


A New Alkaloid from the Endophytic Fungus of *Crocus sativus* L., *Aspergillus fumigatus* Y0107

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Abstract: A new alkaloid (compound **1**), together with 8 known alkaloids (compounds **2-9**), were isolated from *Aspergillus fumigatus* Y0107 extracts from the lateral buds of *Crocus sativus* Linn (saffron). The structure of compound **1** was elucidated with NMR spectroscopy, High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) and compared with its analogues in the literatures. The antibacterial activities of the crude ethyl acetate (EA) extract (CEE) and isolated compounds (compounds **1**, **2**, **6**, **8**) were evaluated against plant pathogenic bacteria. Compound **2** and the CEE demonstrated moderate inhibitory activity with MICs of 100 $\mu\text{g/mL}$ against *Erwinia* sp.

Keywords: *Crocus sativus* L.; endophytic fungus; *Aspergillus fumigatus* Y0107; alkaloids; plant pathogenic bacteria. © 2022 ACG Publications. All rights reserved.

1. Introduction

Crocus sativus L. (saffron) is a flowering plant belonging to the Iridaceae family, and its dry stigma is a precious traditional Chinese Medicines with a long history of application. At present, more than 40 kinds of endophytic fungi have been isolated from different parts of *Crocus sativus* Linn, including *Aspergillus*, *Alternaria*, *Penicillium*, *Trichoderma*, *Fusarium*, *Alternaria*, *Paecilomyces*, *Epicoccum*, *Mortierella*, *Hansfordia*, *Chaetomium*, *Rhizopus* genus and so on [1-2]. To the best of our knowledge, only four studies have documented metabolites of endophytic fungi from *C. sativus*. Zheng, et al. [3] characterized (-)-(1*R*,4*R*)-1,4-(2,3)-indolmethane-1-methyl-2,4-dihydro-1*H*-pyrazino-[2,1-*b*]-quinazoline-3,6-dione, a quinazoline alkaloid, from *Penicillium vinaceum*, an endophytic fungus isolated from *C. sativus* corm. Furthermore, phialomustin A-D with an azaphilone derived skeleton and a crocin with an unascertained structure were isolated from endophytic fungi, *Phialophora mustea* and

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Fusarium sp., respectively [4-5]. In addition, Luo, et al. [6] extracted beauvericin, prolipyrone C and ergosta-5 α ,8 α -epidioxy-6,22-dien-3 β -ol from an undocumented endophytic fungus (CSL-13). However, there has been no reports on the novel secondary metabolites of *Aspergillus* genus endophytic fungi from the plant *C. sativus* so far.

Accordingly, in our ongoing work, with the chemical investigation of *Aspergillus fumigatus* Y0107 extract from *Crocus sativus* L., a new alkaloid (compound **1**) together with 8 known alkaloids (**2-9**) (Figure 1) was isolated and structurally characterized. Importantly, compound **2** and CEE exhibited moderate inhibitory activity against plant pathogenic bacteria, *Erwinia* sp. In a nutshell, we isolated and elucidated the structure of a new alkaloid, and evaluated the bioactivity of several isolates from *Aspergillus fumigatus* Y0107. Herein, the isolation, structure elucidation and bioactivity assessment of the alkaloid compounds are described.

