DOI 10.1007/s10600-023-04140-2
Chemistry of Natural Compounds, Vol. 59, No. 5, September, 2023

CARBOLINE ALKALOIDS FROM THE CIGAR TOBACCO-DERIVED FUNGI *Aspergillus* sp. AND THEIR ANTI-TMV ACTIVITY

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Two new β*-carboline alkaloids, 1-acetyl-6-hydroxymethyl-7-methoxy-*β*-carboline (**1**) and 1-acetyl-6-hydroxymethyl-8-methoxy-*β*-carboline (**2**), together with four known ones (**3–6**) were isolated from the cigar tobacco (a variety of* Nicotiana tabacum *L.)-derived endophytic fungi* Aspergillus *sp. Their structures were determined by means of HR-ESI-MS and extensive 1D and 2D NMR spectroscopic studies. The anti-TMV activities test revealed that compounds **1** and **2** showed potential anti-TMV activities with inhibition rates of 31.8 and 35.5%, respectively. These rates are close to that of positive control. Compounds **3–6** also showed anti-TMV activities with inhibition rates in the range of 21.5–28.8%, respectively. The isolation of the aforementioned* β*-carboline alkaloids may provide materials for the screening of anti-TMV activity inhibitors and contribute to the utilization of cigar tobacco-derived endophytic fungi.*

Keywords: *β*-carboline alkaloids, fungi *Aspergillus* sp., anti-tobacco mosaic virus (anti-TMV) activity.

Plant diseases, caused by viruses seriously damage the yield and quality of crops and bring huge losses to agricultural production [1]. Plant viruses, accounting for almost half of all plant diseases, cause losses of up to US$ 30 billion each year worldwide [2]. Tobacco mosaic virus (TMV) is one of the most widely studied plant viruses, which can cause deformation and stunting of the leaves, flowers and fruits of infected plants [3]; additionally it can infect more than 885 species of plants belonging to 65 families [4]. There are few available antiviral agents against TMV on the market, except ningnanmycin and ribavirin [5]; therefore, there is an urgent need to develop anti-TMV agents with novel structures and remarkable effects, and which are environmentally friendly.

The previous studies showed that endophytic fungi represent a promising and huge natural products pool with valuable biological potential for applications in medicine, agriculture and industry [6, 7]. Among the numerous existing endophytic fungi, *Aspergillus* strains constitute one of the most prolific sources of secondary metabolites with diverse chemical classes and interesting biological activities [8, 9]. In our previous works, some anti-TMV agents, such as alkaloids [10–12], butyrolactones [13, 14], isocoumarins [15, 16], and the like, have been isolated from the genus of this fungus.

Cigar tobacco (a variety of *Nicotiana tabacum* L.) is an important economic crop that is widely cultivated in Yunnan Province. In addition, the unique special geographical environment of Yunnan also supplies a unique microbial population in cigar tobacco [17]. As a matter of course, the unique microbial population and rich microbial species in Yunnan cigar tobacco also provide a new source for the discovery of bioactivity metabolites [18–20]. In the present work, detailed phytochemical studies on the culture broth of the endophytic fungi *Aspergillus* sp. obtained from cigar tobacco was carried out.

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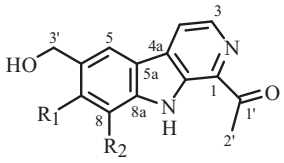
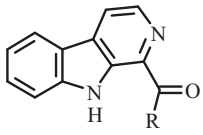
TABLE 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data of Compounds **1** and **2** ($CDCl_3$, δ, ppm, J/Hz)

