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Secondary metabolites produced by Fusarium sp. 2TnP1-2, an endophytic fungus from Trewia nudiflora

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Abstract: Aim To study antifungal and antibacterial secondary metabolites of *Fusarium* sp. 2TnP1-2, an endophytic fungus isolated from *Trewia nudiflora*. **Methods** PDA fermentation extract was isolated by bio-assay guided fractionation and different column chromatography methods including silica gel column, Sephadex LH-20 column and preparative thin layer chromatography. Structures of these compounds were identified on the basis of spectroscopic analysis of 1D, 2D-NMR, MS and comparison of chemical and physical data with authentic samples reported in literatures. The antibacterial and antifungal activities of the isolated compounds were measured using the disc diffusion method. **Results** Three compounds were isolated and their structures were determined to be trichosetin (*N*-demethyl equisetin, **1**), lateritin (4-methyl-6-(1-methylethyl)-3-phenylmethyl-1,4-perhydrooxazine-2,5-dione, **2**), 5α,6α-epoxy-24(*R*)-methylcholesta-7,22-dien-3β-ol (**3**). **Conclusion** All the compounds were obtained from *Fusarium* for the first time. Trichosetin and lateritin possess antibacterial activity against *Staphylococcus aureus*.

Key words: *Trewia nudiflora*; *Fusarium*; antibacteria; tetramic acid

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滑桃树内生真菌 Fusarium sp. 2TnP1-2 次生代谢产物研究

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摘 要:**目的** 对滑桃树内生真菌 *Fusarium* sp. 2TnP1-2 的抗菌活性次生代谢产物进行研究。**方法** 采用硅胶柱色谱、Sephadex LH-20、制备性薄层色谱等对次生代谢产物进行分离纯化。根据理化性质和波谱分析进行结构鉴定。利用纸片扩散法对这些化合物的抗真菌及抗细菌活性进行测试。**结果** 从该菌株 PDA 培养基发酵产物中分离得到 3 个化合物,分别鉴定为: trichosetin (*N*-demethyl equisetin, **1**), lateritin (4-methyl-6-(1-methylethyl)-3-phenylmethyl-1,4-perhydrooxazine-2,5-dione, **2**), 5α,6α-epoxy-24(*R*)-methylcholesta-7,22-dien-3β-ol (**3**)。**结论** 3 个化合物均为首次从该真菌的代谢物中分离得到,trichosetin 和 lateritin 具有抗金黄色葡萄球菌活性。

关键词:滑桃树;镰刀菌属;抗菌; tetramic acid

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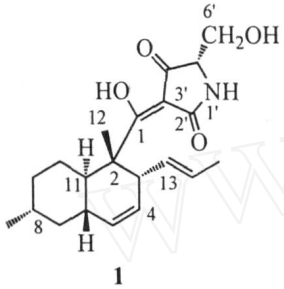
Endophytes, microorganisms that reside in the tissues of living plants, are relatively unstudied as potential sources of novel natural products for exploitation in medicine, agriculture and industry[1-2]. Based on the methods and rationale used to provide the best opportunities to isolate novel endophytic microorganisms at the genus or species[3-4], the tropical plant *Trewia nudiflora* L. (Euphorbiaceae) was selected to isolate endophytes and search for bioactive compounds from their metabolites. Fungi are the most commonly isolated endophytic microorganisms from the plant. During the course of antifungal activity survey of the endophytes from *T. nudiflora*, a fungus *Fusarium* sp. 2TnP1-2 was selected for further investigation. Bioassay guided fractionation led to isolation of three compounds. Theses compounds were elucidated to be trichosetin (*N*-demethyl equisetin, **1**), lateritin (4-methyl-6-(1-methylethyl)-3-phenylmethyl-1, 4-perhydrooxazine-2, 5-dione, **2**), 5α, 6α-epoxy-24(*R*)-methylcholesta-7, 22-dien-3β-ol (**3**). Their antibacterial and antifungal activities were tested by disk diffusion method.