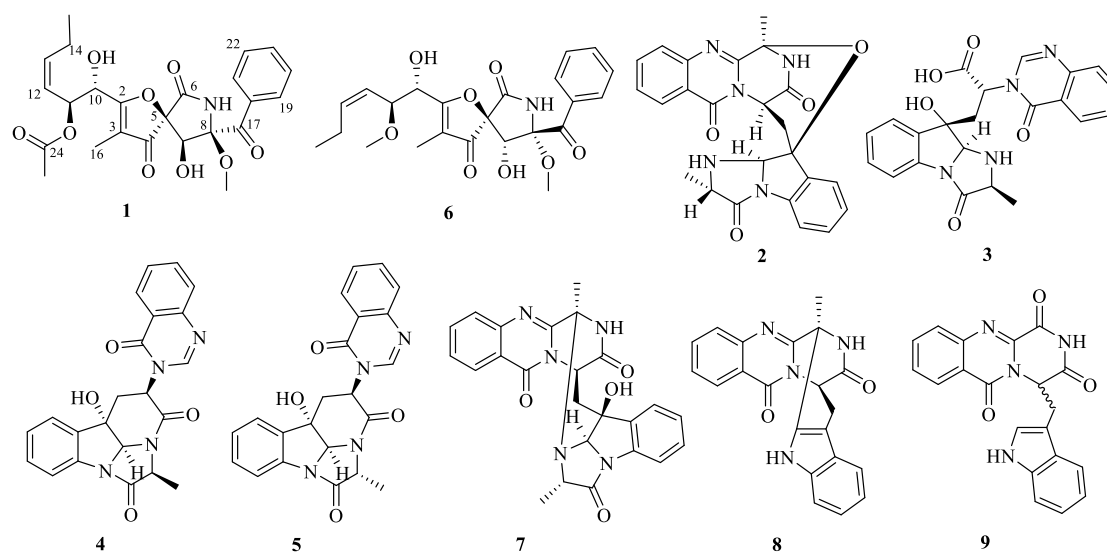


Figure 1. Chemical structures of alkaloid compounds from *Aspergillus fumigatus* Y0107

2. Materials and Methods

2.1. General Experimental Procedures

The optical rotation was measured by a Jasco P-2000 digital polarimeter. The NMR spectra (including ^1H NMR, ^{13}C NMR, ^1H - ^1H COSY, HSQC, and HMBC) were recorded on a Bruker AVANCE III-400 spectrometer. The 77.160 ppm and 7.260 ppm resonances of CDCl_3 and the 206.260 ppm and 2.050 ppm resonances of acetone- d_6 , were used as internal references to ascertain ^{13}C and ^1H chemical shifts, respectively. A Waters Synapt G2 type UHPLC-q-TOF mass spectrometer was used to obtain HR-ESI-MS data. Column chromatography (CC) was performed with silica gel (Qingdao Marine Chemical Co., Ltd, China) and Sephadex LH-20 (Pharmacia, Sweden), and thin layer chromatography was performed using silica gel 60 GF 254 (TLC, Qingdao Marine Chemical Co., Ltd, China). A reversed-phased C 18 column (5 μm , 10 mm \times 250 mm, YMC-Pack ODS-A column) was used to carry out on an Agilent 1260 Infinity II HPLC (Aligent Technologies Inc., Germany).

2.2. Fungal Material

The endophytic fungus strain was isolated from lateral buds of *Crocus sativus* L. (purchased from Jiande Sandu Saffron Professional Cooperative) on May 7th, 2020, and stored in a -80 $^\circ\text{C}$ cryopreservation box (ThermoFisher Scientific (China) Co. Ltd). Morphology analysis and 18S rRNA gene sequencing were performed to assign the strain as *Aspergillus fumigatus* (GenBank accession no. MZ854147) and named as Y0107.

2.3. Extraction and Isolation

The seed culture with *Aspergillus fumigatus* Y0107 strains was cut into small pieces and inoculated into a solid medium (rice 120 g of 150 mL ultrapure water in 1 L erlenmeyer flask, 140 bottles) for stationary fermentation at 26 °C for 21 days. After cultivation, the crude extract (182.1 g) of the fermented rice medium was obtained by soaking ethyl acetate (EA, 18 + 4 × 10 L) overnight. The crude extract was dispersed into 2.5 L ultrapure water, and then extracted with 2 L petroleum ether (PE), EA, and *n*-butanol three times in turn. Then EA layers were collected and concentrated by vacuum to yield a crude EA extract (CEE, 49.3 g).

