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SHORT REPORT

Anti-phytopathogenic himeic acid derivatives produced by the *Nicotiana tabacum* symbiotic fungus *Aspergillus* sp. TE-65L

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Abstract: In our study, chemical exploration of the *Nicotiana tabacum* L. symbiotic fungus *Aspergillus* sp. TE-65L led to the discovery of a new himeic acid derivative, japonimeic acid J (**1**), along with two previously reported compounds **2** and **3**. The structure of the new compound was unequivocally elucidated using nuclear magnetic resonance spectroscopy and high-resolution electrospray ionization mass spectrometry. Furthermore, compounds **1** and **2** displayed strong anti-phytopathogenic potency against *Alternaria alternata* and *Botrytis cinerea*, with a minimum inhibitory concentration of 4 µg/mL. This result suggests that they have considerable potential as new, naturally sourced agro-antibiotics for controlling fungal plant diseases.

Keywords: Symbiotic fungus, natural product, secondary metabolite, himeic acids, anti-phytopathogenic potency

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1 Fungal Source

The fungal strain used in this study for chemical exploration was *Aspergillus* sp. TE-65L, which is preserved in Zhengzhou University of Light Industry. This strain was isolated from the healthy leaves of *Nicotiana tabacum* L. collected from Hubei, China, in August 2016. Based on the morphological observation (specifically its mycelium and spores) and molecular techniques (specifically the amplification and analysis of the internal transcribed spacer region of ribosomal DNA), this fungus was identified as *Aspergillus* sp. The sequence was deposited in the GenBank database under accession number OQ629860.

2 Previous Studies

Symbiotic fungi live within plant tissues without causing harm, forming compatible and amicable relationships (Delaux & Schornack, 2021; Tunlid & Talbot, 2002). These fungi produce diverse bioactive secondary metabolites that help the host plant maintain physiological homeostasis; furthermore, they adapt their metabolism to create compounds suited to the specific environmental niches of their host plants (Keller, 2019). Numerous secondary metabolites with diversified chemical structures have been discovered (Bendejacq-Seychelles et al., 2024; Pusztahelyi et al., 2015). Furthermore, fungal secondary metabolites displayed considerable biological activities, leading to their development into life-saving drugs and agrochemicals. Examples include the antifungal drug griseofulvin and the plant growth hormone gibberellic acid. Among various symbiotic fungi, those associated with *N. tabacum* have gained increasing attention. For example, three new isochromenes isolated from *Aspergillus versicolor*, an endophytic fungus found in *N. tabacum*, showed strong inhibitory activity against tobacco mosaic virus (Yang et al., 2023). Furthermore, a new benzothiazole derivative isolated from the *N. tabacum* symbiotic fungus *Aspergillus* sp. 1022LEF was found to possess potent insecticidal activity against the fall armyworm *Spodoptera frugiperda* (Yuan et al., 2024). Additionally, chemical exploration of the *N. tabacum* symbiotic fungus *A. japonicus* TE-739D yielded a new prenylated indole alkaloid, which showed promising anti-tumor activity against the gastric cancer MFC cells both in vitro and in vivo (Cao et al., 2024).

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Table 1. ^{1}H and ^{13}C NMR spectroscopic data for compound **1** (measured in DMSO-d_6, δ in ppm)

No.	δ_H (mult., *J* in Hz)	δ_C, type	No.	δ_H (mult., *J* in Hz)	δ_C, type
1		173.4 C	14		177.6 C
2	2.28 t (7.4)	33.3 CH_2	15		115.2 C
3	1.51 m	24.4 CH_2	16	8.41 s	143.6 CH
4	1.27 m	28.5 CH_2	17		163.2 C
5	1.27 m	28.5 CH_2	18		173.2 C
6	1.27 m	28.6 CH_2	19	3.12 m 2.82 m	41.4 CH_2
7	1.27 m	28.7 CH_2	20	2.79 m	34.5 CH
8	1.43 m	28.0 CH_2	21		176.7 C
9	2.22 m	32.4 CH_2	22	1.14 d (7.1)	17.0 CH_3
10	6.72 dt (16.2, 6.9)	139.8 CH	1-OCH_3	3.57 s	51.2 CH_3
11	6.29 d (16.2)	122.4 CH	21-OH	12.28 s	
12		147.0 C	17-NH	13.08 s	
13	6.55 s	116.1 CH			

