

A novel podophyllotoxin derivative with higher anti-tumor activity produced via 4'-demethylepipodophyllotoxin biotransformation by *Penicillium purpurogenum*

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ARTICLE INFO

Keywords:

Podophyllotoxin
Anti-tumor activity
Penicillium purpurogenum
Biotransformation

ABSTRACT

Biological modification of natural products is an important approach to improve the pharmacological properties. 4'-d-β-Demethylepipodophyllotoxin (4'-d-β-DMEP), a typical natural lignin, is used as the aglycon of topoisomerase inhibitor etoposide (VP-16). In this study, 4'-d-β-DMEP was isomerized into the novel product 4'-d-α-DMEP with 43.09% conversion by *P. purpurogenum* ACCC 32170. 4'-d-α-DMEP was largely produced extracellularly in the late fermentation stage, which was accompanied with the decrease of dry cell weight and the increase of culture pH. A constitutively expressed cytosolic enzyme was involved in the transformation and functioned when culture pH ranged from 7.0–10.0. The IC₅₀ value of 4'-d-α-DMEP was around 0.80 μM against tumor cells MCF-7, which was significantly reduced by 11.27 and 17.34 times than 4'-d-β-DMEP (i.e., 9.82 μM) and VP-16 (i.e., 14.67 μM). This study provided the novel podophyllotoxin derivative with higher anti-tumor activity and microbial transformation process, which would promote transferring lignans into the clinic trial.

1. Introduction

Plant natural product has been one common origin for drug discovery. Due to the limitation of plant natural product in anti-tumor activity and toxicity to normal cells, solubility and so on, these products are usually modified based on structure-activity relationship in delivering candidates to the clinic trial [1,2]. Microorganisms demonstrate a magnitude of biodiversity that surpasses those of plants, and can have exceptional metabolic adaptability [3]. Therefore, exploring the modification capacities of microorganisms will drives the biotechnology boom in drug development and stable industrial-scale production.

Lignans, characterized by the coupling of two phenylpropanoid units, show varied structures containing several stereogenic centers and possess a wealth of antiviral, antibacterial, and antineoplastic activities [4,5]. 4-d-β-Demethylepipodophyllotoxin (4'-d-β-DMEP) is a typical natural lignin (Fig. 1) and can be used as the aglycon of the topoisomerase inhibitor etoposide (VP-16), a chemotherapy regimen for various malignancies [6,7]. The structure-activity relationships among podophyllotoxin and its related compounds indicated that the *trans*-

fused lactone ring is required for anti-tumor activity [8,9]. In order to overcome the limitations of 4'-d-β-DMEP and develop new compounds with better anti-tumor activity, the lactone ring was modified with isomerization, opening and so on by plants and microorganisms [9–12]. Notably, the *trans*-fused lactone ring (C-2 α-configuration, C-3 β-configuration) of podophyllotoxin, was isomerized into the *cis*-fused lactone ring (C-2, 3 β-configurations) of picropodophyllin by *Sinopodophyllum emodi*, *P. peltatum* (plant) and *Penicillium melini* (microorganism), and the target was correspondingly changed from tubulin to the insulin-like growth factor-I receptor which triggered cell death [10,13,14]. Relative to the production by plant facing long production times and the intensive resource requirements associated with growing the natural plant hosts, microbial transformation for producing the desired compounds has the advantage of spending less time, convenience and high conversion [15–17]. Thus, transforming 4'-d-β-DMEP into novel compounds with higher anti-tumor activity via the lactone ring modification by microorganisms was conducted in this study.

Besides isomerizing the lactone ring, *Penicillium* also modified C-4 of

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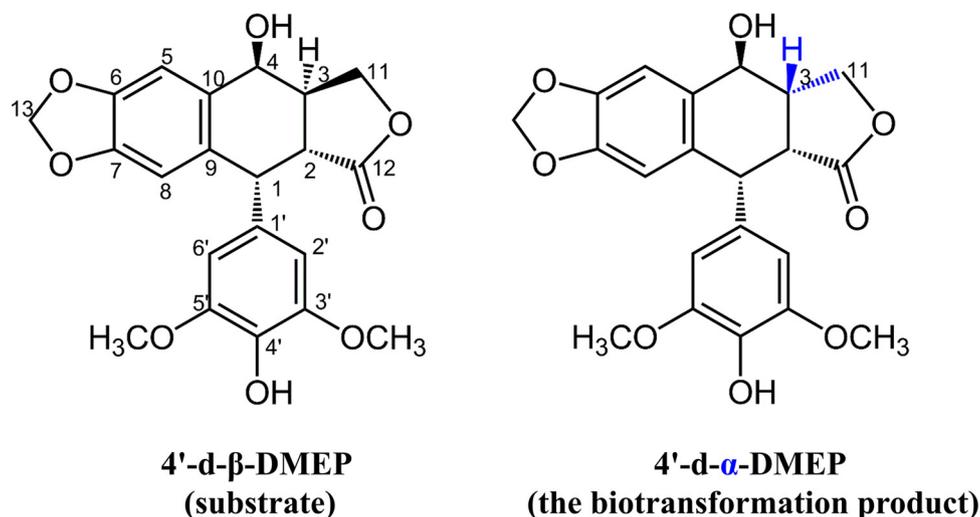


Fig. 1. Structures of 4'-d-β-DMEP (Demethylepipodophyllotoxin) and 4'-d-α-DMEP.

Table 1

Biotransformation of 4'-d-β-DMEP into 4'-d-α-DMEP by *Penicillium purpurogenum*.

<i>Penicillium purpurogenum</i>	Accession numbers	Conversion (%)
ACCC 32170	MN094405	43.09
ACCC 32414	MN094406	26.11
ACCC 32182	MN094404	11.72
CGMCC 3.7800	MN094396	19.94
CGMCC 3.8206	MN094399	19.86
CGMCC 3.8023	MN094398	18.32
CGMCC 3.5184	MN094391	17.55
CGMCC 3.5198	MN094394	16.09
CGMCC 3.5692	MN094395	13.44
CGMCC 3.7915	MN094397	11.81
CGMCC 3.5189	MN094393	10.70
CGMCC 3.5188	MN094392	8.21
CGMCC 3.5168	MN094390	8.21
CGMCC 3.8909	MN094400	0
CGMCC 3.4363	MN094388	0
CFCC 87382	MN094408	8.81
CCTCC AF93079	MN094408	7.79

Note: ACCC, purchased from Agricultural Culture Collection of China; CGMCC, purchased from China General Microbiological Culture Collection Center; CCTCC, purchased from China Center for Type Culture Collection; CFCC, purchased from China Forestry Microbial Species Preservation Center.