The CEE was subjected to silica gel CC eluted with a gradient eluant of CH₂Cl₂/EA (CH₂Cl₂, 50:1 to EA, v/v) and CH₂Cl₂/MeOH (10:1 to MeOH neat) to yield eleven fractions (Fr. 1~11). Fr. 4 (2.93 g) and Fr. 5 (1.66 g) were merged and eluted with different solvents to yield Fr. 45-1 (3.25 g). Moreover, the fraction was separated by silica gel CC (PE/EA, 2:1 to 1:1) to afford Fr. 45-1-1~45-1-5. Fr. 45-1-3 (975.2 mg) was separated by silica gel CC (PE/EA, 2:1), Sephadex LH-20 CC (CH₂Cl₂/MeOH, 1:1), and repeated TLC (PE/EA, 1:1; CH₂Cl₂/MeOH, 50:1) to yield compound **2** (48.1 mg). Fr. 45-1-4 (724.4 mg) was subjected to silica gel CC (CH₂Cl₂/MeOH, CH₂Cl₂ to 100:1) and Sephadex LH-20 CC (CH₂Cl₂/MeOH, 1:1), and separated by semi-preparative HPLC (50% MeCN/H₂O, v/v, 1.5 mL/min) over a C₁₈ column to obtain compound **3** (1.8 mg, *t_R* = 14.6 min). Fr. 45-1-5 (561.1 mg) was purified by Sephadex LH-20 CC with CH₂Cl₂/MeOH (1:1), silica gel CC with a gradient eluent of CH₂Cl₂/EA (CH₂Cl₂, 10:1 to 1:1), and repeated TLC (PE/EA, 1:2 and 1:4, respectively) to harvest compound **4** (18.9 mg). Fr. 6 (2.16 g) was subjected to silica gel CC, eluted with PE/EA (2:1 to EA neat) and Sephadex LH-20 CC with CH₂Cl₂/MeOH (1:1) to obtain 13 fractions (Fr. 6-4-1~6-4-4, Fr. 6-5-1~6-5-4, Fr. 6-6-1~6-6-5). Fr. 6-4-3 (244.2 mg) was separated via silica gel CC (CH₂Cl₂/MeOH, 50:1 to 1:1), TLC (PE/EA, 1:1) and semi-preparative HPLC (60% MeCN/H₂O, v/v, 1.5 mL/min) to get compound **5** (1.6 mg, *t_R* = 21.6 min). Compounds **1** (1.7 mg, *t_R* = 21.1 min) and **6** (2.5 mg, *t_R* = 25.5 min) were yielded from Fr. 6-5-3 (162.9 mg) by silica gel CC (CH₂Cl₂/MeOH, CH₂Cl₂, 100:1 to 10:1) and semi-preparative HPLC (49% MeCN/H₂O, v/v, 1.5 mL/min). Fr. 6-6-4 (264.3 mg) was separated following silica gel CC (CH₂Cl₂/MeOH CH₂Cl₂, 100:1 to 2:1), and semi-preparative HPLC (45% MeCN/H₂O, v/v, 1.5 mL/min) to afford compound **7** (2.6 mg, *t_R* = 19.5 min). Compounds **8** (14.1 mg, *t_R* = 14.7 min, 60% MeCN/H₂O, v/v, 1.5 mL/min) and **9** (5.2 mg, *t_R* = 15.5 min, 50% MeCN/H₂O, v/v, 1.5 mL/min) were purified by semi-preparative HPLC from Fr. 6-6-5 (309.2 mg) after silica gel CC (PE/EA, 5:1 to EA neat).

11-Acetyl-pseurotin A₂ (**1**): White solid; $[\alpha]_D^{28} = -34.0$ (*c* 0.1, MeOH); HR-ESI-MS *m/z* 496.1582 [M + Na]⁺, calcd 496.1564 for C₂₃H₁₉N₅O₄Na; ¹H NMR (400MHz, TMS, CDCl₃) and ¹³C NMR (100MHz, TMS, CDCl₃) data see Table 1.

2.4. Antibacterial Bioassays

The antibacterial activities of compounds **1**, **2**, **6** and **8**, together with CEE, were determined based on a microbroth dilution method in 96-well culture plates according to the National Committee for Clinical Laboratory Standards [15-16]. The plant pathogenic bacteria, *Agrobacterium tumefaciens*, *Pantoea agglomerans*, *Ralstonia solanacearum*, *Erwinia sp.* were purchased from Ningbo testobio Co., Ltd, Zhejiang, China, and cultured in the NA nutrient medium (1 g yeast extract, 3 g beef extract, 5 g peptone, and 5 g glucose in 1 L sterile water, the pH was adjusted to 7.2 with NaOH) [17]. Streptomycin (USP Grade, Sangon Biotech Co., Ltd, Shanghai, China), an antibacterial drug, was used as the positive control. The tested bacteria were placed in a constant temperature oscillation incubator (100 rpm) for 12 h at 30 °C. The NA nutrient broth was used to adjust the bacterial concentration to 1×10⁵-1×10⁶ cfu/mL by observing the OD600 values of each bacterial dilution. The concentration of monomers, positive control and CEE initial solution in DMSO/H₂O was 200, 200 and 400 μg/mL, respectively. First of all, the diluted bacterial solution was added into each well of a 96-well culture plate (50 μL per well). Then, the initial solution (50 μL) was added into the first well and mixed evenly, from which 50 μL of solution was transferred into the second well and mixed well following the two fold dilution method. Serial dilutions were repeated from the third to the twelfth well in triplicate. The minimal inhibitory concentrations (MIC) were determined after 24 h of incubation.