3 Present Study

In our initial screening, the fungal strain *Aspergillus* sp. TE-65L was found to possess promising antifungal properties. To discover novel secondary metabolites produced by *Aspergillus* sp. TE-65L isolated from *N. tabacum*, an in-depth exploration of the metabolic products of this fungal strain was conducted. The strain was subjected to static fermentation using the rice medium (comprising 100 g of rice and 100 mL of distilled water). The fermentation process lasted for 25 days at approximately 28°C and a 12-hour light/12-hour dark cycle under natural light. After that, the fermented rice medium was collected and extracted with ethyl acetate (EtOAc) three times. The EtOAc phase was collected and concentrated in a vacuum to yield 26 g of brown extract. Then, the obtained extract was separated by silica gel column chromatography using a stepwise gradient elution of petroleum ether (PE) and EtOAc. The extract was eluted with a gradient of PE–EtOAc from 20:1 to 1:1 (v/v) to yield five fractions (A–E). Fraction C (1.5 g), eluted with PE-EtOAc 5:1, was further separated on a LiChroprep RP-18 gel column using a gradient of MeOH–H_2O mixture (from 30% to 100%, v/v) to yield seven subfractions labeled C1 through C7. Compounds **1** (3.1 mg, t_R = 9.5 min) and **2** (2.0 mg, t_R = 8.0 min) were isolated from fraction C3 using semipreparative high-performance liquid chromatography (HPLC) with MeCN–H_2O in a 40:60 ratio, while compound **3** (10.2 mg, t_R = 11.6 min) was isolated from fraction C4 using semipreparative HPLC with MeCN–H_2O in a 55:45 ratio.

*Japonimeic acid J (**1**):* yellow amorphous solid; $[\alpha]^{26}_D$ +22.8 (c = 0.07, MeOH); UV (MeOH): λ_{max} 253.1 nm; ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; HRESIMS: *m/z* 449.2295 $[M + H]^+$ (calcd for $C_{23}H_{33}N_2O_7^+$, 449.2282).

Anti-phytopathogenic activity assay: The following five agricultural pathogenic fungi obtained from BeNa Culture Collection (BNCC) were used for the anti-phytopathogenic activity assay: *Alternaria alternata* WH1-21 BNCC, *Botrytis cinerea* BNCC 338228, *Fusarium oxysporum* BNCC 120618,

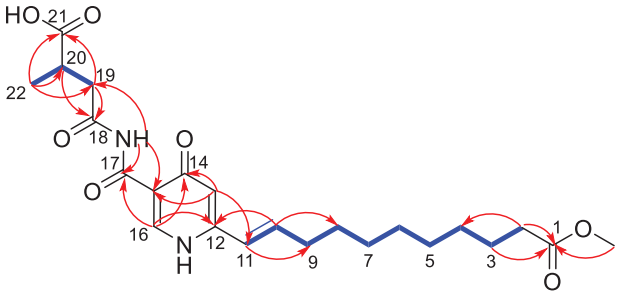

Figure 1. Key ^{1}H-^{1}H COSY and HMBC correlations for **1**

Rhizoctonia cerealis BNCC 357868, and *Colletotrichum gloeosporioides* BNCC 116357. The anti-phytopathogenic activity assay against the above-mentioned pathogens was performed using 96-well microplates with a modified broth microdilution method (Wang et al., 2016). Carbendazim, a common chemical pesticide used in agriculture, was chosen as a positive control. Briefly, the tested compounds (10 µL) were added to a 96-well microplate, and PDB medium (50 µL) was then continuously diluted in the plate. A fungal suspension (50 µL) was prepared by mixing fungi with sterile water; the final concentrations of the tested compounds were adjusted to be within the range of 1–64 µg/mL.

