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Biotransformation of Neoandrographolide by Endophytic Fungus from *Dendrobium officinale* Kimura et Migo

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Neoandrographolide is one of the principal constituents of ent-labdane diterpenoid lactones isolated from the aerial parts of the traditional herbal medicine *Andrographis paniculata* (Burm. F.) Nees. To obtain more types of diterpenoid derivatives and acquire compounds with better activities than the substrate, we used endophytic fungi to transform neoandrographolide for the first time. An endophytic fungus, namely, 2T12J01A, which was identified as *Fusarium oxysporum* based on 18S ribosomal DNA and the inte DNAl transcribed spacer of ribosomal DNA, was used to transform neoandrographolide and obtain four compounds: (**2**) isoneoandrographolide, (**3**) 14-deoxyandrographolide, (**4**) 8α,17β-epoxy-3,14-dideoxyandrographolide and (**5**) andrograpanin. The structural elucidation of compounds was achieved mainly by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR), distortionless enhancement by polarization transfer (DEPT), heteronuclear singular quantum correlation (HSQC), ^{1}H detected heteronuclear multiple quantum coherence (HMQC), nuclear overhauser spectroscopy and high-resolution electrospray ionization mass spectrometry experiments. Finally, the proposed biosynthetic pathways of neoandrographolide by 2T12J01A were established.

Keywords: Biotransformation, Neoandrographolide, *Endophytic fungus*, *Fusarium oxysporum*.

INTRODUCTION

Neoandrographolide is one of the principal constituents of ent-labdane diterpenoid lactones and has antiinflammatory[1,2], antiviral[3], anti-radical[4], hepatoprotective[5], and antihuman immunodeficiency *virus* properties[6]. Studies on the structural modifications of neoandrographolide have been increasingly conducted to improve its activities and solubility. Microbial transformation is an important approach for modifying bioactive substrates by microorganism enzymes. Microbial transformation presents advantages such as high stereoselectivity or high regio-selectivity and mild reaction conditions over chemical synthesis. Microbial transformation is considered a useful *in vitro* model for identifying metabolites *in vivo*[7]. The microbial biotransformation of neoandrographolide by *Aspergillus niger* (AS 3.739)[8] and *Mucor spinosus* (AS 3.2450)[9] have recently been reported. To obtain more types of diterpenoid derivatives and acquire compounds with better activities than the substrate, we used endophytic fungi to transform neoandrographolide for the first time.

Endophytes are bacterial or fungal microorganisms that colonize healthy plant tissues intercellularly and/or intracellularly without causing any apparent disease symptoms[10]. Endophytes have been isolated from almost every host plant studied so far. The production of highly bioactive metabolites such as taxol[11] and ambuic acid[12] was reported from several endophytes[3]. An adapted potential of biodegradation and a set of specific enzymes allow endophytes to transform natural products such as thioridazine[14], 2-benzoxazolinone and 2-hydroxy-1,4-benzoxazin-3-one[15].

The biotransformation of neoandrographolide was screened from more than 100 kinds of fungi, which were isolated from the medicinal plants *Andrographis paniculata* (Burm.f.) Nees, *Huperzia serrata* and *Dendrobium officinale* Kimura et Migo. 2T12J01A was selected as the biocatalyst for scaled-up biotransformation because of its good ability in converting neoandrographolide. In this work, the biotransformation of Compound **1** by 2T12J01A was investigated. Four metabolites (Fig. 1) were isolated and identified. The extraction, isolation and identification of the metabolites were also discussed.

EXPERIMENTAL

The NMR spectrum was recorded on a Bruker ARX-400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C) in MeOH-d_4