3. Results and Discussion

3.1. Structure Elucidation

Compound **1** was isolated as a white solid and characterized as a dark spot under UV light at 254 nm and a brown spot after spraying with 10% H₂SO₄ in EtOH with heating. Its molecular formula was determined to be C₂₄H₂₇NO₉ from the [M + Na]⁺ ion at *m/z* 496.1582 (calculated 496.1564) in HR-ESI-MS, requiring 12 degrees of unsaturation.

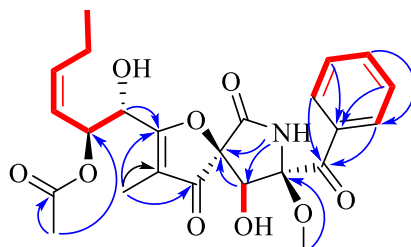


Figure 2. Key ¹H-¹H COSY and HBMBC correlations of compound **1**

The ¹³C NMR/HSQC spectrum (Table 1), showed 24 carbon resonances, assigned to four electrophilic groups (two carbonyls at δ_c 197.8 (C-4) and 194.5 (C-17), an ester group at δ_c 170.5 (C-24) and an acylamino at δ_c 165.2 (C-6)), and four aromatic chemical shifts. Ten and four carbon signals were ascribed to sp³ and sp² hybridized carbon atoms, respectively. The ¹H NMR spectroscopy (Table 1) revealed one oxymethyl and three methyl groups. The heteronuclear single quantum correlation (HSQC) is a powerful sequence widely used to correlate ¹H nuclei one-bond coupled with ¹³C nuclei. The ¹H NMR and HSQC spectra and distortionless enhancement by polarization transfer (DEPT)-135 displayed signals of three oxygenated methines (δ_H 5.80, H-11; 4.77, H-10; 4.62, H-9), one methylene (δ_H 2.26, H_a-14; 2.15, H_b-14), two exchangeable protons (δ_H 7.38, NH; 4.03, 9-OH), as well as two olefinic protons (δ_H 5.75, H-13; 5.38, H-12), according to their chemical shifts. The five aromatic protons (δ_H 8.28, d, *J* = 7.2 Hz, H-23, H-19; 7.64, t, *J* = 7.6 Hz, H-21; 7.49, d, *J* = 8 Hz, H-22, H-20) were identified as mono substituted benzene protons according to the typical coupling constants, number of split peaks and ¹H-¹H chemical shift correlation spectroscopy (COSY) correlations (H-19/H-20/H-21/H-22/H-23), as seen in Figure 2.

Except the aromatic protons of mono substituted benzene, ¹H-¹H COSY revealed the two proton-proton spin systems of H-10/H-11/H-12/H-13/H-14/H-15 and H-9/H-9-OH (Figure 2). HMBC (Figure 2) confirmed the correlation of C-18 with H-20 and H-22, and C-17 with H-19 and H-23, indicating that a carbonyl was connected to a mono substituted benzene. Furthermore, C-5 exhibited correlations to H-9 and NH-7, while C-9 also correlated to NH-7, suggesting that there was a *N*-containing five-membered heterocyclic ring in compound **1**. The correlations between H-16 and C-2, C-3 and C-4 uncovered the position of CH₃-16, while protons of oxymethyl that correlated to C-8, revealed the position of the oxymethyl group. Moreover, H-25 exhibited correlations with C-24 and C-11, while H-11 was correlated to C-24, which indicated the position C-24 and CH₃-25. And the correlation between H-10 and C-2 displayed the connection of C-2 and C-10. The presence of a *cis* double bond was confirmed by the typical *J* coupling constant of 10 Hz at H-12. The above data further established the planar structure of compound **1**, possessing a 1-oxa-7-azaspiro[4.4]non-2-ene-4,6-dione skeleton.