C-ring in 4'-d-β-DMEP. *Penicillium purpurogenum* Y.J. Tang catalyzed the ligation of 4'-d-β-DMEP with the sulfur-containing compounds via C-S bond formation, and the product 4β-(1,2,4-triazole-3-yl)sulfanyl-4-deoxy-podophyllotoxin exhibited stronger anti-tumor activity (approximately 200-fold higher than VP-16) [18]. In view to the strong modifying capacity of *Penicillium purpurogenum*, we purchased 17 strains of *P. purpurogenum* from the centers collecting microbial culture in China and tested their capacities to modify the lactone ring of 4'-d-β-DMEP. Subsequently, the biotransformation process of the new product was analyzed and the transformation model was provided. Finally, the anti-tumor activity and toxicity to normal cells of the new product were measured. The results will promote the discovery of novel podophyllotoxin derivatives with higher anti-tumor activity and transferring lignans into the clinic trial.

2. Materials and methods

2.1. Media and chemicals

The slant and the preculture have been prepared by the methods

described previously [12,18]. Liquid seed medium was consisted of glucose (30 g/L), NaNO₃ (2 g/L), K₂HPO₄ (1 g/L), NaCl (0.5 g/L), MgSO₄·7H₂O (0.5 g/L) and FeSO₄·7H₂O (0.01 g/L), and the pH was adjusted to 6.6 with 2.0 M HCl. The biotransformation experiments were carried out in the above mentioned liquid seed medium and glucose was replaced by the substrate 4'-d-β-DMEP (0.1 g/L) used as the sole carbon source. The standard 4'-d-β-DMEP (pure 98%) were purchased from Shanxi Huisheng Medicament Technology Company, Ltd. (Shanxi, China). Acetonitrile was HPLC (high-performance liquid chromatography)-grade, and all other chemicals used for extraction and isolation were analytic grade and commercially available.

2.2. Microorganisms

P. purpurogenum ACCC 32170, ACCC 32414 and ACCC 32182 were purchased from Agricultural Culture Collection of China (ACCC, Beijing, China), *P. purpurogenum* CGMCC 3.7800, 3.8206, 3.8023, 3.5184, 3.5198, 3.5692, 3.7915, 3.5189, 3.5188, 3.5168, 3.8909 and 3.4363 were purchased from China General Microbiological Culture Collection (CGMCC) Center (Beijing, China), *P. purpurogenum* CFCC 87382 was purchased from China Forestry Microbial Species Preservation Center (Beijing, China), *P. purpurogenum* CCTCC AF 93079 was purchased from China Center for Type Culture Collection (CCTCC, Wuhan, China). ITS (the internal transcribed spacer) sequences of these fungal strains were amplified by using the primers TCCGTAGGTGAAC CTGCGG and TCCTCCGCTTATTGATATGC [19,20], and these sequencing data were submitted to NCBI for obtaining the accession numbers (Table 1).

2.3. Identification of the new products

To test the ability of *P. purpurogenum* to transform 4'-d-β-DMEP, a biotransformation experiment was carried out in a 250 mL Erlenmeyer flask containing 90 mL liquid seed medium, 10-mL aliquot of liquid seed culture and 4'-d-β-DMEP with a final concentration of 0.1 g/L. The culture was incubated for 9 d at 28 °C on a rotary shaker at 120 rpm, a 2 mL aliquot of liquid seed culture was sampled dynamically for detecting the new product. The broth was broken by 5-s pulsed sonication for 23 min, and then extracted by using dichloromethane. The organic layer was collected and evaporated in vacuum at 45 °C, the remainder was dissolved in acetonitrile and analyzed periodically for detecting the decrease of 4'-d-β-DMEP and the appearance of new products on a Reprosil-Pur Basic C18 column (4.6 mm × 250 mm × 5 μm) from Dr. Maisch GmbH (Germany) by using a liquid chromatography DGU-20A_{5R} system (Shimadzu Corporation, Japan). The optimized mobile

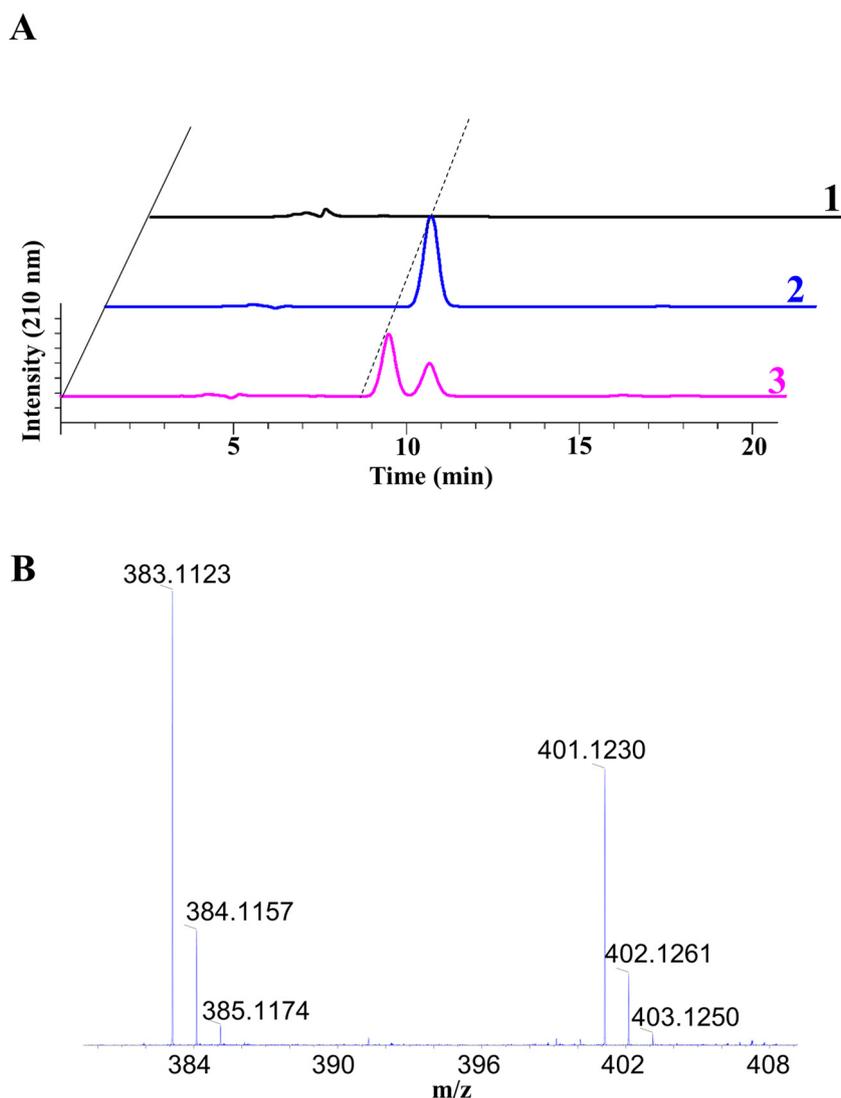
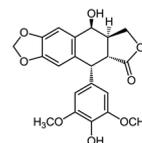