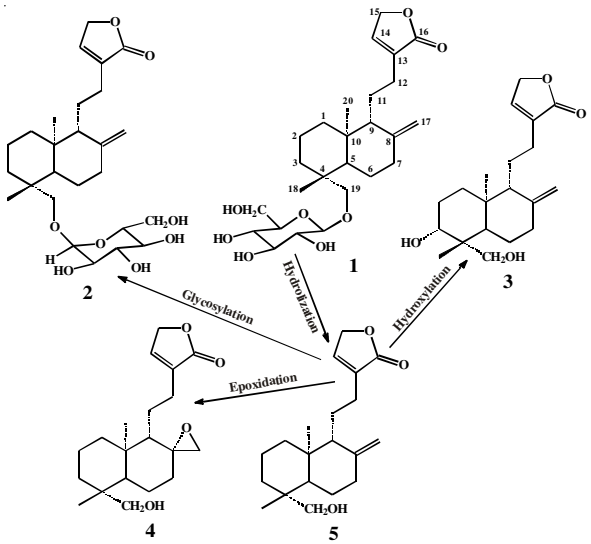

Fig. 1. Compound structures and their proposed biotransformation pathways

with TMS as the inte DNAl standard. Chemical shifts were expressed in δ (ppm) and coupling constants (J) were reported in hertz (Hz). Ultra-violet (UV) spectra were measured with a Shimadzu TU-1901 double beam UV-visible spectrophotometer (Beijing Purkinje General Instrument Co., Ltd, China). High-resolution electrospray ionization mass spectrometry (HRESIMS) spectra were obtained by using an Agilent 6210 time-of-flight mass spectrometer in *m/z*. Sample analysis were performed on an Agilent 1260 series high-performance liquid chromatography (HPLC) equipped with a CAPCELL PAK C18 MG S5 column (SHISEIDO CO., LTD., Japan) at 4.6 × 250 mm (5 µm) and UV a detector at 205 nm. The mixture of MeOH and H_2O (65:35, v/v) was used as the mobile phase and was held for 40 min. The flow rate was 1 mL/min. Preparative HPLC was performed with a CAPCELL PAK C18 MG S5 column (SHISEIDO CO., LTD. Japan) at 20 × 250 mm (5 µm) in a LC 3000 high liquid chromatograph apparatus equipped with a UV3000 detector (Beijing Innovation Hengtong Technology Co., Ltd., China) at 205 nm. The mixture of MeOH and H_2O (65:35, v/v) was used as the mobile phase and was held for 40 min. The flow rate was 16 mL/min. MeOH was HPLC grade and H_2O was double distilled in our laboratory. Thin layer chromatography (TLC) was conducted on silica gel HSGF254 and the spots were visualized by spraying with Legal and Kedde reagents. All analytic reagents were analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (China).

Substrate: Substrate neoandrographolide (with purity > 98 %) was purchased from Shanghai Yuanye Biological Technology Co., Ltd. (China). The structure of the substrate neoandrographolide was characterized by comparing its ^1H- and ^{13}C NMR data with reported data in literature.

Microorganism: 2T12J01A was isolated with a method described by Strobel *et al.*[16] with slight modifications. The leaves and stems of the plant were washed under running water for 6 h and by supersonic means. The leaves and stems were then rinsed five times with sterile distilled water. The leaves were sterilized with 75 % EtOH (v/v) for 1 min and 0.1 % mercuric chloride (v/v) for 5 min. The stems were sterilized with 75 % EtOH (v/v) for 3 min and 0.1 % mercuric chloride (v/v) for 8 min. Finally, the samples were rinsed six times in sterilized water and were cut into small sections by using a sterilized knife. The cut segments were placed on Petri dishes containing potato dextrose agar (PDA) and incubated at 28 °C in darkness. The Petri dishes with the last sterile water as the control were incubated under the same conditions. Pure cultures were then transformed to PDA plates. The purified fungi were stored in the PDA slant at 4 °C.

Medium: All culture and biotransformation experiments were performed in a potato medium, which was produced by the following procedure. Approximately 200 g of minced husked potato was boiled in H_2O for 0.5 h. The extract was then filtered and the filtrate was added with H_2O to 1 L after the addition of 20 g of glucose. The broth was autoclaved in individual Erlenmeyer flask at 121 °C and 15 psi for 20 min and cooled before incubation.

Culture and biotransformation procedures: The screening scale biotransformation of neoandrographolide was performed in 100 mL Erlenmeyer flasks containing 40 mL of potato medium. Microorganisms were transferred into the flasks from the slants. The flasks were placed on rotary shakers operating at 150 rpm at 28 °C. The substrate was dissolved in MeOH with a concentration of 10 mg/mL. After 48 h of culture, 0.4 mL of the substrate solution was added into the fermentation flasks. These flasks were maintained under the same conditions for an additional 120 h. Culture control consisted of fermentation blanks, in which microorganisms were grown without substrates but with the same amount of MeOH. Substrate control involved the use of a sterile medium containing the same amount of substrate without microorganisms, as well as incubation under the same conditions. The broths were filtered after fermentation and the filtrates were extracted with the same volume of ethyl acetate three times. The cells were extracted with MeOH by supersonic means. All extracts were evaporated to dryness under reduced pressure and then combined. The residues were dissolved in MeOH. The solutions were spotted on silica gel plates, which were developed by using $CHCl_3$/MeOH (9:1) and visualized by spraying with Legal's and Kedde's reagents. Both HPLC (Fig. 2) and TLC analyses revealed that 2T12J01A could biotransform the substrate.

