) c	50.2 (± 2.8) b
12 (C_{10})	81.3 (± 2.7) a	65.9 (± 0.8) c	40.7 (± 1.5) dc
13 (C_{12})	55.5 (± 0.6) d	46.7 (± 3.1) d	31.2 (± 2.5) fe
14 (C_{16})	40.5 (± 4.2) e	29.2 (± 2.9) g	8.7 (± 2.3) i
15 (C_{18})	27.1 (± 3.4) gfh	8.6 (± 1.3) i	6.6 (± 1.0) i

[a]The data are expressed as means and the standard errors arise from three independent experiments.

***In vitro* Activity against Phytopathogenic Fungi**. In the survival experiment, the homologous alkyl gallates acquired were tested for their fungicidal activities against *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, and *Fusarium graminearum* using the mycelial growth inhibition test method (Table 1). The results revealed that these phytopathogenic fungi all showed susceptibilities to these compounds; in particular, the fungicidal action of these alkyl gallates was much greater than that of the parent compound gallic acid. In the case against *S. sclerotiorum*, compound **12** was found to be more effective, followed by compounds **9**, **11**, and **10**, with inhibition rates of 81.3, 74.4, 72.6, and 71.1% at 50 μg mL^{-1}, respectively. In the case against *R. solani*, compound **9** proved to be more potent, with the inhibition of mycelial growth as high as 81.5%, followed by compounds **10**, **7**, and **11**, for which the inhibition rates of mycelia were 76.7, 72.2, and 68.6%, respectively. In contrast, the fungicidal activities of compounds **8** (C_7) and **15** (C_{18}) are obviously decreased. This is a so-called cut off phenomenon. In the case against *F. graminearum*, compound **10** (C_9) was seen to display the best fungicidal activity with an inhibition rate of 73.7%. Compounds **7** (C_7), **9** (C_8), and **11** (C_{10}) also exhibited promising inhibitions of mycelial growth of 54.6, 52.1, and 50.2%, but to a lesser extent compared to compound **10** (C_9). However, it is noteworthy that as the length of the alkyl chains increased, the fungicidal activities of the gallates against *S. sclerotiorum*, *R. solani,* and *F. graminearum* were not distinctly reinforced.

In the mycelial growth inhibition test, compounds **7**, **9**, **10**, **11**, **12**, and **13** were further investigated for their antifungal activities against a selected eight phytopathogenic fungi including *Venturia nashicola*, *Fulvia fulva*, *Alternaria alternata*, *Fusarium oxysporium*, *Gaeumannomyces graminis*, *S. sclerotiorum*, *R. solani*, and *F. graminearum*. Compounds **9**, **10**, and **11** were found to exhibit a broad antimicrobial spectrum except *G. graminis*. Phytopathogenic fungi such as *F. graminearum*, *R. solani*, and *S. sclerotiorum* are more susceptible to alkyl gallates, for which the inhibition rates are higher than 50%. Notably, the antifungal activities of the compounds are a parabolic function of their lipophilicity, and for nearly all of them, the C_8 and C_9 compounds displayed maximum activity with alkyl chains lengthening between C_7–C_{12}, whereas the antifungal action of the other compounds of this series of gallates is relatively weak and irrelevant to the alkyl chains, as demonstrated by the inhibition rates in Table 1.

Overall, with respect to the phytopathogenic fungi tested, **9** and **10** were confirmed to be the most active compounds.

In vivo* Activity against *Erysiphe graminis. The activities of this series of alkyl gallates were also tested for their control effects on wheat powdery mildew in the greenhouse. The fungicidal action of these synthesized compounds against *E. graminis* was determined *in vivo*, and the results are presented in Table 2. Among them, compounds **1–5** (C_1–C_5) displayed remarkable inhibition towards *Erysiphe graminis* and, in particular, compound **5** was found to be the most potent, with preventive and curative effects of 83%, 83% (8 days) and 78%, 77% (10 days), whereas compounds **6–14** (C_6–C_{16}) were almost inactive, and only compound **15** exhibited moderate preventive and curative actions.