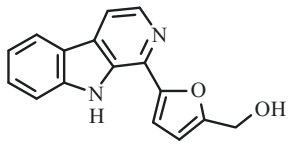
C atom	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
1	–	137.5 (C)	–	137.3 (C)
3	8.56 (d, J = 4.8)	140.7 (CH)	8.58 (d, J = 4.8)	140.5 (CH)
4	8.41 (d, J = 4.8)	119.3 (CH)	8.40 (d, J = 4.8)	118.7 (CH)
5	8.16 (s)	121.9 (CH)	7.84 (d, J = 1.8)	112.5 (CH)
6	–	124.9 (C)	–	136.7 (C)
7	–	151.4 (C)	6.69 (d, J = 1.8)	104.5 (CH)
8	7.13 (s)	97.5 (CH)	–	148.8 (C)
1a	–	133.3 (C)	–	132.4 (C)
4a	–	130.7 (C)	–	129.3 (C)
5a	–	119.9 (C)	–	130.6 (C)
8a	–	139.0 (C)	–	128.6 (C)
1′	–	198.1 (C)	–	198.0 (C)
2′	2.55 (s)	26.5 (CH_3)	2.56 (s)	26.4 (CH_3)
3′	4.33 (s)	62.4 (CH_2)	4.42 (s)	66.9 (CH_2)
MeO	3.76 (s)	56.2 (CH_3)	3.84 (s)	56.3 (CH_3)
NH	10.28 (s)		10.25 (s)	



1, 2



3, 4



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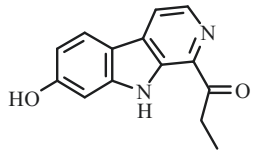

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1: R_1 = OMe, R_2 = H; **2:** R_1 = H, R_2 = OMe; **3:** R = CH_3, **4:** R = OMe

As a result, two new (**1** and **2**) and four known *β*-carboline alkaloids (**3–6**) were isolated. Their structures were determined by means of HR-ESI-MS and extensive 1D and 2D NMR spectroscopic studies. The anti-TMV activity test revealed that compounds **1–6** showed potential anti-TMV activity. The isolation of the aforementioned *β*-carboline alkaloids may provide materials for the screening of anti-TMV activity inhibitors and contribute to the utilization of cigar tobacco-derived endophytic fungi.

The whole culture broth of *Aspergillus* sp. was extracted with EtOAc. The extract was partitioned between EtOAc and 3% tartaric acid. The aqueous layer was adjusted to pH 9.5 with saturated Na_2CO_3 aq. and extracted again with EtOAc. The EtOAc-soluble alkaloidal materials were subjected repeatedly to column chromatography on silica gel, MCI, RP-18 and preparative HPLC to afford compounds **1–6**, including two new *β*-carboline alkaloids, 1-acetyl-6-hydroxymethyl-7-methoxy-*β*-carboline (**1**) and 1-acetyl-6-hydroxymethyl-8-methoxy-*β*-carboline (**2**), along with four known ones (**3–6**). ^{1}H and ^{13}C NMR data of **1** and **2** were listed in Table 1. The known compounds – 1-acetyl-*β*-carboline (**3**) [21], methoxycarbonyl-*β*-carboline (**4**) [22], perlolyrin (**5**) [23], and trichocarboline C (**6**) [24] were identified by the comparison of their spectroscopic data with the literature.

Compound **1** was isolated as a pale-brown oil. Its molecular formula was determined to be $C_{15}H_{14}N_2O_3$ by HR-ESI-MS at *m/z* 293.0907 $[M + Na]^+$ (calcd for $C_{15}H_{14}N_2NaO_3$, 293.0902), having 10 degrees of unsaturation. Strong absorption bands accounting for hydroxy (3387 cm^{-1}), carbonyl (1715 cm^{-1}), and aromatic (1618, 1447, 1326 cm^{-1}) groups can be observed in its IR spectrum. Its UV spectrum showed maximum absorption at 215, 256, and 364 nm, suggesting the existence of an aromatic structure. Its ^{1}H, ^{13}C, and DEPT NMR data (Table 1) showed resonances for 15 carbons and 14 hydrogen atoms. These signals can be attributed to a 1,2,4,5-tetrasubstituted benzene ring (C-5–C-8, C-5a, C-8a, H-5, and H-8), a 1,2,3-trisubstituted pyridine ring (C-1–C-4, C-1a, C-4a, H-3, and H-4), an acetyl group (C-1′, C-2′, and H_3-2′), a hydroxymethyl group (C-3′ and H_2-3′), one methoxy group (δ_C 56.2 q, δ_H 3.76 s), and an amine (-NH-, δ 10.28). By further analysis of the preceding NMR data, the pyridine ring and amine should be incorporated with the benzene ring to form a five number ring to support the existence of 10 degrees of unsaturation, and compound **1** should be a *β*-carboline alkaloid. This deduction can also be confirmed by the HMBC correlations (Fig. 1) from H-3 to C-1/C-4/C-4a, from H-4 to C-1a/C-4a/C-5a, from H-5 to C-4a/C-5a/C-8a, from -NH to C-1/C-8/C-1a/C-4a/C-5a/C-8a, and the comparison of NMR data with known compounds, 1-acetyl-*β*-carboline [21] and trichocarboline C [24].