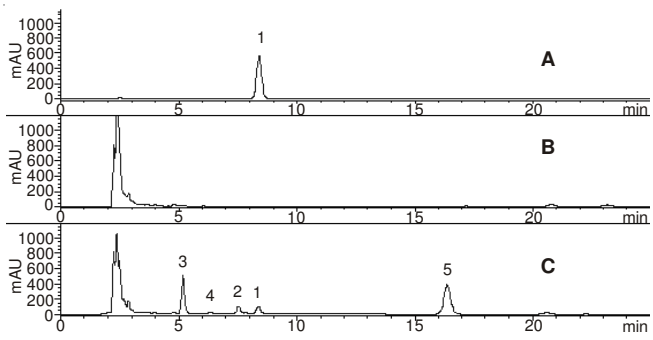

Fig. 2. HPLC chromatograms of biotransformation of neoandrographolide

Preparative scale biotransformation of neoandrographolide by 2T12J01A was conducted in 1000 mL Erlenmeyer flasks containing 400 mL potato medium. A total of 1 g neoandrographolide was transformed by the strain. Other procedures were the same as screening scale biotransformation.

Isolation of biotransformed products: Approximately 200 mL of brown residue was obtained from the fermented broth of 2T12J01A. The residue was developed on preparative HPLC with a CAPCELL PAK C18 MG S5 column at 20 × 250 mm (5 µm) (SHISEIDO CO., LTD., Japan) and eluted with MeOH/H_2O (65:35). Thereafter, Compounds **2** (56 mg), **4** (49 mg), **3** (128 mg) and **5** (163 mg) were obtained and 17 mg of Compound **1** remained after biotransformation.

RESULTS AND DISCUSSION

Identification of biotransformation products: Compound **1** was a white amorphous powder: UV (MeOH) λ_{max} (log ε) 206 (4.18) nm. The molecular formula was determined to be $C_{26}H_{40}O_8$ based on the HRESIMS peak at *m/z* 503.2630 ([M + Na]$^+$, $C_{26}H_{40}O_8Na^+$; calc. 503.2621). The infrared (IR) spectrum showed the presence of hydroxyl (3375 cm^{-1}), α,β-unsaturated-γ-lactone (1742 and 1648 cm^{-1}) and exo-methylene (904 cm^{-1}) groups. The ^{1}H- and ^{13}C NMR spectra of Compound **1** (Tables 1 and 2) were consistent with those of the neoandrographolide[17]. Therefore, compound **1** was identified as neoandrographolide.

Compound **2** was a white amorphous powder and was positive for Legal and Kedde reactions, thus suggesting the presence of an α,β-unsaturated-γ-lactone. TLC analyses showed that compound **2** was more polar than the substrate. The molecular formula was determined to be $C_{26}H_{40}O_8$ based on the HRESIMS peak at *m/z* 503.2628 ([M + Na]$^+$, $C_{26}H_{40}O_8Na^+$; calc. 503.2621). The ^{1}H and ^{13}C NMR spectra of compound **2**

