Stereo and region-selective biosynthesis of two new dihydroartemisinic acid glycosides by suspension-cultured cells of *Artemisia annua*

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ABSTRACT

Background: The system of plant-cultured cells is one of the optimal systems to investigate biosynthesis pathway and their bioactive intermediates. **Objective:** To study the biosynthesis of dihydroartemisinic acid (1) by suspension-cultured cells of *Artemisia annua*. **Materials and Methods:** Substrate (compound 1) was administered into the suspension-cultured cells of *A. annua* and co-cultured for 2 days. The methanol extract was separated on various column chromatography methods and the structures of two biosynthesis products were elucidated based on the analysis of ^{1}H NMR, ^{13}C NMR, 2D NMR, and ESI-MS. Time-course curve was also established. Furthermore, in vitro antitumor activities of compounds 1-3 against HepG2, K562, and A549 cell lines were evaluated by MTT assay. **Results:** Two new compounds were obtained, namely 3α-hydroxy-dihydroartemisinic acid-α-D-glucopyranosyl ester (2) and 15-hydroxy-cadin-4-en-12-oic acid-β-d-glucopyranosyl ester (3). The results demonstrated that the cultured cells of A. annua possessed the abilities to stereo-selective hydroxylate and region-selective glycosylate sesquiterpene compounds in a highly efficient manner. Inhibitory effects of compounds 1-3 on proliferation of HepG2, K562, and A549 cell lines *in vitro* were also investigated. **Conclusion:** Two new dihydroartemisinic acid glycosides were obtained by stereo- and region-selective biosynthesis with cultured cells of *A. annua*.

Key words: Antitumor, *Artemisia annua*, biosynthesis, dihydroartemisinic acid, glycosylation

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INTRODUCTION

Artemisinin, a sesquiterpene lactone with a peroxide bridge extracted from Chinese medicinal herb (*Artemisia annua*, Qinghao), is famous for its bioactivity against both chloroquine-resistant and sensitive strains of *Plasmodium falciparum* as well as cerebral malaria with high safety profile.[1,2] In addition to their antimalarial activity, artemisinins were reported in recent decades as potential reagents against cancer cells.[3] Since artemisinin was found, its biosynthesis pathway attracted more and more attention. To use biotechnology method to produce or increase the yield of artemisin, many scientists have devoted themselves to elucidate the biosynthesis pathway.[4-8]

Dihydroartemisinic acid (1), one of the precursors of artemisinin,[7] attracted increasing attention after it was confirmed to transform to artemisinin *in vitro* by a nonenzymatic process.[7,8] Many researchers have been carried out to investigate the biosynthesis pathway from dihydroartemisinic acid to artemisinin.[5,6] However, the process is still incompletely understood, particularly within the plant cells.

Over the past few decades, biotransformation has been extensively studied because it is considered to be an important method for converting inexpensive and plentiful substances into expensive and scarce ones. Recently, plant cell cultures have been studied as potential agents during biotransformation reactions, especially for obtaining chiral alcohols, which are intermediates of pharmaceutical, and other potential compounds in industrial scale. Plant cell cultures as important biotransformation systems have been used widely.[9-12] To elucidate and evaluate the biosynthesis pathways of artemisinins, our research group has been screening tens of plant culture cell systems and has got some encouraging scientific information.[13-18]

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To date, there is no report on the biotransformation of dihydroartemisinic acid by plant-cultured cells except the report from our research group.[13,14] As a continuation work to explore the plant cell biotransformation of the precursor of artemisinin, and with the aim to enrich the metabolites of dihydroartemisinic acid, and to find out novel artemisinin derivatives, which might possess good antimalaria and/or antitumor activities, the biotransformation of dihydroartemisinic acid by suspension-cultured cells of *Artemisia annua* was investigated in the present paper.