TABLE 2. *In vivo* Control of *Erysiphe graminis* with Protective and Curative Spray Applications of Alkyl Gallates **1–15** (Dose 500 μg/mL)

Compound (Number of carbon in R)	Protective effects, % (± SD)[a]		Curative effects, % (± SD)[a]	
	8 days	10 days	8 days	10 days
Gallic acid (0)	33 (± 5.4) d	42 (± 2.3) D	49 (± 8.3)b	38 (± 4.9) D
1 (C_1)	54 (± 3.8) c	45 (± 0.9) D	39 (± 7.6)c	50 (± 4.6) C
2 (C_2)	39 (± 5.7) d	33 (± 4.7) E	55 (± 6.1) b	52 (± 3.4) C
3 (C_3)	60 (± 7.9)bc	53 (± 2.1) C	17 (± 5.9) ed	36 (± 1.0) D
4 (C_4)	57 (± 6.5) bc	73 (± 5.7) B	77 (± 8.4) a	66 (± 1.1) B
5 (C_5)	83 (± 3.2) a	83 (± 4.0) A	78 (± 8.5) a	77 (± 4.6) A
6 (C_6)	3 (± 6.0) g	5 (± 2.3) GH	7 (± 0.5)ef	10 (± 6.8) EF
7 (C_7)	22 (± 6.0) e	17 (± 4.1) F	0 f	17 (± 4.1) E
8 (C_7)	15 (± 3.0) e	10 (± 5.9) GF	11 (± 1.9) ed	10 (± 5.6) EF
9 (C_8)	0 f	0 H	0 f	0 G
10 (C_9)	7 (± 3.7) f	7 (± 6.3) GH	17 (± 2.9) ed	7 (± 6.4) FG
11 (C_{10})	8 (± 2.6) f	7 (± 3.2) GH	15 (± 2.2) ed	6 (± 3.4) FG
12 (C_{10})	2 (± 3.0) f	2 (± 2.6)GH	20 (± 6.8)d	4 (± 5.5) FG
13 (C_{12})	5 (± 4.4)f	7 (± 7.3) GH	0 f	7 (± 7.3) FG
14 (C_{16})	7 (± 2.5) f	9 (± 4.5) G	19 (± 4.2) d	9 (± 4.5) EFG
15 (C_{18})	38 (± 5.6) d	39 (± 5.6) DE	11 (± 7.8) ed	39 (± 5.6) D
Triadifeon*	64 (± 4.1) b	76 (± 4.7) AB	82 (± 1.6) a	72 (± 5.3) AB

[a]The data are expressed as means and the standard errors arise from three independent experiments; *dose 250 μg/mL.

This is in contrast to the results obtained *in vitro*, which are probably due to the better water solubility of compounds **1–5** than that of compounds **6–15** along with the increase in the alkyl chain length. The data indicate that the preventive and curative effects of compound **5** at a concentration of 500 μg mL^{-1} are comparable and even superior to Triadimefon, for which the preventive and curative effects are 64% and 82% with 8 days treatment and 76% and 72% after 10 days management at a concentration of 250 μg mL^{-1}. The control plants that were treated with distilled water and acetone for 10 days displayed the serious disease of wheat powdery mildew; the plants that were treated with Triadimefon are slightly infected by wheat powdery mildew, while the plants that were treated with compound **5** are almost uninfected by this pathogen.

A series of novel alkyl gallates was synthesized and evaluated for their antifungal activities against plant pathogenic fungi. *In vitro*, all of the compounds are obviously active against the selected fungi except *G. graminis*.

For the compounds C_7–C_{12}, the antifungal activity is a parabola function of their lipophilicity. In general, among the compounds tested, compounds **9** and **10** exhibited the best activity. *In vivo*, however, compound **5** is the most effective against *E. graminis*, which is even superior to Triadimefon. Overall, the length of the alkyl chain in the alkyl gallates is not a major contributor but is significantly associated with their antifungal activities. The broad antifungal activities of these alkyl gallates may be of great value for designing effective antifungal agents.

EXPERIMENTAL

General. Alkanols were purchased from Shanghai Chemical Factory (China). Gallic acid and dicyclohexylcarbodiimide (DCC) were purchased from Aldrich Chemical Co. (Milwaukee, WI). The organic solvents were purified and dried using appropriate procedures. EI-MS spectra were measured with a VG Autospec3000 mass spectrometer (VG, England). ^1H and ^{13}C NMR spectra were recorded on Bruker AM-400 and DRX-500 spectrometers (Karlsruhe, Germany). Chemical shifts were reported in ppm (δ) relative to the internal standard tetramethylsilane (TMS). Air- and/or moisture-sensitive reactions were carried out under an argon or nitrogen atmosphere. The water solubility of the prepared alkyl gallates was estimated by Discovery Studio 4.0.