TABLE 2. Anti-TMV Activities of Compounds **1–6** on *N. glutinosa* Leaf*

Compound	% Inhibition at 20 μM	IC_{50}, μM	Compound	% Inhibition at 20 μM	IC_{50}, μM
1	31.8 ± 2.5	29.9	**5**	28.4 ± 2.3	37.8
2	35.5 ± 2.4	24.6	**6**	21.5 ± 2.4	64.9
3	28.8 ± 2.6	36.8	Ningnanmycin	33.6 ± 2.4	31.8
4	24.6 ± 2.2	52.4			

* All results are expressed as mean ± SD; n = 3.

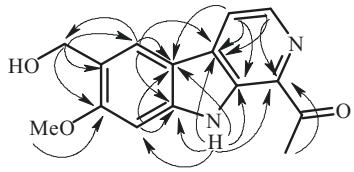

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Fig. 1. Key HMBC correlations of **1**.

Since the skeleton of the compound is determined, the positions of substituents (acetyl, hydroxymethyl, and methoxy groups) can also be determined by further analysis of its HMBC data. The HMBC correlations from the methoxy protons (δ 3.76) to C-7 indicated that the methoxy group was located at C-7. The hydroxymethyl group located at C-6 was supported by the HMBC correlations from H_2-3′ to C-5/C-6/C-7, and from H-5 to C-3′. Finally, the acetyl group located at C-1 was supported by the HMBC correlations from H_3-2′ to C-1. Thus, in this way, the structure of **1** was established and given the systematic name of 1-acetyl-6-hydroxymethyl-7-methoxy-*β*-carboline.

1-Acetyl-6-hydroxymethyl-8-methoxy-*β*-carboline (**2**) was also obtained as a pale-brown oil, and its molecular formula was determined to be $C_{15}H_{14}N_2O_3$ by HR-ESI-MS *m/z* 293.0910 $[M + Na]^+$. The ^1H and ^{13}C spectral data of **2** are depicted as similar to those of compound **1** in C-1–C-4, C-1a, C-4a. The obvious chemical shift differences result from the disappearance of two aromatic singlets (δ_H 8.16, 7.13) and the appearance of two doublets δ_H 7.84 (J = 1.8 Hz), 6.69 (J = 1.8 Hz), indicating that the positions of substituents on the benzene ring should be changed. In addition, the position of a hydroxylmethyl group at C-6 and a methoxy group at C-8 can also be determined by further analysis of its HMBC correlations. Therefore, in this way, the structure of **2** was determined.

Since certain *β*-carboline alkaloids exhibit potential antiviral activity [25–27], compounds **1–6** were tested for their anti-TMV activities. The anti-TMV activities were tested by the half-leaf method, using ningnanmycin ($C_{16}H_{25}N_7O_8$, CAS#: 156410-09-2, a commercial product for plant disease in China, with an inhibition rate of 33.6%) as a positive control [28, 29]. The results revealed that compounds **1** and **2** showed potential anti-TMV activities with inhibition rates of 31.8 and 35.5%, respectively. These rates are close to that of the positive control. Compounds **3–6** also showed anti-TMV activities with inhibition rates in the range of 21.5–28.8%, respectively.