MATERIALS AND METHODS

General

^{1}H and ^{13}C nuclear magnetic resonance (NMR) and 2D NMR spectra were recorded on a Bruker DRX-400 spectrometer, the chemical shifts (δ) were given in ppm relative to TMS as an internal standard, and coupling constants were given in Hz. ESI-MS data were obtained with a 4000 Q TRAP LC/MS/MS system by direct inlet using MeOH as solvent. HR-TOF-MS were recorded on SYNAPT™ G2 HDMS, Waters, Manchester, U.K. Silica gel (100-200 mesh and 200-300 mesh) used for column chromatography (CC), and silica GF_{254} (10-40 μ) for TLC were supplied by the Qingdao Marine Chemical Factory, China. ODS (YMC Co., Ltd., Japan) and Sephadex LH-20 (Pharmacia Co.) were also used for separation. HPLC analysis was performed on a Agilent 1200 liquid chromatography system (Palo Alto, CA, USA), equipped with vacuum degasser, quaternary gradient pump, auto-sampler, and DAD, connected to a Agilent ChemStation software. An Agilent Hypersil ODS column (ϕ4.6 mm × 250 mm, 5 μm) and guard column (4.6 mm × 12.5 mm, 5 μm) were used. A binary gradient elution system consisted of water (A) and methanol (B) and separation was achieved using the following gradient program: 0-5 min 40-50% B; 5-10 min 50-60% B; 10-15 min 60-70% B; 15-20 min 70-85% B; 20-25 min 100% B, and finally, reconditioning the column with 40% B isocratic for 2 min. The flow rate was 0.8 ml/min, and the system operated at 30ºC. The detection wavelength was set at 210 nm.

Substrate

Dihydroartemisinic acid (1) was extracted and isolated from *A. annua* by our research group according to the referenced protocol.[19] The structure was determined by MS and NMR. Its purity was >98% by HPLC analysis.

Plant cell cultures

The cells of *A. annua* have been subcultured routinely every 3 weeks using MS medium containing 2,4-dichlorophenoxyacetic acid (2,4-D 0.5 mg/L) and 6-benzylaminopurine (6-BA 1 mg/L). Prior to being used for biotransformation experiments, the cultured cells were transferred to a 500-ml conical flask containing 200 ml of medium and cultured on a rotary shaker (110 rpm) for 13 days at 25°C in the dark.

Biotransformation of dihydroartemisinic acid (1)

Dihydroartemisinic acid (1, 110 mg) was dissolved in 1.1 ml of ethanol and distributed among 22 Erlenmeyer flasks of 13-day-old cultures and incubated for an additional 2 days. After incubation, the cultures and media were separated by filtration with suction. The dried cultures were extracted with methanol for four times by ultrasound-assisted extraction. Each of the MeOH fractions was concentrated and partitioned between H_2O and EtOAc. The EtOAc fractions were combined and further purified on column chromatography by silica gel, sephadex LH-20, and ODS to afford products 2 and 3. The same culture was repeated 5 times.

3α-Hydroxydihydroartemisinic acid-α-D-glucopyranosyl ester (2)

^{1}H NMR (pyridine-d_5, 400 MHz) δ: 0.8 (3H, d, J = 4.4 Hz), 1.2 (3H, d, J = 6.8 Hz), 2.0 (3H, s), 2.61 (1H, br. s), 5.31 (1H, s), 6.32 (1H, d, J = 3.6 Hz); ^{13}C-NMR (pyridine-d_5,100 MHz) δ: 42.4 (C-1), 35.2 (C-2), 67.0 (C-3), 139.6 (C-4), 121.7 (C-5), 37.1 (C-6), 44.6 (C-7), 27.4 (C-8), 37.0 (C-9), 28.8 (C-10), 43.7 (C-11), 175.9 (C-12), 14.9 (C-13), 19.6 (C-14), 20.2 (C-15), 95.6 (C-1′), 73.9 (C-2′), 78.3 (C-3′), 70.8 (C-4′), 79.1 (C-5′), 61.9 (C-6′); ESI-MS: m/z 413 $[M-H]^-$, 437 $[M+Na]^+$. HR-TOF-MS (m/z = 437.2154 $[M+Na]^+$, calcd. for $C_{21}H_{34}O_8Na^+$, 437.2151).