Synthesis. To a solution of gallic acid (2.00 mM) and the corresponding alcohol (2.00 mM) in THF (10 mL) cooled at 0°C was added a solution of DCC (4.2 mM) in THF (10 mL). After stirring for 10 h, the solvent of the resulted mixture was removed under reduced pressure. The residue was extracted with ethyl acetate five times and filtered. The filter was washed

successively with 4 M HCl solution, saturated $NaHCO_3$ solution, and water, and then dried over Na_2SO_4 and evaporated. The crude products were purified by column chromatography on silica gel with petroleum ether–ethyl acetate (4:1) as eluent. The structures of the synthesized esters **1–15** were established by spectroscopic methods. Among them, **6**, **8**, and **12** are new compounds, and the others are known compounds.

Hexan-2-yl 3,4,5-trihydroxybenzoate (6), $C_{13}H_{18}O_5$, obtained in 45% yield as a colorless solid. EI-MS (*m*/*z*, $I_{rel.}$, %): 254 $[M]^+$ (26), 224 (10), 197 (4), 170 (100), 153 (69), 125 (14), 107 (3), 79 (8), 56 (19). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 7.25 (2H, s, H-1, 5), 5.05 (1H, m, CH), 1.86, 1.30, 1.28 (each 2H, m, CH_2), 1.27 (3H, d, J = 6.1, CH_3), 0.86 (3H, t, J = 7.3, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 167.3 (CO), 143.9 (C), 143.8 (C), 136.8 (C), 121.9 (C), 109.9 (CH), 109.9 (CH), 72.4 (OCH), 35.6 (CH_2), 27.5 (CH_2), 22.5 (CH_2), 19.9 (CH_3), 13.9 (CH_3).

Benzyl 3,4,5-trihydroxybenzoate (8), $C_{14}H_{12}O_5$, obtained in 52% yield as a colorless solid. EI-MS (*m*/*z*, $I_{rel.}$, %): 260 $[M]^+$ (16), 224 (33), 153 (40), 143 (34), 125 (5), 107 (4), 99 (43), 83 (10), 70 (23), 56 (100). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 7.13 (2H, s, H-2, 6), 7.37–7.29 (5H, m, ArH), 5.24 (2H, s, OCH_2). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 167.0 (CO), 144.5 (C), 144.5 (C), 137.4 (C), 136.1 (C), 128.4 (CH), 128.4 (CH), 128.0 (CH), 128.0 (CH), 128.0 (CH), 120.9 (C), 109.4 (CH), 109.4 (CH), 66.4 (OCH_2).

2-Isopropyl-5-methylcyclohexyl 3,4,5-trihydroxybenzoate (12), $C_{17}H_{24}O_5$, obtained in yield 54% as a colorless solid. ESI-MS *m/z* 307 $[M - 1]^-$. ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm, J/Hz): 7.11 (2H, s, ArH), 3.41, 2.02 (each 1H, m, CH), 1.87 (2H, br, CH_2), 1.84 (1H, m, CH), 1.66 (1H, m, CH), 1.62, 1.27 (each 2H, m, CH_2), 0.85 (6H, d, J = 7.7, CH_3), 0.72 (3H, d, J = 6.9, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 166.8 (C), 144.5 (C), 144.5 (C), 137.0 (C), 121.7 (C), 109.3 (CH), 109.3 (CH), 74.1 (CH), 47.2 (CH), 40.9 (CH_2), 33.7 (CH_2), 23.5 (CH_2), 31.4 (CH), 26.3 (CH), 23.5 (CH_3), 22.0 (CH_3), 20.7 (CH_3).

The known compounds **1–5**, **7**, **9–11**, **13–15** were characterized as methyl 3,4,5-trihydroxybenzoate (**1**, $C_8H_8O_5$, yield 93%) [8, 10], ethyl 3,4,5-trihydroxybenzoate (**2**, $C_9H_{10}O_5$, yield 86%) [8, 10], isopropyl 3,4,5-trihydroxybenzoate (**3**, $C_{10}H_{12}O_5$, yield 81%) [9], isobutyl 3,4,5-trihydroxybenzoate (**4**, $C_{11}H_{14}O_5$, yield 87%) [9], isopentyl 3,4,5-trihydroxybenzoate (**5**, $C_{12}H_{16}O_5$, yield 77%) [8], heptyl 3,4,5-trihydroxybenzoate (**7**, $C_{14}H_{20}O_5$, yield 63%) [8, 10, 11], octyl 3,4,5-trihydroxybenzoate (**9**, $C_{15}H_{22}O_5$, yield 71%) [7, 10, 11], nonyl 3,4,5-trihydroxybenzoate (**10**, $C_{16}H_{24}O_5$, yield 80%) [8, 10, 11], decyl 3,4,5-trihydroxybenzoate (**11**, $C_{17}H_{26}O_5$, yield 68%) [8, 10, 11], dodecyl 3,4,5-trihydoxybenzoate (**13**, $C_{19}H_{30}O_5$, yield 72%) [7, 10, 11], hexadecyl 3,4,5-trihydroxybenzoate (**14**, $C_{23}H_{38}O_5$, yield 73%) [10], and octadecyl 3,4,5-trihydroxybenzoate (**15**, $C_{25}H_{42}O_5$, yield 61%) [10] by comparison of their ^{1}H NMR, ^{13}C NMR, and MS data with those reported.