Fig. 2. 4'-d-β-DMEP was transformed into its isomer. (A) Detection of the new product transformed from the substrate 4'-d-β-DMEP. The new product with the retention time of 10.67 min was detected in the microbial transformation system 3 added with 0.25 mM 4'-d-β-DMEP relative to the substrate control 2 (the substrate was with the retention time 9.49 min) and pure culture of *Penicillium purpurogenum* 1; (B) Identifying the new product as the molecular formula of C₂₁H₂₀O₈ by high-resolution mass spectrometry.

phase consisted of acetonitrile-water (40:60, v/v). The column oven temperature was set to 40 °C, and the flow rate was 0.6 mL/min. The detection wavelength was 210 nm. For preparing the new product, the biotransformation samples were treated using the above mentioned method and separated on a Reprosil-Pur Basic C18 column (10 mm × 250 mm × 5 μm) from Dr. Maisch GmbH (Germany) by using AutoPurification HPLC/MS (Waters, USA). The optimized mobile phase consisted of acetonitrile-water (30:70, v/v), and the flow rate was 8 mL/min. Purities of the new product were measured by careful integration of all peaks detected by the above mentioned liquid chromatography DGU-20A_{SR} system and then the standard curve was established. The conversion rate of the new product was calculated by measuring the molar concentration ratio of the product to the initial substrate. In addition, NMR (nuclear magnetic resonance) spectra were obtained on a Bruker Ascend 400 (Billerica, MA) spectrometer, chemical shifts are reported as parts per million (ppm) relative to TMS (tetramethyl silane) in DMSO (dimethyl sulfoxide); HR-ESI-MS (high resolution electrospray ionisation mass spectrometry) spectrum was obtained with Thermo Scientific™ Orbitrap Fusion™ (Thermo Fisher Scientific, USA). Melting points of the substrate and new product were measured by automatic melting point apparatus MP300 (Suzhou

Jiangdong Precision Instrument CO., Ltd, China).

Substrate 4'-d-β-Demethylepipodophyllotoxin was a white solid [21], the melting point was 246–248 °C.



¹H NMR δ_H (400 MHz, Dimethyl sulfoxide-d₆) 8.21 (s, 1 H), 6.90 (s, 1 H), 6.46 (s, 1 H), 6.17 (s, 2 H), 5.95 (d, *J* = 8 Hz, 2 H), 5.38 (d, 1 H), 4.69 (dd, *J* = 6.0, 3.6 Hz, 1 H), 4.45 (d, *J* = 5.2, 1 H), 4.27 (t, *J* = 3.9 Hz, 1 H), 4.13 (dd, *J* = 10.8, 8.3, 1 H), 3.58 (s, 6 H), 3.18 (dd, *J* = 14.2, 5.2 Hz), 2.73 (dddd, *J* = 14.4, 11.2, 7.7, 3.5, 1 H). ¹³C NMR δ_C (101 MHz, Dimethyl sulfoxide-d₆) 175.27 (C), 147.46 (C), 147.46 (C), 146.49 (C), 146.49 (C), 135.11 (C), 133.56 (C), 131.80 (C), 130.59 (C), 110.19 (CH), 109.80 (CH), 108.87 (CH), 108.87 (CH), 101.49 (CH₂), 67.95 (CH), 65.23 (CH₂), 56.30 (CH₃), 56.30 (CH₃), 43.37 (CH), 38.29 (CH), 38.29 (CH).

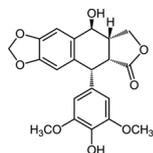
The new product purified was a white solid, the yield was 11.33%,

Table 2
 ^1H and ^{13}C NMR data of 4'-d- β -DMEP and its biotransformation product (δ values in ppm).