15-Hydroxy-cadin-4-en-12-oic acid-β-d-glucopyranosyl ester (3)

^{1}H NMR (pyridine-d_5, 400 MHz) δ: 0.744 (3H, d, J = 6.4 Hz), 1.21 (3H, d, J = 6.8 Hz), 1.79 (3H, s), 2.57 (1H, br, s), 4.30 (2H, s), 5.89 (1H, s), 6.32 (1H, d, J = 8.0 Hz); ^{13}C-NMR (pyridine-d_5,100 MHz) δ: 43.5 (C-1), 27.0 (C-2), 23.8 (C-3), 142.1 (C-4), 120.7 (C-5), 37.9 (C-6), 45.2 (C-7), 28.9 (C-8), 36.7 (C-9), 28.9 (C-10), 43.9 (C-11), 177.4 (C-12), 16.5 (C-13), 21.0 (C-14), 67.9 (C-15), 97.1 (C-1′), 75.4 (C-2′), 79.9 (C-3′), 72.3 (C-4′), 80.6 (C-5′), 63.5 (C-6′); ESI-MS: m/z 413 $[M-H]^-$, 437 $[M + Na]^+$, HR-TOF-MS (m/z = 437.2144 $[M + Na]^+$, calcd. for $C_{21}H_{34}O_8Na^+$, 437.2151).

Time-course of biotransformation

Cultured cells of *A. annua* (10 g) were transferred to a 500-ml Erlenmeyer flask containing 200 ml medium, and cultured by continuous shaking for 13 days at 25°C. Compound 1 (5 mg/flask) was added to the suspension cultures and incubated at 25°C in a rotary shaker (110 rpm). At 1-day intervals, three of the flasks were taken out from the rotary shaker, and the cells and media were separated by

filtration. The extraction and analysis procedures were the same as those described earlier. The yields of the products were calculated on the basis of the peak area from HPLC using calibration curves prepared by HPLC-DAD and were expressed as relative percentages to the total amount of whole biosynthesis products.

MTT-cell proliferation assay

The inhibitory effects of 1-3 on the proliferation of HepG2, K562, and A549 cells were evaluated *in vitro* by MTT [3-(4,5-dimethylthiazole-2-yl)-2,5- diphenyl-tetrazoliumbromide] assay, which was performed as described in the literature.[20] The concentrations of biosynthesis products on the selected cell lines were in the range of 0.0185-0.5000 μmol/ml.

RESULTS

Structural elucidation of biotransformation products

Two new compounds were obtained after dihydroartemisinic acid (1) was incubated with the plant-cultured cells of *A. annua* for 2 days. Their structures were elucidated to be 3α-hydroxy-dihydroartemisinic acid-α-d-glucopyranosyl ester (2) and 15-hydroxy-cadin-4-en-12-oic acid-β-d-glucopyranosyl ester (3) [Figure 1].

Compound 2 was obtained as an amorphous powder. It displayed a quasimolecular ion peak at m/z 413 $[M-H]^-$ and 437 $[M+Na]^+$ in ESI-MS, indicating that the molecular weight of product 2 was 414, 178 more than that of dihydroartemisinic acid. The molecular formula of 2 was determined as $C_{21}H_{34}O_8$ on the basis of HR-TOF-MS (m/z = 437.2154 $[M+Na]^+$, calcd. for $C_{21}H_{34}O_8Na^+$, 437.2151). This information, together with the ^{13}C NMR spectrum, suggested that compound 2 might be a molecule that has a glucose attached to hydroxyl-dihydroartemisinic acid. In the ^1H NMR spectrum of 2, in addition to the signals for dihydroartemisinic acid moiety, it showed one set of α-glucopyranosyl signals, with the resonance for the anomeric proton at δ 6.32 (1H, d, J = 3.6 Hz). Its ^{13}C NMR spectrum exhibited 21 carbon signals, including one anomeric carbon signal at δ 95.6, suggesting that 2 was larger than 1 by one hexose moiety and a hydroxyl group. A comparison of ^{13}C NMR spectra of 2 with that of 1 showed that carboxyl carbon signal was shifted upfield by 7.9 ppm (183.8 → 175.9). This suggested that 2 was an ester. The carbon signal at C-15 in 2 was shifted upfield (δ 23.7 → 20.2), and one carbon signal was shifted markedly downfield (δ 26.6 → 67.0). These data suggested that the upfield shift of the C-15 signal was caused by a γ-effect due to hydroxylation of 1 at the position of C-3. The ^{13}C NMR signal of the aglycone in 3 was coincident with that of 3α-hydroxy-dihydroartemisinic acid.[9] HMBC spectrum showed that δ 175.9 (C_{12}) was correlated with δ_H 6.32 (1H, d, J = 3.6 Hz), suggesting that sugar moiety was linked to carbonyl group of 1. Therefore, the structure of 2 was proposed to be 3α-hydroxy-dihydroartemisinic acid-α-d-glucopyranosyl ester. Biotransformation product 2 is a new compound.