The ¹H NMR data of compound **1**, pseurotin A₂ (2011) [18], pseurotin A₂ (2016) [18] and pseurotin A [19] were seen in Table S1. The NMR chemical shifts of compound **1** and pseurotin A₂ (2011) were more similar than that of the other two compounds. The chemical shift of 7-NH (δ_H 7.38) in compound **1** was close to pseurotin A₂ (2011), which might indicate the configuration of C-8 was same as that of pseurotin A₂ (2011). While their chemical shifts of 9-OH were all strongly shielded, the reason of which might be instrument effect. When compared with chemical shifts of pseurotin A₂ [18], the deshielded chemical shift ($\Delta\delta$ = 1.10 ppm, Table S1) of H-11 indicated that the acetyl group moiety was located at 11-OH, consistent with HMBC cross peaks. And the chemical shift ($\Delta\delta$ = 0.22 ppm) of H-10 was also deshielded due to the long-range correlations. Thus, it could be considered that the difference between compound **1** and pseurotin A₂ was the 11-position (-¹¹CH(CH₃COO)- in compound **1** and -¹¹CH(OH)- in pseurotin A₂). In the meantime, compound **1** had the exactly same chiral carbon

in the same positions (C-5, C-8, C-9, C-10 and C-11) with pseurotin A₂, and the optical rotation value of compound **1** ($[\alpha]_{\text{D}}^{28}$ -34.0 (c 0.1, MeOH)) was closed to that of pseurotin A₂ ($[\alpha]_{\text{D}}^{23}$ -30.6 (c 0.1, MeOH)) [20]. Accordingly, we found that compound **1** possessed the same absolute configuration as pseurotin A₂, the absolute configuration of which had been previously determined (5*S*, 8*R*, 9*S*, 10*S*, 11*S*) by ECD analysis and was named 11-acetyl-pseurotin A₂.

Table 1. NMR spectral data for compound **1** in CDCl₃

Position	δ_{H} (mult., <i>J</i> in Hz) ^a	δ_{C} ^b	HMBC (H#→C#) ^c
2		185.1	
3		113.6	
4		197.8	
5		91.9	
6		165.2	
7-NH	7.38 (s)		5, 9
8		89.7	
9	4.62 (d, 11.2)	74.0	5
10	4.77 (d, 5.6)	70.7	2, 13
11	5.80 (m)	71.1	24
12	5.38 (t, 10)	122.3	
13	5.75 (m)	140.2	
14	2.26 (m), 2.15 (m)	21.6	
15	0.97 (t, 7.6)	14.1	13, 14
16	1.80 (s)	5.8	2, 3, 4
17		194.5	
18		132.5	
19/23	8.28 (d, 7.2)	130.7	23, 21, 17
20/22	7.48 (t, 8.0)	128.9	18, 22
21	7.64 (t, 7.6)	134.8	19
24		170.5	
25	2.04 (s)	21.2	24, 11
8-OMe	3.38 (s)	51.8	8
9-OH	4.03 (d, 12.4)		

^a Recorded at 400MHz. ^b Recorded at 100MHz. ^c Recorded at 400MHz (¹H dimension), showing the correlations of protons with indicated carbon.