position	4'-d- β -Demethylepipodophyllotoxin		Biotransformation product	
	^1H (ppm)	^{13}C (ppm)	^1H (ppm)	^{13}C (ppm)
1	4.45 ($J=5.2$ Hz)	43.37	4.23 ($J=2.8$ Hz, $J=8.8$ Hz)	45.13
2	3.18 ($J=14.2, 5.2$ Hz)	38.29	3.68 ($J=10.6, 3.2$ Hz)	43.34
3	2.73 ($J=14.4, 11.2, 7.7, 3.5$ Hz)	38.29	3.16 ($J=9.7, 7.4, 3.6$ Hz)	43.34
4	4.69 ($J=6.0, 3.6$ Hz)	67.95	4.48 ($J=4.8$ Hz)	67.63
4-OH	5.38	–	5.55	–
5	6.90	109.80	6.95	106.05
6	–	146.49	–	146.51
7	–	146.49	–	146.51
8	6.46	110.19	6.71	109.43
9	–	130.59	–	133.27
10	–	133.56	–	131.74
11	4.27 ($J=3.9$ Hz), 4.13 ($J=10.8, 8.3$ Hz)	65.23	4.21, 4.07 ($J=9.4, 3.6$ Hz)	65.44
12	–	175.27	–	179.22
13	5.95, 5.95 ($J=8$ Hz)	101.49	5.95, 5.95 ($J=4$ Hz)	101.15
1'	–	135.11	–	134.65
2'	6.17	108.87	6.38	105.70
3'	–	147.46	–	148.25
3'-OMe	3.58	56.30	3.65	56.57
4'	–	131.80	–	131.09
4'-OH	8.21	–	8.25	–
5'	–	147.46	–	148.25
5'-OMe	3.58	56.30	3.65	56.57
6'	6.17	108.87	6.38	105.70

^1H and ^{13}C NMR spectra were obtained with dimethyl sulfoxide (DMSO).

the melting point was 246–248 °C.



^1H NMR δ_{H} (400 MHz, Dimethyl sulfoxide- d_6) 8.25 (s, 1 H), 6.95 (s, 1 H), 6.71 (s, 1 H), 6.38 (s, 2 H), 5.95 (d, $J = 4$ Hz, 2 H), 5.55 (d, 1 H), 4.48 (t, $J = 4.8$ Hz, 1 H), 4.23 (d, $J = 2.8$, t, $J = 8.8$ Hz, 2 H), 4.07 (dd,

$J = 9.4, 3.6$ Hz, 1 H), 3.68 (dd, $J = 10.6, 3.2$ Hz, 1 H), 3.65 (s, 6 H), 3.16 (tdd, $J = 9.7, 7.4, 3.6$ Hz). ^{13}C NMR δ_{C} (101 MHz, Dimethyl sulfoxide- d_6) 179.22 (C), 148.25 (C), 148.25 (C), 146.51 (C), 146.51 (C), 134.65 (C), 133.27 (C), 131.74 (C), 131.09 (C), 109.43 (CH), 106.05 (CH), 105.70 (CH), 105.70 (CH), 101.15 (CH₂), 67.63 (CH), 65.44 (CH₂), 56.57 (CH₃), 56.57 (CH₃), 45.13 (CH), 43.34 (CH), 43.34 (CH). HRMS (ESI) m/z calcd. for C₂₁H₂₀O₈⁺ 401.1222 [M+H]⁺, found 401.1230.

2.4. The biotransformation of 4'-d- β -DMEP into 4'-d- α -DMEP

To locate and characterize the enzyme system responsible for 4'-d- β -DMEP biotransformation, two batches of cells were grown in the presence and absence of 0.25 mM 4'-d- β -DMEP, substrate control was run with the addition of 0.25 mM 4'-d- β -DMEP and without inoculation of *P. purpurogenum*. At day 8, the cells were harvested by filtration through 4 layers of cheese cloth and washed three times with cold ultrapure water. Mycelial pellets were cooled in liquid nitrogen, vigorously grinded and were suspended in Tris–HCl buffer (pH 7.0, 20 mL). The suspensions were centrifuged at 9000 g for 20 min to remove cell debris and undisturbed cells, and then the pellet (membrane protein fraction) and supernatant (soluble protein fraction) were separated following the method described previously [22]. Each fraction was adjusted to 20 mL with Tris–HCl buffer (pH 7.0) and the abilities to transform 4'-d- β -DMEP into 4'-d- α -DMEP were tested. The reaction mixture (1 mL) contained 0.25 mM 4'-d- β -DMEP, 1.0 mg protein (cell-free extraction), and 50 mM Tris–HCl buffer, pH 7.0. Reactions were performed at 30 °C. Samples were analyzed by using a liquid chromatography DGU-20A_{SR} system. The enzymatic activities for transforming 4'-d- β -DMEP into 4'-d- α -DMEP in 50 mM citric acid buffer (pH 4.0–5.0), PIPES buffer (pH 6.0–7.0), Tris–HCl buffer (pH 7.5–9.0) or Na₂CO₃–NaHCO₃ buffer (pH 9.5–10.0) were measured. Total protein was determined with bovine serum albumin as a standard, the experiments were performed in duplicate. One unit of enzyme defined as the amount that catalyzes the formation of 1 nmol 4'-d- α -DMEP per min.

To analyze the effect of glucose addition on the biotransformation of 4'-d- α -DMEP, glucose with the concentrations of 0 g/L, 5 g/L, 10 g/L, 15 g/L and 20 g/L was added into the liquid seed media. Subsequently, dry cell weight, the concentrations of glucose, the substrate and product and medium pH were monitored dynamically [23]. Each data point is an average of three independent experiments with an error bar. Significant difference between the biotransformation systems added with 0 g/L and 5 g/L glucose were measured by using the *t*-test for independent samples. An asterisk indicates significant ($P < 0.05$)

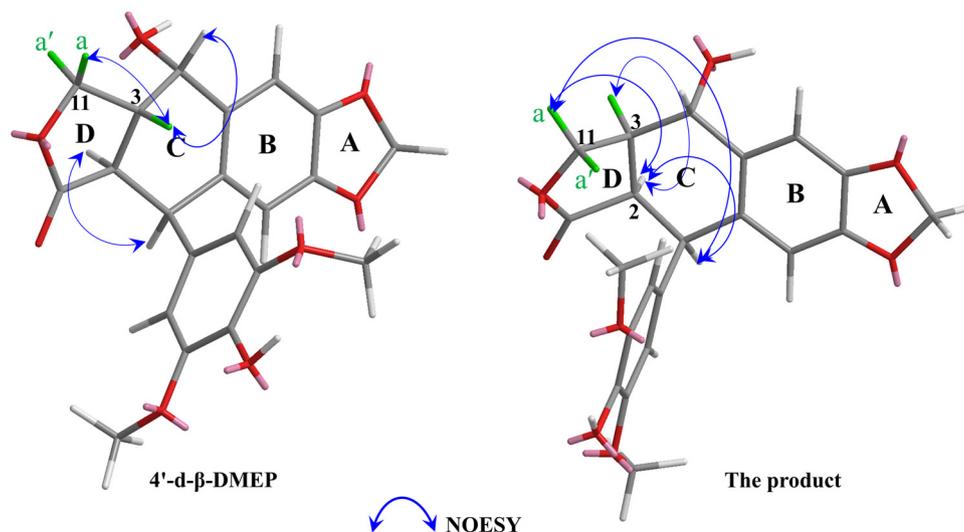


Fig. 3. The key NOESY correlations of the substrate and product.