TABLE-1
^{13}C NMR (δ) SPECTROSCOPIC DATA (100 MHz, MeOH-d_4) OF METABOLITES AND NEOANDROGRAPHOLIDE

Carbon	Comp. **1**	Comp. **2**	Comp. **3**	Comp. **4**	Comp. **5**
1	39.7	40.2	36.5	39.8	40.2
2	23.0	20.1	34.6	19.4	20.0
3	37.2	37.0	74.8	36.5	36.5
4	40.7	39.2	40.2	40.2	40.6
5	57.7	57.7	55.0	57.0	57.6
6	25.5	25.7	25.2	22.8	25.5
7	39.4	39.6	39.8	37.9	39.7
8	149.2	149.1	151.2	60.1	149.2
9	57.9	57.7	55.9	54.9	57.8
10	40.2	40.6	40.0	41.4	40.0
11	20.0	22.9	20.0	21.0	23.0
12	25.6	25.4	27.8	27.8	25.4
13	134.8	134.8	134.7	134.5	134.8
14	147.6	147.6	147.7	147.8	147.6
15	72.1	72.0	72.1	72.1	72.0
16	177.0	176.9	176.9	177.3	176.9
17	107.3	107.3	104.2	51.5	107.3
18	28.3	28.5	22.7	27.9	27.9
19	71.7	71.6	64.8	64.8	64.8
20	15.8	15.8	15.8	15.8	15.8
glc-1′	105.1	100.7	–	–	–
glc-2′	75.3	73.7	–	–	–
glc-3′	77.8	75.1	–	–	–
glc-4′	73.4	71.9	–	–	–
glc-5′	78.3	73.8	–	–	–
glc-6′	62.8	62.7	–	–	–

TABLE-2
^{1}H NMR (δ) SPECTROSCOPIC DATA (400 MHz, MeOH-d_4) OF METABOLITES AND NEOANDROGRAPHOLIDE

Carbon	Compound **1**	Compound **2**	Compound **3**	Compound **4**	Compound **5**
1	1.82 br d(13.2)	1.83 br d (13.2)	1.05 td (12.7 5.1)	1.80 br d (13.2)	0.98 td (13.3 13.7)
	1.10 td(13.2 13.7)	1.11 td (13.2 13.7)	1.68 m	0.97 td (13.2 13.7)	1.85 m
2	1.65 ddt(13.9 8.1)	1.62 ddt (13.9 8.1)	2.10 td (12.9 5.4)	1.58 ddt (13.9 8.1)	1.36 m
	1.47 m	1.51 m	1.88 m	1.35 m	1.59 ddt (13.9 8.2)
3	1.98 br d(13.9)	2.05 br d (13.9)	3.35 o	2.26 br d (13.9)	1.40 br d (13.9)
	0.97 br d(13.9)	1.02 br d (13.9)		1.01 br d (13.9)	2.42 br d (13.9)
5	1.28 m	1.29 m	1.05 dd (12.9 2.6)	1.08 m	1.08 dd
6	1.89m	1.90 m	1.71 m	1.80 m	1.75 m
	1.41m	1.41 m	1.39 qd (12.9 4.3)		1.36 qd (12.8 4.4)
7	2.44dt(12.9 4.3)	2.44 dt (12.9 4.3)	2.39 dt (12.9 4.3)	1.88 m	1.95 m
	1.99m	2.00 m	1.92 m	1.36 m	2.42 dt (12.8 4.4)
9	1.70t	1.69 t	1.59 t (4.9)	1.30 br s	1.62 t
11	1.83m	1.80 m	1.72 m	1.46 m	1.48 m
	1.69m	1.65 m	1.52 m	1.09 m	1.78 m
12	2.42 m	2.42 m	2.40 m	2.31 br t	2.10 m
	2.12 m	2.12 m	2.12 m	7.6	2.38 m
14	7.36 s	7.36 s	7.40 t (1.7)	7.35 o	7.35 m
15	4.84 brs	4.84 brs	4.81 d (1.7)	4.80 br s	4.82 m
17	4.88 brs	4.89 brs	5.25 br s	2.79 d (4.3)	4.62 s
	4.65brs	4.66 brs	5.05 br s	2.30 d (4.3)	4.85 s
18	1.05 s	1.06 s	1.35 s	1.29 s	0.95 s
19	4.12 d (9.6)	3.60 d (9.6)	4.78 d (10.0)	3.72 dd (10.7 4.8)	3.32 d (11.1)
	3.23 d (9.6)	3.48 d (9.6)	3.85 o	3.34 dd (10.7 4.7)	3.73 d (11.1)
20	0.73 s	0.74 s	0.67 s	1.03 s	0.68 S
glc-1′	4.19 d (7.7)	4.68 d (3.7)			
glc-2′	3.17 m	3.37 dd (3.7 9.7)			
glc-3′	3.34 m	3.62 dd (9.7 9.6)			
glc-4′	3.31 m	3.24 dd (9.5 9.3)			
glc-5′	3.25m	3.55 ddd (9.3 5.6 2.2)			
glc-6′	3.87 dd (2.2 11.8)	3.77 dd (2.2 11.8)			
	3.69 dd (5.3 11.8)	3.64 dd (5.6 11.8)			