Compound 3 possessed the same molecular weight as that of 2. The molecular formula of 3 was determined as $C_{21}H_{34}O_8$ on the basis of HR-TOF-MS (m/z = 437.2144 $[M + Na]^+$, calcd. for $C_{21}H_{34}O_8Na^+$, 437.2151). The main difference

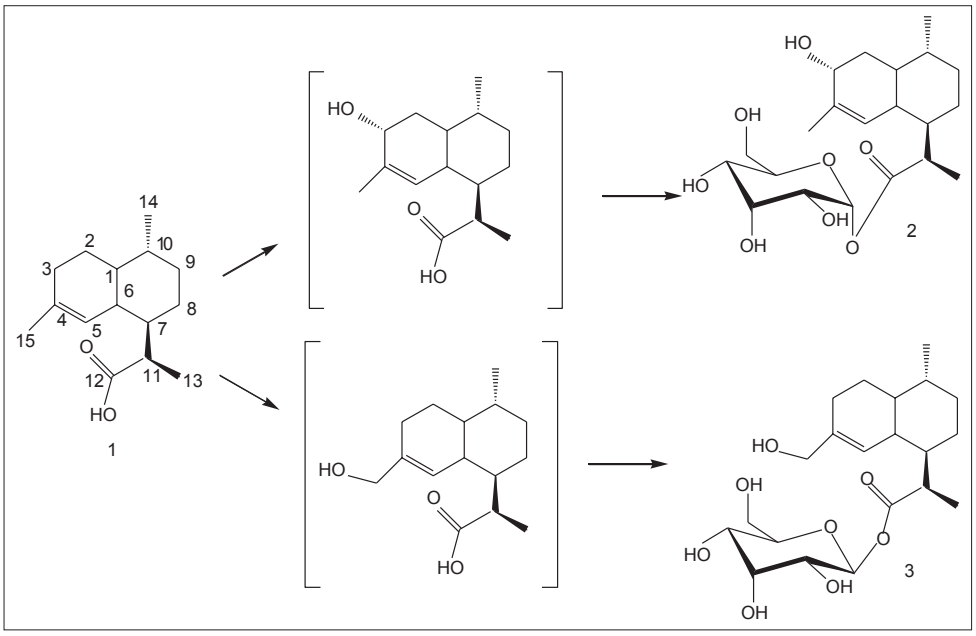

Figure 1: Biotransformation pathway of dihydroartemisinic acid

between 3 and 2 in ^{1}H NMR was that the configuration of glucopyranosyl in 3 was β-form with the resonance for the anomeric proton at δ 6.32 (1 H, d, J = 8.0 Hz), while there was an α-glucopyranosyl in 2. Furthermore, there was a single peak in 3 at δ 4.30. The main difference between 3 and 2 in ^{13}C NMR was that δ value at the position of C-2 in 2 was downshift to 35.2 because of the γ-effect of hydroxylation of 1 at C-3, but 3 did not show this phenomenon. On the contrary, δ value at the position of C-4 in 3 was shifted downfield (δ 139.6 → 142.1), and δ value at the position of C-15 was shifted markedly downfield (δ20.2 → 67.9) when compared to 2. This information suggested that product 3 has a hydroxyl group at C-15. The ^{13}C NMR signal of the aglycone in 3 was coincident with that of 15-hydroxy-cadin-4-en-12-oic acid.[9] HMBC spectrum showed that δ H-15 was correlated with δ 142.1 (C-4) and δ 120.7 (C-5). The contour plot was much stronger between H-15 and C-4 than between H-15 and C-5. Therefore, 3 was determined to be 15-hydroxy-cadin-4-en-12-oic acid-β-D-glucopyranosyl ester. Biotransformation product 3 is also a new compound.

Establishment of time-course curve of substrate 1

[Figure 2] showed the results of the biotransformation products of compound 1 by cultured cells of *A. annua*. As indicated in [Figure 2], compound 1 disappeared after 3 days' culture, meaning that 1 was completely transformed. A possible biosynthesis pathway in the cultures was proposed in [Figure 1]. As shown in [Figure 1], hydroxylation firstly happened at C_3 and C_{15}. Then, glycosylation took place at the carbonyl group of C_{12} to form compounds 2 and 3.