Biological Assay. Mycelial Growth Inhibition Test *in vitro*. The synthesized compounds were dissolved in acetone and tested for antifungal activities *in vitro* by a poison food technique [26]. Potato dextrose agar (PDA) was used as the medium for all test fungi. The media incorporating test compounds at a concentration of 50 μg mL^{-1} was inoculated at the center of the test fungi in agar discs (4 mm diameter). Three replicate plates for each fungi were incubated at 26 ± 2°C. Control plates containing media mixed with acetone (1 mL) were included. After incubation for 2–6 days, the mycelial growth of fungi (mm) in both treated (T) and control (C) Petri dishes were measured diametrically in three different directions until the fungal growth in the control dishes was almost complete. The percentage of growth inhibition (*I*) was calculated using the formula

$$I\,(\%) = [(C - T)/C] \times 100 \quad (1)$$

The corrected inhibition (IC) was then calculated as follows:

$$IC = [(I - \mathrm{CF})/(100 - CF)] \times 100 \quad (2),$$

where $CF = [(90 - C_0)/C_0 \times 100]$, 90 is the diameter (mm) of the Petri dish, and C_0 is the growth (mm) of the fungus in the control.

Analysis of variance was performed on the data with the PROC GLM procedure (SAS Institute, Cary, NC, USA). If the value of P > F was less than 0.01, the means were separated with the least significant different (LSD) test at the p = 0.05 level.

***In vivo* Assay**. In order to further investigate the *in vivo* antifungal activities of the synthesized compounds, such as the duration of protection and curative activity, the plant disease of wheat powdery mildew (*E. graminis*) was used in the test. The effects of the test compounds on disease development and spread were determined using potted plants in a greenhouse. The potted plants were arranged randomly in two groups in a greenhouse and watered twice daily with tap water. The potted plant seedlings were sprayed with solutions of the test compounds in water–acetone (95:5) that contained Tween 20 (250 g mL^{-1}) as wetter and allowed to stand for 24 h.

For the test of preventive effects, the plants in the first group were inoculated with the pathogen of the plant disease one day after being sprayed with either the test compounds or a standard fungicide at a dose 500/250 μg mL^{-1}. For the test of

curative effects, the plants in the second group were first inoculated with the plant pathogenic fungi one day before the application of the test compounds and with a standard fungicide at a dose 500/250 μg mL^{-1}. Control plants in each group were similarly treated with distilled water–acetone containing Tween 20.

For the development of wheat powdery mildew, the treated wheat seedlings at the first stage were inoculated with *E. graminis* by shaking the infected leaves over them. The inoculated wheat seedlings were incubated for 8 days at 20 ± 1°C and 60% RH (relative humidity) of the day and 18 ± 1°C and 60% RH of the night with 16 h of daylight per day in artificial climate chambers (RP-300, R. P. China), and then the disease severity was determined. The disease severity was recorded on a 0–5 scale, where 0 = no colonies visible to the unaided eye; 1 = few scattered, small discrete colonies; 2 = larger, but still discrete colonies; 3 = colonies merging to form larger mildew lesions; 4 = mildew covering half the total leaf surface; and 5 = mildew covering the total leaf surface [27].

The experiment was conducted three times, and the mean value of the three estimates for each treatment was converted into percentage fungal control by the equation

$$\text{Control (\%)} = 100 \times [(A - B)/A] \quad (3)$$

where A = disease incidence (%) on leaves or sheaths steam-sprayed with Tween 20 solution alone, and B = disease incidence (%) on treated leaves or sheaths.

The percentage disease incidence was determined using the formula

$$\text{Disease incidence (\%)} = [(\Sigma\text{scale} \times \text{number of plant leaves infected})/(\text{highest scale} \times \text{total number of leaves})] \times 100 \quad (4)$$

Analysis of variance was performed on the data with the PROC GLM procedure (SAS Institute, Cary, NC, USA). If the value of P > F was less than 0.01, the means were separated with the least significant different (LSD) test at the p = 0.05 level.

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