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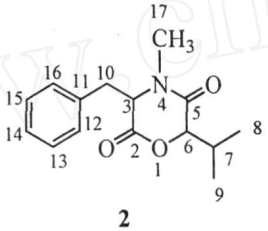

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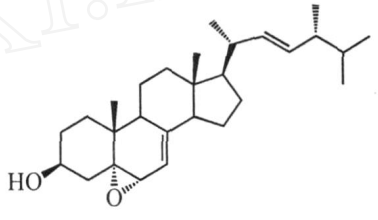

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1 Material and methods

1.1 General

Optical rotations were determined on a JASCO DIP370 digital Polarimeter. MS were measured on a VG AutoSpec 3000 mass spectrometer. All the NMR data were obtained at room temperature on AM-400 and DRX-500 spectrometer (TMS as internal reference, chemical shift in δ). Thin layer chromatography was conducted on silica gel F_{254} plates (Qingdao Meigao Chemical Co. Ltd.). TLC developing agent is 5% (*V*/*V*) sulphuric acid in ethanol. Column chromatography was carried out on silica gel (50-71 μm; Qingdao Marine Chemical Factory), Sephadex LH-20 (GE Healthcare Bio-Sciences AB), RP-18 gel (40-63 μm; Merck, Darmstadt, Germany). Preparative TLC was carried out on silica gel F_{254} (Qingdao Meigao Chemical Co. Ltd.).

1.2 Biological material

The fungal strain was isolated from the petioles of *Trewia nudiflora* L. (Euphorbiaceae), which were collected from greenhouse of Kunming Institute of Botany, People's Republic of China. The petioles were washed in running tap water and cut into 5 mm pieces. These small pieces were surface sterilized successively with 0.1% (*V*/*V*) Tween-20 for 30 s, 1% (*V*/*V*) sodium hypochlorite for 5 min, sterilized water for 5 min then 75% (*V*/*V*) ethanol for 5 min. The surface sterilized pieces were incubated at 26 ℃ on PDA supplemented with 100 mg·L^{-1} nalidixic acid to suppress the bacterial growth and incubated at 26 ℃ until colony or mycelium appeared surrounding the pieces. Hyphal tips originating from segments were transferred to Petri dishes containing fresh PDA medium free of antibiotics. Each isolates was then grown and examined to ascertain that it originated from a single organism. During the antifungal bioassay test, the strain 2TnP1-2, whose extract afforded antifungal activity, was selected for further investigation. The strain was then identified as a *Fusarium* sp. by Prof. LUO Yun-long, Yunnan Agriculture University, Kunming, China, and deposited in Kunming Institute of Botany, Chinese Academy of Science, Kunming, China.

1.3 Fermentation, extraction and fractionation

Agar fermentation was performed with PDA medium (2 L) for 14 d. The fermentation product was chopped and extracted four times with ethyl acetate-methanol-formic acid (80∶15∶5, *V*/*V*) exhaustively. The combined extract solution was evaporated in vacuo at 45 ℃ to give a residue. The residue was extracted by methanol to give 1.6 g crude extract.

The crude extract (FM) was chromatographed on vacuum liquid chromatography (VLC) (37 g silica gel), eluted with a PE-EtOAc (20∶1 - 0∶100, *V*/*V*) and EtOAc-MeOH gradient system, to afford 12 sub fractions (FM1 - FM12). Antifungal test showed that FM5 and FM6 were active. Combined FM5 and FM6 (340 mg) was subjected on VLC RP-18 (10 g), eluted with a MeOH-H_2O gradient system, to afford three main fractions (FM5c1 - FM5c3). FM5c1 was isolated by Sephadex LH-20 in acetone and further purified by prepared TLC to obtain compound **1** (30 mg). FM7 was isolated by VLC RP-18 (4 g), eluted with a MeOH-H_2O (50∶50 - 95∶5) to afford compound **2** (29 mg). FM8 was purified by Sephadex LH-20 in methanol and silica gel to give compound **3** (4 mg).

1.4 Bioassays

Antifungal activities against *Canidia albicans*, *Saccharomyces cerevisiae*, and antibacterial activities against *Staphylococcus aureus*, *Mycobacterium tuberculosis* were carried out by disk diffusion assay on agar plates as described[5]. Results were expressed by minimal inhibition amount (μg/disc) and diameter of inhibition zone. Rifampicin was used as positive control.