Japonimeic acid J (**1**) was obtained as a yellow amorphous solid. Its molecular formula was determined to be $C_{23}H_{32}N_2O_7$ from high-resolution electrospray ionization mass spectrometry (*m/z* 449.2295 $[M + H]^+$, calcd for $C_{23}H_{33}N_2O_7^+$, 449.2282). Analysis of the proton nuclear magnetic resonance (^{1}H NMR) and heteronuclear single quantum coherence (HSQC) spectra (Table 1) of **1** revealed the existence of two olefinic protons resonating as singlets (δ_H 8.41 and 6.55), two mutually coupled olefinic protons (δ_H 6.72 and 6.27), one methoxy group (δ_H 3.57), nine aliphatic methylene protons (δ_H 1.27–3.12), and one methine proton

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(δ_H 2.79). The ^{1}H NMR spectrum displayed two exchangeable proton singlets at δ_H 13.08 (1H, s, CONH) and 12.28 (1H, s, 4'-OH). The former assignment was confirmed by heteronuclear multiple bond correlation (HMBC), while the latter assignment was supported by the molecular formula of **1**. The ^{13}C NMR spectrum (Table 1) of **1** exhibited 23 resonances corresponding to five ester carbonyl carbons (δ_C 177.6, 176.7, 173.4, 173.2, and 163.2), six olefinic carbons (δ_C 147.0, 143.6, 139.8, 122.4, 116.1, and 115.2), nine methylene carbons (δ_C 41.4, 33.3, 32.4, 28.7, 28.6, 28.5, 28.5, 28.0, and 24.4), one methine carbon (δ_C 34.5), and two methyl carbons (δ_C 51.2 and 17.0).

The planar structure of **1** was elucidated through detailed analysis of ^{1}H-^{1}H COSY and HMBC data (Figure 1). The presence of a pyridine-4(1*H*)-one moiety was established through HMBCs from H-13 to C-14/C-15 and from H-16 to C-12/C-14, combined with the characteristic downfield chemical shifts of C-16 (δ_C 143.6) and C-12 (δ_C 147.0). A long-chain aliphatic acid unit was identified through a ^{1}H-^{1}H COSY experiment, with the spin system extending from H_2-2 to H_2-11. This chain was linked to the pyridine ring via C-12, as evidenced by key HMBCs from H_2-10 to C-12 and from H-13 to C-11. The HMBC correlation from the methoxy protons (δ_H 3.57, 1-OCH$_3$) to C-1 secured its position esterifying the carboxylic acid terminus. Furthermore, HMBCs from H_3-22 to C-19/C-20/C-21, from H-20 to C-18, and from the amide proton NH-17 (δ_H 13.08) to C-15/C-17/C-19 demonstrated that the 4-formamido-2-methyl-4-oxobutanoic acid residue was directly connected to the identified carbons. This unit was confirmed to be attached to C-15 based on the aforementioned HMBC correlation from NH-17 to C-15. Based on these collective data, the planar structure of **1** was determined (Figure 2). Compound **1** possessed a single chiral center. Its α ($[\alpha]^{26}_D$ +22.8, *c* 0.07, MeOH) was consistent with that reported for japonimeic acids A–I, suggesting an identical 20*R* absolute configuration (Dong et al., 2025; Sowemimo et al., 2008).

The known compounds isolated were identified as japonimeic acid B (**2**) (Dong et al., 2025) and microsphaerone C (**3**) (Sowemimo et al., 2008) by comparing their NMR and HRESIMS data with reported data.