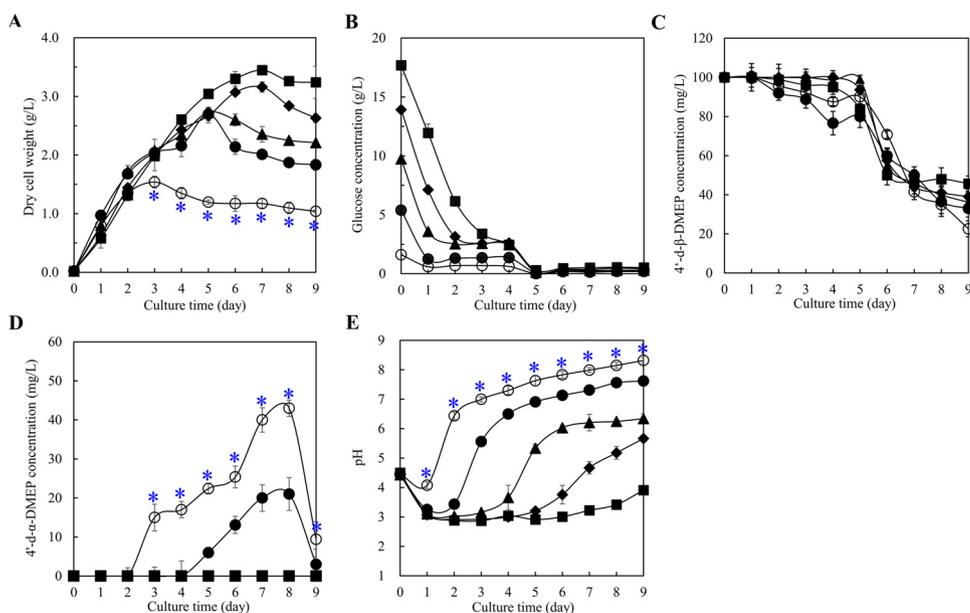


Fig. 4. Identifying the key factors impacting the biotransformation of 4'-d-β-DMEP into 4'-d-α-DMEP. (A) The effect of glucose concentrations on the growth of *Penicillium purpurogenum*. Microbial transformation system without glucose addition, ○; microbial transformation system with the addition 5 g/L glucose, ●; with the addition of 10 g/L glucose, ▲; with the addition of 15 g/L glucose, ◆; with the addition 20 g/L glucose, ■. Dynamic changes of glucose concentrations (B), medium pH (C), and 4'-d-β-DMEP concentrations (D) during the culture process. (E) The effect of glucose concentrations on the production of 4'-d-α-DMEP.

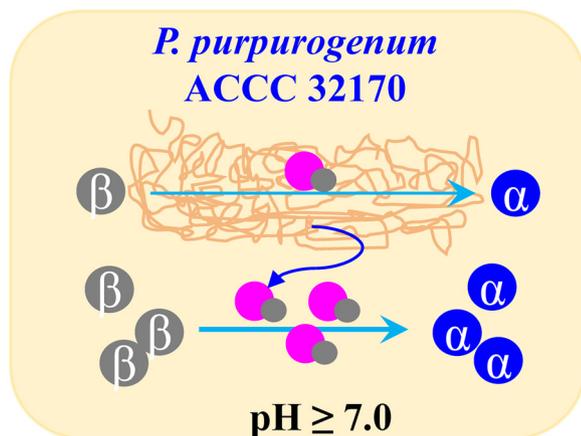


Fig. 5. The model for the biotransformation of 4'-d-β-DMEP into 4'-d-α-DMEP by *Penicillium purpurogenum*. The enzyme responsible for the biotransformation of 4'-d-β-DMEP into 4'-d-α-DMEP was constitutively expressed and located intracellularly. The degeneration of *Penicillium purpurogenum* increased the medium pH and resulted in the leakage of the intracellular enzyme, which triggered the large production of 4'-d-α-DMEP.

difference from the corresponding the biotransformation system added with 5 g/L glucose. Statistical analyses were conducted by using SPSS software package 19.0 [24].

To determine the extracellular and intracellular 4'-d-β-DMEP and 4'-d-α-DMEP, the fermentation broths after 8 days cultivation were separated by filtrating through 4 layers of cheese cloth, the media were extracted with CH_2Cl_2 (the extracellular 4'-d-β-DMEP and 4'-d-α-DMEP); the mycelia were cooled in liquid nitrogen, vigorously grinded and then extracted with CH_2Cl_2 (the intracellular 4'-d-β-DMEP and 4'-d-

α-DMEP). The organic layers were evaporated, the remainders were dissolved in acetonitrile and then chromatographed by the method described above, respectively.

2.5. Anti-tumor activity and cytotoxicity assays

Human hepatocellular liver carcinoma (HepG2), human cervical cancer cells (HeLa), human breast cancer cells (MCF-7) and human liver cells (HL-7702), immortalized human cervical epithelial cells (H8), human breast epithelial cells (HMEC) were adopted in this study for evaluating the anti-tumor activity and cytotoxicity of 4'-d-α-DMEP, which followed the method described by Zhang [25]. All the above mentioned cells were grown in tissue culture T-25 flasks in a Thermo Scientific CO_2 incubator in a humidified environment at 37 °C and 5% CO_2 . HepG2, HeLa, HL-7702 and H8 cells were cultured in RPMI 1640 medium (Gibco, Thermo Fisher) containing 10% fetal calf serum (Sigma) and 1% penicillin C/streptomycin (Biosharp); MCF-7 and HMEC were cultured in high-glucose DMEM (HyClone™) containing 10% fetal calf serum and 1% penicillin C/streptomycin. After these cells were freshly trypsinized in the logarithmic growth phase, cell suspensions were seeded in a 96-well microtiter plate and treated with 4'-d-β-DMEP, 4'-d-α-DMEP and VP-16 with varying concentrations (10-fold dilutions starting from 100 mM). After 48 h, the attached cells were treated with 100 mL of MTT (2 mg/mL) solution for 4 h at 37 °C, and the absorbance of each sample was measured at 492 nm using a microplate reader (Cytation 3, BioTek) with Gen5 software (BioTek). IC_{50} values were calculated by using Orange 8 software and the average of triplicate experiments.