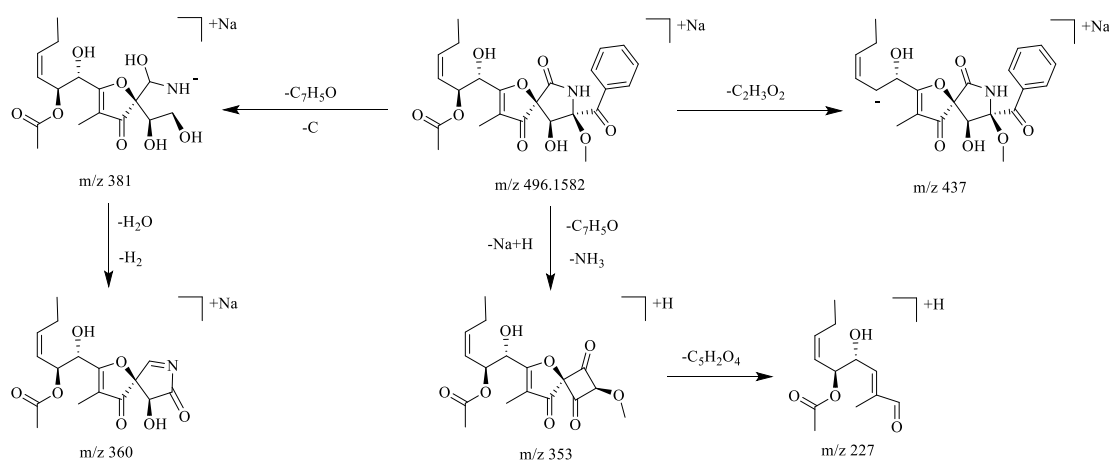


Figure 3. Proposed fragmentation pathways of positive ions for compound **1**

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The MS/MS fragmentation pathways of $[M+Na]^+$ and $[M+H]^+$ ions for compound **1** were suggested in Figure 3. Under collision voltage of mass spectrum, various characteristic ions were formed from compound **1** owing to the successive losses of CH_3COOH , H_2O , H_2 , NH_3 , CO and/or C_6H_6 molecules. The dissociation processes included de-esterification (m/z 437 $[M+Na-C_2H_3O_2]$), decarbonization (m/z 381 $[M+Na-C_7H_5O-C]$), dehydrogenation, dehydration (m/z 360 $[M+Na-C_7H_5O-C-H_2-H_2O]$), deamination (m/z 353 $[M+H-C_7H_5O-NH_3]$) and decarbonylation (m/z 227 $[M+H-C_7H_5O-NH_3-C_5H_2O_4]$) (seen in Figure S1).

The eight documented alkaloid compounds were identified to be 18-*epi*-fumiquinazolin C (**2**) [7], fumigatoside F (**3**) [8], chaetominine (**4**) [9], (-)-11-*epi*-chaetominine (**5**) [10-11], 11-*O*-methylpseurotin A (**6**) [12], 2'-*epi*-fumiquinazoline D (**7**) [7], fumiquinazoline J (**8**) [13], and oxoglyantrypine (**9**) [14] by spectroscopic methods comparison of NMR and MS data with that of compounds reported in the literatures.

3.2. Antibacterial Activity

Several isolated compounds and CEE were tested for antibacterial activity against plant pathogenic bacteria. As shown in Table 2, compound **2** (MIC 100 $\mu\text{g/mL}$) and CEE (MIC 100 $\mu\text{g/mL}$) exhibited moderate antibacterial activity against *Erwinia* sp., which was weaker than the positive control (streptomycin, MIC 25 $\mu\text{g/mL}$), while compounds **1**, **6** and **8** showed no activity against this strain when their concentration range was within 100 $\mu\text{g/mL}$. Unfortunately, none of the tested samples displayed inhibition against the strains, *A. tumefaciens*, *P. agglomerans* and *R. solanacearum* when their concentration range was within 100 $\mu\text{g/mL}$, except the positive control.

Table 2. Antibacterial activity data of compounds **1**, **2**, **6**, **8** and CEE

Samples	MIC ($\mu\text{g/mL}$)			
	<i>A. tumefaciens</i>	<i>P. agglomerans</i>	<i>R. solanacearum</i>	<i>Erwinia</i> sp.
1	> 100	> 100	> 100	> 100
2	> 100	> 100	> 100	100
6	> 100	> 100	> 100	> 100
8	> 100	> 100	> 100	> 100
CEE	> 100	> 100	> 100	100
streptomycin	100	50	50	25

4. Conclusions

In this work, a new alkaloid compound, namely 11-acetyl-pseurotin A₂ (**1**) together with eight known alkaloids were isolated from CEE of *Aspergillus fumigatus* Y0107, an endophytic fungus of the lateral buds of *Crocus sativus* Linn. The structure of compound **1** was elucidated with various spectroscopies, HR-ESI-MS analysis and comparison with its analogues in the literatures. Among them, compound **2** and CEE displayed antimicrobial activities against *Erwinia* sp., while no significant effects against the other three plant pathogenic bacteria were observed when their concentration range was within 100 $\mu\text{g/mL}$. Additionally, compounds **1**, **6** and **8** showed no markedly inhibition against the abovementioned plant pathogenic bacteria when their concentration range was within 100 $\mu\text{g/mL}$.

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Supporting Information

Supporting information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

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