(Tables 1 and 2) indicated the presence of glucose and andrograpanin moieties. The NMR data of compound **2** were closely similar to those of compound **1** except for the α-glucose moiety of compound **2** replacing the β-glucose moiety of compound **1**. The existence of the α-glucosyl moiety was inferred from the anomeric proton at δ 4.68 (d, *J* = 3.7 Hz, H-1') and was confirmed by the presence of one set of glucose carbon signals. The coupling constant (JH1', H2' = 3.7 Hz) of the anomeric proton proved the α-configuration of the sugar unit. This result suggested that compound **2** was andrograpanin-19-O-α-D-glucopyranoside, which was supported by the HMBC cross peak of the glucose anomeric proton (δ_H 4.68) and andrograpanin nucleus C-19 (δ_C 71.6) C. The configuration of compound **2** was elucidated by NOESY and was determined to be the same as those of compound **1** with α-orientations of H-19, H-20 and β-orientations of H-9, H-18. Thus, compound **2** was assigned and named isoneoandrographolide.

Compound **3** was a white amorphous powder and was positive for Legal and Kedde reactions, thus suggesting the presence of an α,β -unsaturated lactone. TLC analyses showed that Compound **3** was more polar than the substrate. The ^{1}H NMR and ^{13}C NMR spectra of compound **3** (Tables 1 and 2) were consistent with those of 14-deoxyandrographolide[18]. Therefore, Compound **3** was identified as 14-deoxyandro-grapholide.

Compound **4** was a white powder and was positive for Legal and Kedde reactions, thus suggesting the presence of an α,β-unsaturated lactone. TLC analyses showed that compound **4** was more polar than the substrate. The ^{1}H NMR and ^{13}C NMR spectra of compound **4** (Tables 1 and 2) were consistent with those of 8α,17β-epoxy-3,14-dideoxyandrographolide[9]. Therefore, compound **4** was identified as 8α,17β-epoxy-3,14-dideoxyandrographolide .

Compound **5** was a white amorphous powder and was positive for Legal and Kedde reactions, thus suggesting the presence of an α,β-unsaturated lactone. TLC analyses showed that Compound **5** was less polar than the substrate. The ^{1}H NMR and ^{13}C NMR spectra of compound **5** (Table- 1 and 2) were consistent with those of andrograpanin[19,20]. Therefore, compound **5** was identified as andrograpanin.

Identification of endophytic fungus

Fungal 18S ribosomal DNA (rDNA) and ribosomal DNA-inteDNAl transcribed spacer (rDNA-ITS) amplification and sequencing: The identities of the organisms were deter-mined based on partial or nearly full-length 18S rDNA and rDNA-ITS gene sequence analysis. Primers NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS8 (5'-TCCGCAGGTTCACCTACGGA-3') were used for the amplification of 18S rDNA. The universal primers ITS1 (5'-AACTCGGCCATTTAGAGGAAGT-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the ITS regions from the DNA extract. Fungal DNA was extracted from pure cultures by using a genomic DNA miniprep kit (Shanghai GeneRay Biotech Co., LTD., China) according to the manufacturer's instructions. Polymerase chain reaction was performed with the following cycles: 30 cycles of 94 °C for 10 s, 52 °C for 15 s and 72 °C for 10 s.

Phylogenetic analysis: The final sequence sets were then subjected to BLAST analysis and identities of ≥ 99 % were considered conspecific. 2T12J01A was identified as *Fusarium oxysporum*.

Nucleotide sequence accession numbers: The 18S rDNA and rDNA-ITS gene sequences of 2T12J01A were deposited in the GenBank database under accession numbers KC493355.1 and KC429789.1, respectively.

Conclusion

2T12J01A exhibited a good ability of transforming neoandrographolide. The biotransformation reaction involved hydrolization, hydroxylation, glycosylation and epoxidation. These processes are rarely reported in the microbial transformation process of natural coumarine. It might be related to the physical and chemical properties of neoandrographolide and the diversity of enzymes in the fungi. Based on the structures of the isolated metabolites, the possible biotransformation pathways were speculated (Fig. 1).

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