2.6. Cell lysis and immunoblotting analysis

MCF-7 cells were seeded at a density of 2×10^5 cells per well and

Table 3
In vitro anti-tumor activity and toxicity of 4'-d-α-DMEP, 4'-d-β-DMEP and VP-16.

Compound	IC_{50} (μM)					
		HepG2	HL-7702	HeLa	H8	MCF-7
4'-d-α-DMEP	0.23 ± 0.01	45.07 ± 0.02	2.50 ± 0.02	43.05 ± 0.27	0.80 ± 0.04	45.52 ± 0.03
4'-d-β-DMEP	0.40 ± 0.03	50.82 ± 0.11	9.81 ± 0.06	42.86 ± 0.09	9.82 ± 0.02	50.48 ± 0.12
VP-16	17.29 ± 0.12	23.42 ± 0.08	18.23 ± 0.21	18.23 ± 0.04	14.67 ± 0.29	30.13 ± 0.23

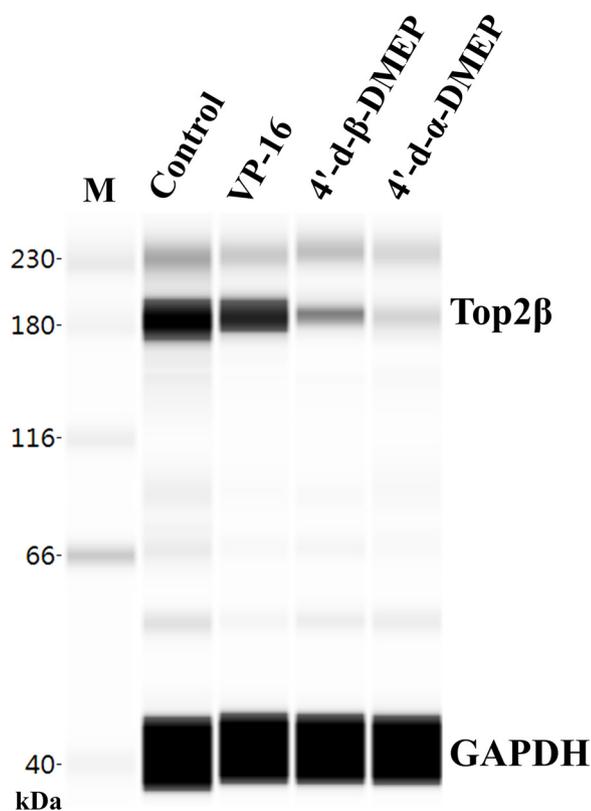


Fig. 6. 4'-d- α -DMEP, 4'-d- β -DMEP and VP-16 induce a decrease in the hTOP2 β levels in MCF-7 cells. Western blot assays show the effect of inhibitor addition on hTOP2 β expression levels in MCF-7 cells. MCF-7 cells were treated with 10 μ M 4'-d- α -DMEP, 4'-d- β -DMEP and VP-16 for 48 h and lysed with the alkaline lysis procedure as described under "Materials and methods". Lysates were analyzed by immunoblotting with anti-hTOP2 β .

then treated with 10 μ M VP-16, 4'-d- β -DMEP and 4'-d- α -DMEP at 37 $^{\circ}$ C for 48 h. The cells were collected, washed with 2 mL PBS (phosphate buffer saline, pH 7.4) and then lysed with 250 μ L RIPA lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM sodium pyrophosphate, 25 mM β -glycerophosphate, 1 mM EDTA, 1 mM Na₃VO₄, 0.5 μ g/mL leupeptin). The samples were cooled on ice and centrifuged for 5 min (4 $^{\circ}$ C and 12,000 rpm). The supernatants were then analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted with hTOP2 β antibody [26–28].

3. Results

3.1. *P. purpurogenum* ACCC 32170 with high capacity for isomerizing 4'-d- β -DMEP into 4'-d- α -DMEP with high capacity for isomerizing 4'-d- β -DMEP into 4'-d- α -DMEP

In consideration of the strong capacity of *P. purpurogenum* to transform podophyllotoxins, we collected 17 strains of *P. purpurogenum* from the centers collecting microbial culture in China and then tested their transformation capacities. 4'-d- β -DMEP, the aglycon of the topoisomerase inhibitor VP-16 used in dozens of chemotherapy regimens for various malignancies [6,29], was the substrate (Fig. 1). *Penicillium purpurogenum* transformed 4'-d- β -DMEP into a new product, for which is accompanied with the decrease of 4'-d- β -DMEP (the retention time is 9.49 min) in the microbial transformation system (Fig. 2A). Notably, the new product with the retention time of 10.67 min was found in the microbial transformation systems except the systems inoculated with CGMCC 3.8909 and CGMCC 3.4363 (Table 1). The new product was isolated, analyzed by the high-resolution mass spectrometry and then

its molecular formula was identified as C₂₁H₂₀O₈ (m/z [M+H]⁺ 401.1230, calculated 401.1222), the same as the substrate 4'-d- β -DMEP (Fig. 2B). This may be indicative of the isomerization of the substrate.

ACCC 32170 was found to be with the highest transformation ability and the conversion reached 43.09% (Table 1). Therefore, the new product was largely synthesized by using ACCC 32170 as the biocatalyst. In total, 1.50 g of 4'-d- β -DMEP was used to prepare the new product at the beginning of the biotransformation process, and the production reached its maximum value of 43.09 mg/L. After the biotransformation broth was lysed, extracted and isolated, a total of 0.17 g white solid residue was obtained, and its purity was 96.13% detected by the liquid chromatography.