The inhibitory effects of the isolated himeic acid derivatives **1**–**3** on the growth of plant pathogenic fungi were tested using the anti-phytopathogenic activity assay. The following five agricultural pathogenic fungi were chosen as the testing strains due to their damaging effects on essential food and economic crops: *Alternaria alternata*, *Botrytis cinerea*, *Fusarium oxysporum*, *Rhizoctonia cerealis*, and *Colletotrichum gloeosporioides*. As illustrated in Table 2, compounds **1** and **2** showed promising activity against *A. alternate*, with minimum inhibitory concentrations (MICs) of 16 and 4 μg/mL, respectively. Importantly, compound **2** showed stronger activity than the positive control (carbendazim, MIC = 8 μg/mL). Moreover, compounds **1** and **2** (MIC = 4 μg/mL) showed strong activity against *B. cinerea* compared with carbendazim (MIC = 8 μg/mL). Notably, the structural differences between compounds **1**, **2**, and **3** revealed critical functional group dependencies for antifungal activity. The presence of the C-1 methoxy group in compound **1** significantly affected its antifungal activity. Specifically, the removal of the C-1 methoxy group led to a 4-fold increase in the activity of compound **1** against *A. alternata* (MIC decreasing from 16 μg/mL to 4 μg/mL) while maintaining equivalent potency against *B. cinerea*. Furthermore, the C-1' substituent ($C_5H_6O_3$ moiety) proved essential for broad-spectrum efficacy, as evidenced by compound **3** (with the absence of this group) exhibiting a >16-fold reduction in its activity against both pathogens (MIC > 64 μg/mL). This result clearly indicates that this fragment serves as a key pharmacophore.

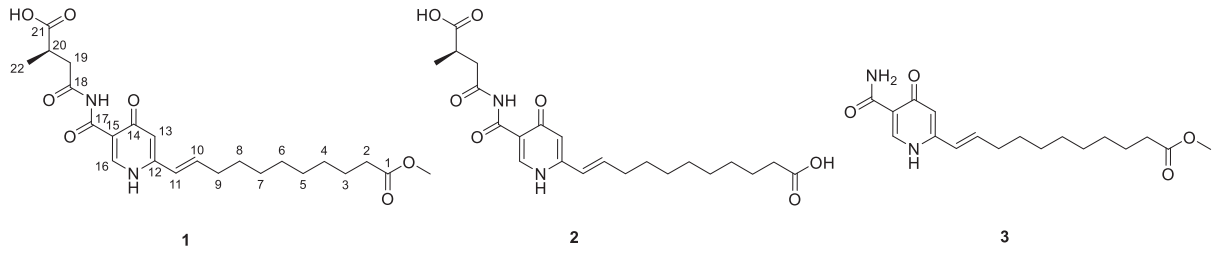

Figure 2. Structures of himeic acid derivatives (compounds **1**–**3**)

Table 2. Inhibitory effects of compounds **1**–**3** on five phytopathogens

Compounds	Phytopathogens (MIC, μg/mL)				
	A. alternate	***B. cinerea***	***F. oxysporum***	***R. cerealis***	***C. gloeosporioides***
1	16	4	>64	>64	>64
2	4	4	>64	>64	>64
3	>64	>64	>64	>64	8
Carbendazim	8	8	16	8	4

Compounds **1** and **2** produced by the fungus *Aspergillus* sp. TE-65L have substantial antifungal activity, which necessitates consideration of their environmental implications. This concept is rooted in microbial chemical ecology, the study of chemical communication between microorganisms and their role in mediating ecosystem processes. First, the fungus producing a toxic substance likely possesses its own genetic tools and strategies to protect itself from being harmed by the very compound it creates. Second, the narrow-spectrum activity of *Aspergillus* sp. suggests a specialized role in mutualistic symbiosis: By suppressing pathogens threatening *N. tabacum*, *Aspergillus* sp. TE-65L safeguards its host habitat and secures its own nutritional niche. Thus, the antifungal properties of himeic acid derivatives produced by *Aspergillus* sp. TE-65L reflect an evolved ecological adaptation.

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Author Contributions

Investigation, G.L. Xi, Q.F. Wang, Y.Z. Zhao, and C.T. Lu; resources, C.T. Lu, H.Y. Chen, L.K. Zhang, and Y.B. Ning; writing—original draft preparation, G.L. Xi and P.F. Yang; writing—review and editing, P.F. Yang and Z.F. Chen; project administration, Z.F. Chen; funding acquisition, P.F. Yang. All authors have read and agreed to the published version of the manuscript.

Availability of Data and Materials

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Conflicts of Interest

The authors declare that they do not have any conflict of interest.

Supporting Information

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