EXPERIMENTAL

General. UV spectra were obtained using a Shimadzu UV-1900 spectrophotometer. A Bio-Rad FTS185 spectrophotometer was used for scanning the IR spectra. 1D and 2D NMR spectroscopic data were recorded on a DRX-500 NMR spectrometer with TMS as the internal standard. ESI-MS and HR-ESI-MS analyses were measured on Agilent 1290 UPLC/6540 Q-TOF mass spectrometer. Preparative HPLC was performed on an Agilent 1260 preparative liquid chromatograph with Zorbax PrepHT GF (2.12 cm × 25 cm) or Venusil MP C_{18} (2.0 cm × 25 cm) columns. Column chromatography was performed using silica gel (200–300 mesh, Qingdao Marine Chemical, Inc., Qingdao, China), Lichroprep RP-18 gel (40–63 μm, Merck, Darmstadt, Germany), Sephadex LH-20 (Sigma-Aldrich, Inc., USA), or MCI gel (75–150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan). Column fractions were monitored by TLC visualized by spraying with 5% H_2SO_4 in ethanol and heating.

Fungal Material. The culture of *Aspergillus* sp. YATAS-21-18 was isolated from the leaves of cigar tobacco, which was collected from the fermentation plant of Yuanjiang County, Yuxi Prefecture, Yunnan Province in 2021. The strain was identified by Dr. Yin-Ke Li based on the analysis of its ITS sequence. It was cultivated at room temperature for 7 days on potato dextrose agar at 28°C. Agar plugs were inoculated into 250 mL Erlenmeyer flasks each containing 100 mL potato dextrose broth and cultured at 28°C on a rotary shaker at 180 rpm for five days. Large-scale fermentation was carried out in 100 Fernbach flasks (1.0 L) each containing 500 g of rice and 300 mL nutrient solution (glucose 5%; peptone 0.15%; yeast 0.5%; KH_2PO_4 0.05%; urea 0.1%; $MgSO_4$ 0.05% in 1.0 L of deionized water; pH 6.5 before autoclaving). Each flask was inoculated with 5.0 mL of cultured broth and incubated at 27°C for 20 days.

Extraction and Isolation. The whole culture broth of *Aspergillus* sp. was extracted four times with EtOH (4 × 25 L) at room temperature and filtered. The extract was partitioned between EtOAc and 3% tartaric acid. The aqueous layer was adjusted to pH 9.5 with saturated Na_2CO_3 aq. and extracted again with EtOAc. The crude extract (72.6 g) was applied to silica gel column chromatography, eluting with a $CHCl_3$–MeOH gradient system (9.5:0.5, 8:2, 7:3, 6:4, 5:5). Five fractions were obtained from the silica gel column and individually decolorized on MCI gel to yield fractions A–E. The further separation of Fr. B (8:2, 7.23 g) by silica gel column chromatography, eluted with $CHCl_3$–$(Me)_2CO$ (9:1, 8:2, 7:3, 6:4, 1:1), yielded mixtures subfractions B1–B5. Subfraction B2 (8:2, 1.63 g) was subjected to RP-18 column chromatography (MeOH–H_2O, 40:60–70:30 gradient) and HPLC to give **3** (12.9 mg), **4** (16.8 mg), and **5** (14.4 mg). Subfraction B3 (7:3, 1.28 g) was subjected to RP-18 column chromatography (MeOH–H_2O, 30:70–65:35 gradient) and HPLC to give **1** (15.4 mg), **2** (16.8 mg), **6** (17.3 mg).

Anti-TMV Assays. The anti-TMV activity was tested using the half-leaf method [28, 29], and ningnanmycin (a commercial product for plant disease in China) was used as a positive control. The virus was inhibited by mixing with the solution of tested compounds. After 30 min, the mixture was inoculated on the left side of the leaves of *Nicotiana glutinosa*, whereas the right side of the leaves was inoculated with the mixture of DMSO solution and the virus as a control. The local lesion numbers were recorded 3–4 days after inoculation. Three repetitions were conducted for each compound. The inhibition rates were calculated according to the formula:

$$\text{Inhibition rate (\%)} = [(C - T)/C] \times 100\%,$$

where C is the average number of local lesions of the control and T is the average number of local lesions of the treatment. Ningnanmycin, a commercial virucide for plant disease in China, was used as a positive control.