The new product was analyzed by ¹H and ¹³C NMR and then these spectral data were compared with those of 4'-d- β -DMEP (Fig. S1–2). The aromatic units (δ_{H} 8.25, 6.95, 6.71, 6.38; δ_{C} 148.25, 146.51, 134.65, 133.27, 131.74, 131.09, 109.43, 106.05, 105.70), methylenedioxy (δ_{H} 5.95, δ_{C} 179.22) and methoxyl groups (δ_{H} 3.65, δ_{C} 56.57) have no obvious changes, while the aliphatic unit (δ_{H} 4.48, 4.23, 4.07, 3.68, 3.16; δ_{C} 67.63, 65.44, 45.13, 43.34) changed significantly relative to the ¹H and ¹³C NMR of 4'-d- β -DMEP (Table 2). Hence, the coupling constants of both the substrate and product in the aliphatic unit were calculated and compared (Fig. S3). Obvious changes had taken place in the H-3 and H-11. The coupling constants of H-3 in 4'-d- β -DMEP (δ_{H} 2.73, dddd, J = 14.4, 11.2, 7.7, 3.5) were changed into 9.7, 7.4 and 3.6 in the product (δ_{H} 3.16, tdd); and the coupling constants of H-11a (δ_{H} 4.13, dd, J = 10.8, 8.3) were changed into 9.4 and 3.6 (δ_{H} 4.07, dd). More importantly, we found that H-3 and H-11a were correlated with H-2 in the NOESY (nuclear overhauser effect spectroscopy) spectrum of the product. While, the correlation ship does not existed in 4'-d- β -DMEP (Fig. 3). These data reflected the reversion of bonding configuration at the interface between C-3 and C-11 from β (the substrate) to α (the product).

3.2. The model for transforming 4'-d- β -DMEP into 4'-d- α -DMEP by *P. purpurogenum* ACCC 32170

The isomerization is a more direct, convenient and efficient process relative to *de novo* biosynthesis and chemical synthesis. The enzyme responsible for 4'-d- α -DMEP production was located in the cytosolic compartment for the cytosolic soluble enzyme (24.17 U/mL) retained 94.71% of the total 4'-d- β -DMEP biotransformation activities present in the cell crude extract (25.52 U/mL). Additionally, cells pretreated with 4'-d- β -DMEP and non-pretreated cells showed the biotransformation activities of 3.63 \pm 0.15 and 3.55 \pm 0.07 U/mg protein, and 4'-d- α -DMEP was not detected in the substrate control. This indicated that a constitutive cytosolic enzyme may be responsible for the biotransformation of 4'-d- β -DMEP into 4'-d- α -DMEP.

According to the cytosolic location and constitutive activity of the biotransformation enzyme, we put forward a hypothesis: whether the increase of cell intensity would promote the biotransformation of 4'-d- β -DMEP into 4'-d- α -DMEP. Thus, different concentrations of glucose were added into the biotransformation media containing 0.1 g/L 4'-d- β -DMEP for increasing cell intensity. We found that glucose addition indeed enhanced cell density as depicted in Fig. 4A, and glucose in different treatments was almost completely consumed at day 5 (Fig. 4B). Corresponding to the glucose profiles, the concentrations of 4'-d- β -DMEP decreased sharply after 5 days cultivation of *P. purpurogenum* ACCC 32170 (Fig. 4C). While we only detected the accumulation of 4'-d- α -DMEP in the treatments of no glucose addition and 5 g/L glucose addition, and the former produced more 4'-d- α -DMEP (Fig. 4D). These data indicated the production of 4'-d- α -DMEP was not correlated with cell density.

In the treatments of no glucose addition and 5 g/L glucose addition, we noticed that medium pH increased after 1 day cultivation and approached to neutral or alkaline conditions at the late stage of fermentation (Fig. 4E). This was accompanied with the decrease of cell density

and the accumulation of 4'-d- α -DMEP, suggesting that medium pH and cell degeneration were closely related with the production of 4'-d- α -DMEP (Fig. 4A-E). Subsequently, we found that the concentrations of extracellular 4'-d- β -DMEP and 4'-d- α -DMEP (19.53 ± 1.65 and 14.57 ± 0.45 mg/L) was 2.03 and 47.57 folds higher than those of the intracellular 4'-d- β -DMEP and 4'-d- α -DMEP (6.44 ± 0.79 and 0.13 ± 0.01 mg/L) in the treatment of no glucose addition, which indicated the biotransformation of 4'-d- β -DMEP into 4'-d- α -DMEP mostly occurred extracellularly. Buffer pH for enzymatic transformation of 4'-d- β -DMEP into 4'-d- α -DMEP ranged from pH 7.0–10.0 (Fig. S4). Hence, we inferred that the biotransformation of 4'-d- β -DMEP into 4'-d- α -DMEP mostly happened extracellularly under neutral and alkaline conditions, which was achieved by the leakage of the intracellular enzyme into the outside (Fig. 5).

3.3. The anti-tumor activity and toxicity of 4'-d- α -DMEP

The structure-activity relationships among podophyllotoxin and the related compounds have suggested that the *trans*-fused lactone ring is required for anti-tumor activity [8,9]. To investigate the influence of the reversion of bonding configuration on anti-tumor activity, *in vitro* cytotoxicity of 4'-d- α -DMEP, 4'-d- β -DMEP and VP-16 on three human tumor cell lines (i.e., HepG2, HeLa, and MCF-7) and normal human cell lines (i.e., HL-7702, H8 and HMEC) was tested. Relative to the reference compounds 4'-d- β -DMEP and VP-16, the new product 4'-d- α -DMEP exhibited higher inhibitory effect on the three human tumor cell lines; especially on MCF-7, the IC₅₀ value of 4'-d- α -DMEP was around 0.80 μ M on tumor cells MCF-7, which was significantly reduced by 11.27 and 17.34 times than 4'-d- β -DMEP (9.82 μ M) and VP-16 (14.67 μ M) (Table 3). While its cytotoxicity to normal human cell lines approached to 4'-d- β -DMEP and was obviously weaker than VP-16 (Table 3). This indicated that the reversion of bonding configuration at the interface between C-3 and C-11 improved the anti-tumor activity.