In vitro antitumor activities

Antitumor activities of 1-3 against the HepG2, K562, and A549 cell lines are shown in [Table 1]. The sensitivity of those three cell lines to compounds 1-3 was quite different. For HepG2 cell line, the inhibitory rate of biotransformation products (2 and 3) was higher than that of compound 1. K562 cell line was not sensitive to both the substrate and the products. Compound 1 showed good inhibitory activity against the A549 cell line while no inhibitory activity appeared for the biotransformation products.

DISCUSSION

The experiment of time-course curve revealed that the yields of compounds 2 and 3 were not high enough while compound **1** was completely transformed. Those might due to the metabolites of dihydroartemisinic acid in cultured cells of *A. annua* were a wide variety. In our previous study, hydroxylation products and artemisinins were isolated as the metabolites of dihydroartemisinic acid by *A. annua*.[14,15]

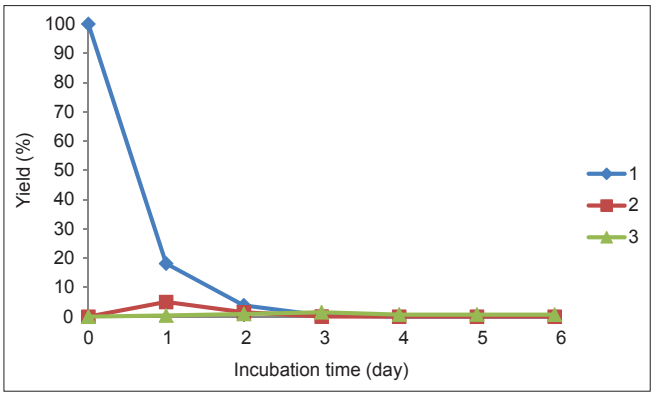

Figure 2: Biotransformation of dihydroartemisinic acid (1) by suspension-cultured cells of *A. annua*

Table 1: Inhibitory effects of compounds 1-3 (0.5 μmol for each) on proliferation of HepG2, K562, and A549 cell lines

Cell lines	Inhibition rate (%)		
	1	2	3
HepG2	37.4±3.7**	78.0±1.1**	70.7±1.5**
K562	50.6±3.9**	25.4±1.6**	7.7±2.5
A549	99.9±0.1**	16.9±2.9**	0±4.8

Values are given as means±standard deviation of three separate experiments.
**Compared to control: P<0.01

Therefore, the metabolism of dihydroartemisinic acid in cultured cells, such as *A. annua*, was very complicated and many metabolites might have existed.

There may be two metabolic pathways for 1 in cells of *A. annua*: To be biosynthesized to complex compounds, such as artemisinin, and to be decomposed to nontoxic constituents. Hydroxylation and glycosylation are supposed to be initial steps to biodegradation of toxicants in plants.[21]

In our previous study, 15-hydroxy-cadin-4-en-12-oic acid was isolated as the metabolite of dihydroartemisinic acid when using crown galls of *Panax quinquefolium* as the biocatalyst system.[9] At the present study, its ester was obtained. This demonstrated that both plant-cultured cells of *A. annua* and crown galls of *P. quinquefolium* could hydroxylate dihydroartemisinic acid at C-15 position.

More importantly, the glycosyltransferase of plant-cultured cells of *A. annua* selectively glycosylated dihydroartemisinic acid with carboxyl group at C-12 position to produce corresponding glycosides though hydroxyl group existed. This information indicated that the glycosyltransferase functioned as a high region-selective enzyme. In addition, α-glycoside was isolated in this experiment. Generally,

α-configuration compound is rarely found when compared with β-configuration products isolated from plant biotransformation system.[22] β-Glycosidase had been isolated from *A. annua* 24 years ago.[23] But the information about α-glycosidases of *A. annua* has not been reported up to now. This is the first report that the administrated substrate was converted into its d-glucopyranoside of α-configuration by cultured cells of *A. annua*. Characterization of enzyme that catalyzes the α-glycosylation is now in progress.

CONCLUSION

In summary, plant-cultured cells of *A. annua* have the ability to stereo-selective hydroxylate and region-selective glycosylate exogenous compounds like sesquiterpene in a highly efficient manner. The two new compounds (2 and 3) demonstrated excellent inhibitory effect on proliferation of HepG2 cell line *in vitro*.

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