2 Results

Compound **1**: white powder, $[\alpha]_D^{17}$ = - 261° (*c* = 4.66, MeOH). IR (KBr) σ: 3443, 2921, 1595, 1448 cm^{-1}. ESI-MS *m*/*z*: 360 $[M + H]^+$ (100), 358 $[M - H]^+$ (100). The molecular formula is $C_{21}H_{29}NO_4$ based on its HR ESI-MS data (*m*/*z*: 382.1983 $[M + Na]^+$, cal: 382.1994). The IR spectrum showed the presence of OH (3443, 2921 cm^{-1}) and C = C (1595 cm^{-1}) goups. The ^{13}C-NMR spectrum showed 21 carbon signals: three CH_3 groups, four CH_2, nine CH groups including four double bonds and five quaternary C-atoms (table 1). Compound similarity search in DNP database indicated that this newly isolated compound has similar skeleton to equisetin. Detailed comparison revealed equisetin has one additional methyl group. It suggested the methyl on *N*-1′ may be substituted by a proton in the moiety of **1**. Determination of the structure of **1** was confirmed by HMQC, HMBC and ^{1}H-^{1}H COSY experiments and comparison of spectra with reference[6-7]. NOESY was useful to establish the relative configuration of stereocenters for **1**. NOESY experiment showed NOE correlations between H-3 (δ 3.94) and H-8 (δ 1.32), H-12 (δ 1.32) and H-13 (δ 5.20), also between H-6 (δ 1.70) and H-8 and H-12. The negative optical rotation of **1** ($[\alpha]_D^{17}$ = - 261°) is very similar to that of equisetin ($[\alpha]_D^{17}$ = - 273°), which suggested **1** has same stereo configuration as *N*-demethyl equisetin (trichosetin).

Compound **2**: The molecular formula is $C_{15}H_{19}NO_3$, colorless crystal. $[\alpha]_D^{17}$ = + 22.3° (*c* = 1.1, $CHCl_3$). EI-MS *m*/*z*: 261 $[M^+]$, (100), 244 (7), 231 (3), 217 (3), 170 (25), 142 (36), 134 (23), 91 (100). The ^{13}C-NMR spectrum showed 15 carbon signals: three CH_3, one CH_2, eight CH groups including one oxygenated carbon and three quaternary C-atoms including two carboxyl carbons (table 2). Compound similarity search in DNP database indicated that NMR data of **2** was a good match for lateritin which that isolated from *Gibberella lateritium* IFO 7188[8]. Kagamizono reported the structure of lateritin might be wrong[9]. In order to confirm the correct structure, 2D-NMR experiments were conducted and the structure was elucidated as 4-methyl-6-(1-methylethyl)-3-phenylmethyl-1, 4-perhydrooxazine-2, 5-dione (lateritin).

Table 1 ^{1}H and ^{13}C-NMR spectroscopic data for compound 1 (in CD_3OD)

Position	δ(C)	δ(H)	HMBC (C→H)	^{1}H-^{1}H COSY
1	202.8 (s)	/	H-12	/
2	51.5 (s)	/	H-12	/
3	44.1 (d)	3.94 (m)	H-5, H-13	H-13
4	130.7 (d)	5.28 (m)	H-3, H-6	H-3
5	128.9 (d)	5.28 (m)	H-3, H-6, H-11	/
6	40.3 (d)	1.70 (m)	H-4, H-5	H-11
7	44.1 (t)	1.72 (m) 0.74 (m)	H-5, H-9, H-16	H-7
8	34.9 (d)	1.32 (m)	H-9, H-16	
9	36.8 (t)	1.47 (m) 0.87 (m)		
10	30.7 (t)	1.26 (m)		
11	42.1 (d)	1.47 (m)	H-12	
12	13.9 (q)	1.32 (s)	H-1, H-2, H-3	
13	126.2 (d)	5.20 (m)	H-15	H-3
14	133.8 (d)	5.14 (m)	H-3, H-15	
15	18.4 (q)	1.47 (d), 3.96	H-13, H-14	
16	23.2 (q)	0.83 (d), 4.4	H-7, H-9	
2′	181.2 (s)	/	/	/
3′	103.5 (s)	/	/	/
4′	192.4 (s)	/	/	/
5′	62.7 (d)	3.57 (m)		
6′	64.5 (t)	3.82 (m) 3.55 (m)	H-6′ H-6′	

Table 2 ^{1}H and ^{13}C-NMR spectroscopic data of compound 2 (in CD_3OD)