The substrate 4'-d- β -DMEP is the immediate precursor of chemotherapeutic VM-26 and VP-16 which induced a decrease of the TOP2 β levels [27]. Hence, the inhibitory effect of the new product 4'-d- α -DMEP on the levels of TOP2 β in MCF-7 was investigated. As shown in Fig. 6, the treatment of 4'-d- β -DMEP, 4'-d- α -DMEP and VP-16 on MCF-7 cells all resulted in the decrease of the TOP2 β levels. Meanwhile the inhibitory effect of 4'-d- α -DMEP on TOP2 β was obviously higher than that of VP-16. These data indicated that the reversion of bonding configuration at the interface between C-3 and C-11 did not change the target.

4. Discussion

Structural modification of 4'-d- β -DMEP by plants has yielded the potent anticancer agent VP-16 [7]. Relative to the modification by plants, microbial transformation for producing the desired compounds has the advantage of time saving, convenience and high conversion [15–17]. In this study, 4'-d- β -DMEP was isomerized into the novel podophyllotoxin derivative 4'-d- α -DMEP. Among the collected 17 strains, *P. purpurogenum* ACCC 32170 was with the highest transformation ability and the conversion reached 43.09%. The key enzyme involved in the transformation process was shown to be a constitutively expressed cytosolic protein which catalyze the production of 4'-d- α -DMEP under neutral and alkaline conditions. Correspondingly, the isomerization of 4'-d- β -DMEP into 4'-d- α -DMEP by *P. purpurogenum* ACCC 32170 occurred under neutral and alkaline conditions and 4'-d- α -DMEP was accumulated at the late-stage of fermentation. Comparing with 4'-d- β -DMEP and VP-16, 4'-d- α -DMEP exhibited higher inhibitory effect on human tumor cell lines especially on MCF-7. While its cytotoxicity to normal human cell lines was weaker than VP-16.

The epimerizations at the lactone ring occurred *in vivo*, such as picrotoposide found in plasma, serum, urine, cerebrospinal fluid, and liver [30]. The epimerizations were inferred to be catalyzed by

isomerase. Enzymatic assays of *P. purpurogenum* ACCC 32170 indicated that the enzyme was constitutively expressed cytosolic protein. While the increase of cell density did not promote the transformation of 4'-d- β -DMEP into 4'-d- α -DMEP. 4'-d- α -DMEP was accumulated rapidly after 2 days of inoculation in the medium without glucose addition, which was accompanied with the increase of medium pH and the decrease of cell density. We also noticed that much more the amount of substrate was located extracellularly. These results suggested that the enzyme entered into the medium in the later stage of culture and thrived the transformation of 4'-d- β -DMEP into 4'-d- α -DMEP. This inference was supported by observing that the medium pH fall into the range of pH 7–10 which is suitable for enzymatic reaction. Additionally, we also observed the accumulation of 4'-d-demethylepipodophyllilic acid, a podophyllotoxin derivative produced under alkaline conditions [11]. The addition of 4'-d-demethylepipodophyllilic acid into the culture broth did not make *P. purpurogenum* ACCC 32170 produce 4'-d- α -DMEP, and the conversion of 4'-d- α -DMEP into 4'-d-demethylepipodophyllilic acid was observed under alkaline condition without *P. purpurogenum* ACCC 32170 or the enzyme. This indicated that the conversion of 4'-d- α -DMEP into 4'-d-demethylepipodophyllilic acid depends on the extracellular medium pH. It could be used to explain the sharp decrease of 4'-d- α -DMEP in the later stage of fermentation (Fig. S5).

Structure modification of podophyllotoxin will thrill the development of new potent inhibitors against cancer cells [9,31–33]. Structure-activity relationship indicated the *trans*-lactone was usually essential for anti-tumor activity [9]. Halogenation of C-2 in podophyllotoxin showed that the β configuration was with higher anti-tumor activity than α configuration [34,35]. Both β configurations of C-2 and C-3 in picropodophyllotoxin made the compound target the insulin-like growth factor-I receptor, which induced cell death [13,14]. Moreover, configuration changes of the *trans*-lactone have been reported for 4'-demethylpicropodophyllotoxin, 4'-demethylisopodophyllotoxin, 4'-demethyldehidropodophyllotoxin, isopropodophyllone, 4'-demethylisopropodophyllone and so on [9]. While 4'-d- α -DMEP with both α configurations at C-2 and C-3 was not reported in the previous studies, and regarded as a novel podophyllotoxin derivative. Notably, it still targeted Top 2 β and was with higher anti-tumor activity than 4'-d- β -DMEP and VP-16. Therefore, it has potential to be used as a leading compound for new drug discovery.

5. Conclusions

A novel podophyllotoxin derivative identified as 4'-d- α -DMEP with higher anti-tumor activity was produced from the biotransformation by *Penicillium purpurogenum*. 4'-d- α -DMEP was produced by constitutively expressed cytosolic enzyme, and the production depends on alkaline condition. This discovery will promote the identification of new podophyllotoxin derivatives with higher anti-tumor activity and delivering lignans into the clinic trial.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

CRediT authorship contribution statement

Kai-Zhi Jia: Investigation, Writing - original draft. **Xia Zhan:** Investigation. **Hong-Mei Li:** Resources. **Shengying Li:** Validation, Resources. **Yuemao Shen:** Software, Formal analysis. **Qingsheng Qi:** Validation, Resources. **Yuming Zhang:** Validation, Resources. **Yuezhong Li:** Validation, Resources. **Ya-Jie Tang:** Conceptualization, Writing - review & editing, Project administration, Supervision.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgements

Financial supports from the National Key R&D Program of China (No. 2019YFA0905700), National Natural Science Foundation for Distinguished Young Scholars (No. 21625602), the National Natural Science Foundation of China (No. 21838002), and Hubei Provincial Science and Technology Innovation Major Project (2017ACA173) are gratefully acknowledged.

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