1-Acetyl-6-hydroxymethyl-7-methoxy-β-carboline (1), $C_{15}H_{14}N_2O_3$, obtained as a pale-brown oil. UV (MeOH, λ_{max}, nm) (log ε): 215 (4.18), 256 (3.95), 364 (3.74). IR (ν_{max}, cm^{-1}): 3387, 2965, 1715, 1662, 1618, 1447, 1326, 1269, 1068, 1016, 812. ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1. ESI-MS *m/z* 293 $[M + Na]^+$; HR-ESI-MS *m/z* 293.0907 (calcd for $C_{15}H_{14}N_2NaO_3$, 293.0902).

1-Acetyl-6-hydroxymethyl-8-methoxy-β-carboline (2), $C_{15}H_{14}N_2O_3$, obtained as a pale-brown oil. UV (MeOH, λ_{max}, nm) (log ε): 215 (4.22), 253 (3.92), 366 (3.77). IR (ν_{max}, cm^{-1}): 3396, 2968, 1716, 1658, 1616, 1453, 1329, 1260, 1059, 1012, 827. ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1. ESI-MS *m/z* 293 $[M + Na]^+$; HR-ESI-MS *m/z* 293.0910 (calcd for $C_{15}H_{14}N_2NaO_3$, 293.0902).

ACKNOWLEDGMENT

This project was supported by the Foundation of the China Tobacco Monopoly Bureau Grants and Yunnan Provincial Tobacco Monopoly Bureau Grants (110202103018, 2022530000241002), the National Natural Science Foundation of China (No. 32260111), and the Foundation of Yunnan Innovative Research Team (2019HC020).

REFERENCES

1. S. Savary, L. Willocquet, S. J. Pethybridge, P. Esker, N. McRoberts, and A. Nelson, *Nat. Ecol. Evol*., **3**, 430 (2019).
2. H. C. Godfray, J. R. Beddington, I. R. Crute, L. Haddad, D. Lawrence, J. F. Muir, J. Pretty, S. Robinson, S. M. Thomas, and C. Toulmin, *Science*, **327**, 812 (2010).
3. V. Nicaise, *Front. Plant Sci*., **5**, 660 (2014).
4. B. A. Song, L. H. Jin, S. Yang, and P. S. Bhadury, *Environment Friendly Anti-plant Viral Agents*, Chinese Chemical Industry Press & Springer Press, Beijing, 2010, p. 252.