Position	δ(C)	δ(H)	HMBC	^{1}H-^{1}H COSY	ROESY
C-2	169.9 (s)	/	H-3, H-6, H-10	/	/
C-3	57.0 (d)	5.58 (1H, dd, J = 4.6, 11.7 Hz)	H-10, H-17	H-10	/
C-5	169.7 (s)	/	H-7, H-10, H-17	/	/
C-6	75.6 (d)	4.88 (1H, d, J = 8.5 Hz)	H-7, H-8, H-9	H-7	H-7, H-17
C-7	29.7 (d)	2.02 (1Hm)	H-6 H-8, H-9	H-6 H-8, H-9	H-6, H-8, H-9
C-8	17.3 (q)	0.40 (3H, d, J = 6.8 Hz)	H-7, H-9	H-7	H-6, H-7, H-9
C-9	18.3 (q)	0.80 (3H, d, J = 6.6 Hz)	H-6, H-8	H-7	H-6, H-7, H-8
C-10	34.7 (d)	3.41 (1H, dd, J = 4.9, 14.6 Hz) 2.99 (1H, dd, J = 12.1, 14.5 Hz)	H-10	H-3	H-10
C-11	136.5 (s)	/	H-10, H-14, H-16	/	/
C-12, 16	128.8 (d)	7.27 (2H, m)	H-10, H-14	/	H-10
C-13, 15	128.5 (d)	7.27 (2H, m)	H-10, H-16	/	H-10
C-14	126.8 (d)	7.19 (1H, m)	H-16	/	/
N-CH_3	32.1 (q)	3.01 (3H, s)	H-3	/	H-6, H-7

Compound **3**: Colorless crystal. FAB$^+$-MS m/z: 413. EI-MS m/z (%): 394 $[M - H_2O]^+$ (12), 376 $[M - 2H_2O]^+$ (53), 361 (10), 251 $[M - 2H_2O - C_9H_{17}]^+$ (100). ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.95 (1H, m, H-3), 3.52 (1H, m, H-6), 5.26 (1H, m, H-7), 0.54 (3H, s, H-18), 1.00 (3H, s, H-19), 0.97 (3H, d, J = 6.6 Hz, H-21), 5.13 (1H, dd, J = 15.3, 8.0 Hz, H-22), 5.18 (1H, dd, J = 15.3, 7.3 Hz, H-23), 0.77 (3H, d, J = 7.2 Hz, H-26), 0.78 (3H, d, J = 7.4 Hz, H-27), 0.86 (3H,

d, J = 6.8 Hz, H-28). ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 32.7 (C-1), 30.4 (C-2), 67.2 (C-3), 38.9 (C-4), 75.8 (C-5), 73.1 (C-6), 117.4 (C-7), 143.4 (C-8), 43.2 (C-9), 36.9 (C-10), 21.2 (C-11), 39.2 (C-12), 43.6 (C-13), 54.6 (C-14), 22.8 (C-15), 27.8 (C-16), 55.9 (C-17), 12.1 (C-18), 18.2 (C-19), 40.2 (C-20), 20.9 (C-21), 135.3 (C-22), 132.0 (C-23), 42.7 (C-24), 33.0 (C-25), 19.7 (C-26), 19.4 (C-27), 17.4 (C-28). The structure was determined as 5α, 6β-epoxy-24 (R)-methylcholesta-7, 22-dien-3β-ol by comparison of spectra data with reference[10].

Compounds **1** and **2** showed antibacterial activity against *Staphylococcus aureus* at 2 μg/disc (inhibition zone = 7 mm), but no activity against other test microorganisms at 40 μg/disc. Compound **3** was inactive against all test microorganisms at 40 μg/disc.

3 Conclusion

Trichosetin (*N*-demethyl equisetin) was first isolated from dual culture of *Trichoderma harzianum* and *Catharanthus roseus* callus as a novel tetramic acid (2,4-pyrrolidinedione)[6]. It has a remarkable antimicrobial activity against the Gram-positive bacterial *Staphylococcus aureus* and *Bacillus subtilis*. Phytotoxicity assays revealed trichosetin inhibited root and shoot growth of all tested plant species[11]. Lateritin was first isolated from the mycelial cake of *Gibberella lateritium* IFO 7188 as a new inhibitior of acyl-CoA:cholesterol acyltransferase (ACAT)[8]. Our bioassay experiment showed it could also inhibit the growth of Gram-positive bacterial *Staphylococcus aureus*. 5α, 6β-epoxy-24 (R)-methylcholesta-7, 22-dien-3β-ol was first reported as an antitumor sterol from mycelia of *Cordyceps sinensis* (dong-chong-xia-cao)[10]. All of three compounds have different biological activities and it supported the opinion that endophytes are potential sources of novel natural products for exploitation in medicine.

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