5. Y. H. Ge, K. X. Liu, J. X. Zhang, S. Z. Mu, and X. J. Hao, *J. Agric. Food Chem.,* **60**, 4289 (2012).
6. R. H. Zheng, S. J. Li, X. Zhang, and C. Q. Zhao, *Int. J. Mol. Sci*., **22**, 959 (2021).
7. N. Rustamova, K. Bozorov, T. Efferth, and A. Yili, *Phytochem. Rev*., **19**, 425 (2020).
8. A. Hagag, M. F. Abdelwahab, A. El-Kader, and M. A. Fouad, *J. Appl. Microbiol*., **132**, 4150 (2022).
9. K. L. Yang, J. Tian, and N. P. Keller, *Environ. Microbiol*., **24**, 2857 (2022).
10. G. Y. Yang, J. M. Dai, Q. L. Mi, Z. J. Li, X. M. Li, J. D. Zhang, J. Wang, Y. K. Li, W. G. Wang, M. Zhou, and Q. F. Hu, *Phytochemistry*, **198**, 113137 (2022).
11. J. M. Dai, Q. L. Mi, X. M. Li, G. Du, G. Y. Yang, J. D. Zhang, J. Wang, Y. K. Li, H. Y. Yang, M. Dong, Z. J. Li, and Q. F. Hu, *Phytochemistry*, **205**, 113485 (2023).
12. Y. N. Zhu, M. X. Liu, B. B. Cai, Y. Li, M. F. Li, H. S. Wang, M. Zhou, G. Y. Yang, Q. F. Hu, and Y. K. Li, *Chem. Nat. Compd*., **58**, 712 (2022).
13. L. Yuan, W. Z. Huang, K. Zhou, Y. D. Wang, W. Dong, G. Du, X. M. Gao, Y. H. Ma, and Q. F. Hu, *Nat. Prod. Res*., **29**, 1914 (2015).
14. M. Zhou, G. Du, H. Y. Yang, C. F. Xia, J. X. Yang, Y. Q. Ye, X. M. Gao, X. N. Li, and Q. F. Hu, *Planta Med*., **81**, 235 (2015).
15. M. Zhou, K. Zhou, P. He, K. M. Wang, R. Z. Zhu, Y. D. Wang, W. Dong, G. P. Li, H. Y. Yang, Y. Q. Ye, G. Du, X. M. Li, and Q. F. Hu, *Planta Med*., **82**, 414 (2016).
16. F. X. Yang, J. M. Dai, H. Y. Liu, Q. L. Mi, J. Wang, J. D. Zhang, X. M. Li, W. G. Wang, M. Zhou, Y. K. Li, and Q. F. Hu, *Nat. Prod. Res*., **37**, 1608 (2023).
17. L. L. Zheng, L. Zhao, X. H. Cai, Z. Chen, Z. S. Chai, and X. D. Shi, *Acta Tab. Sin*., **28**, 65 (2022).
18. M. F. Li, D. Xiao, L. C. Zhu, L. Liu, J. N. Zheng, X. J. Gu, Y. N. Zhu, J. Xie, X. Wang, J. M. Dai, Q. L. Mi, Y. K. Yang, Q. F. Hu, Y. K. Li, and J. Q. Shi, *Chem. Nat. Compd*., **58**, 1093 (2022).
19. J. M. Dai, L. C. Zhu, D. Xiao, J. Xie, X. Wang, Q. L. Mi, J. Q. Shi, G. Y. Yin, Y. K. Yang, G. Y. Yang, Q. F. Hu, and W. Kai, *Chem. Nat. Compd*., **58**, 1005 (2022).
20. Q. F. Hu, L. F. Zhang, G. H. Zhang, M. F. Bao, Y. K. Li, D. Miao, Y. P. Wu, G. Du, and G. H. Kong, *Heterocycles*, **104**, 1661 (2022).
21. D. S. Lee, S. H. Eom, S. Y. Jeong, H. J. Shin, J. Y. Je, E. W. Lee, Y. H. Chung, Y. M. Kim, C. K. Kang, and M. S. Lee, *Environ. Toxicol. Pharmacol*., **35**, 171 (2013).
22. N. Yang, Y. H. Shi, A. Z. Xiong, Y. Zhou, L. H. Gu, R. Wang, L. Yang, and Z. T. Wang, *J. Sep. Sci*., **41**, 3014 (2018).
23. Y. Li, M. M. Zhao, and K. L. Parkin, *J. Agric. Food Chem*., **59**, 2332 (2011).
24. M. J. Hao, P. N. Chen, H. J. Li, F. Wu, G. Y. Zhang, Z. Z. Shao, X. P. Liu, W. Z. Ma, J. Xu, T. Mahmud, and W. J. Lan, *Front. Microbiol*., **13**, 947226 (2022).
25. H. J. Song, Y. X. Liu, Y. X. Liu, Y. Q. Huang, Y. Q. Li, and Q. M. Wang, *Bioorg. Med. Chem. Lett*., **24**, 5228 (2014).
26. X. P. Zhang, W. B. Huang, X. Lu, S. S. Liu, H. Feng, W. N. Yang, J. L. Ye, F. Li, S. Y. Ke, and D. G. Wei, *J. Agric. Food Chem*., **69**, 7458 (2021).
27. M. M. Gonzalez, F. M. Cabrerizo, A. Baiker, R. Erra-Balsells, A. Osterman, Nitschko, H. Nitschko, and M. G. Vizoso-Pinto, *Int. J. Antimicrob. Ag*., **52**, 459 (2018).
28. G. Y. Yang, J. M. Dai, Z. J. Li, J. Wang, F. X. Yang, X. Liu, J. Li, Q. Gao, X. M. Li, Y. K. Li, W. G. Wang, M. Zhou, and Q. F. Hu, *Arch. Pharm. Res*., **45**, 572 (2022).
29. Q. F. Hu, Y. Y. Ma, H. Y. Liu, J. M. Dai, F. X. Yang, J. D. Zhang, J. Wang, X. M. Li, X. Liu, J. Li, Y. K. Li, W. G. Wang, M. Zhou, and G. Y. Yang, *Chem. Biol. Technol. Agric*., **9**, 88 (2022).