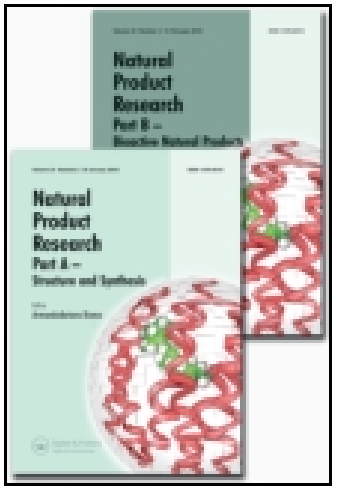

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Three sesquiterpene compounds biosynthesised from artemisinic acid using suspension-cultured cells of *Averrhoa carambola* (*Oxalidaceae*)

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A new sesquiterpene glycoside, artemisinic acid 3-β-O-β-D-glucopyranoside (**3**, 31.24%) and other two biotransformation products, 3-β-hydroxyartemisinic acid (**2**, 36.69%) and 3-β-hydroxyartemisinic acid β-D-glucopyranosyl ester (**4**, 7.03%), were biosynthesised after artemisinic acid (**1**) was administered to the cultured cells of *Averrhoa carambola*. The three biotransformation products were obtained for the first time by using the suspension-cultured cells of *A. carambola* as a new biocatalyst system, and their structures were identified on the basis of the physico-chemical properties, NMR and mass spectral analyses. The results indicate that the cultured cells of *A. carambola* have the abilities to hydroxylate and glycosylate sesquiterpene compounds in a regio- and stereoselective manner. Furthermore, the anti-tumour activity of compounds **3** and **4** was evaluated against K562 and HeLa cell lines. Compound **4** showed strong activity against HeLa cell line, with the IC_{50} value of 0.56 μmol mL^{-1}.

Keywords: *Averrhoa carambola*; suspension-cultured cells; biotransformation; artemisinic acid; regio- and stereoselective hydroxylation; glycosylation

1. Introduction

Artemisinin is a sesquiterpene lactone with a peroxide bridge, which is the functional moiety against both chloroquine-sensitive and multidrug-resistant strains of *Plasmodium falciparum*. Modern pharmacological research also showed that artemisinin possessed very good anti-hepatitis and anti-cancer effects besides anti-malarial activity (Efferth, Dunstan, Sauerbrey, Miyachi, & Chitambar, 2001; Romero et al., 2005). Artemisinic acid (**1**) was proposed to be one of the intermediates in biosynthesis pathway leading to artemisinin in *Artemisia annua* (Zhang et al., 2008; Zhu et al., 2010). The content of **1** was much higher than that of artemisinin in the leaves of *A. annua* (Gupta et al., 1996) and it also showed good anti-tumour activity (Zhou, S.L. Wan, & Y.P. Wan, 2006). Compound **1** has a chemical structure (cadinane-type sesquiterpene) similar to that of artemisinin. Therefore, the utilisation of **1** as a starting material is of practical importance for the synthesis of artemisinin analogues.

Over the past few decades, biotransformation has been extensively studied because it is considered an important method for converting inexpensive and plentiful substances into expensive and scarce ones. Recently, plant cell cultures as an important biotransformation system have been widely used because of their biochemical potential to produce specific

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secondary metabolites that could confer useful elements such as flavours, pigments and agrochemicals (Gren, Wojcieszynska, Guzik, Perkosz, & Hupert-Kocurek, 2010; Ishihara, Hiroki, Toshifumi, & Nobuyoshi, 2003; Liu et al., 2010).

There are some research papers about the biotransformation of artemisinic acid (**1**) (Elmarakby, EI-Feraly, EIsohly, Croom, & Hufford, 1988; Kawamoto, Asada, Sekine, & Furuya, 1998). More recently, we reported a few biotransformation products of artemisinic acid, which were obtained using two biocatalyst systems (the cell suspension cultures of *Catharanthus roseus* and *Panax quinquefolium* crown galls; Zhu et al., 2010).

The suspension culture system of *Averrhoa carambola* L. was established by our research group, and the results of the primary experiment showed that artemisinin derivatives could be biotransformed by this culture system. In this article, artemisinic acid (**1**), a typical sesquiterpene, was used as a substrate in cultured cells of *A. carambola* in order to expand the range of artemisinin derivatives, to find out new reaction types of biotransformation in plant culture cells, and to investigate the bioconversion capability of this new system on important sesquiterpene compounds.

2. Results and discussion

The results demonstrated that artemisinic acid (**1**) was converted into three products (**2**–**4**) by the suspension-cultured cells of *A. carambola*.

The HR–ESI–MS spectrum of **3** included a pseudomolecular ion $[M + Na]^+$ peak at m/z 435.19874, consistent with a molecular formula of $C_{21}H_{32}O_8$ (Calcd for $C_{21}H_{32}Na_{O8}$, 435.19894). The molecular weight of **3** was 162 more than that of substrate **2**, indicating that compound **3** might be the glycosylation product of hydroxyl-artemisinic acid. In the ^{1}H-NMR spectra of **3**, an anomeric proton signal was observed at δ 4.37 (1H, d, $J = 7.6$ Hz), indicating that the configuration of sugar was β type. This result was further confirmed by the study of acid hydrolysis of **3**. The sugar generated by acid hydrolysis was compared with the standard of β-D-glucose by paper chromatography, and found that the R_f value of both were the same. In addition, the component sugar in **3** was indicated to be β-glucopyranosyl on the basis of the patterns of the carbon and proton signals caused by the sugar moiety in NMR spectra. There were 21 carbon signals in the ^{13}C-NMR spectra. On comparison of the data of **1** and **3**, the carbon signal at C-15 in **3** was shifted upfield (δ 23.6 $\rightarrow$ 21.2), and one carbon signal was shifted markedly downfield (δ 26.3 $\rightarrow$ 77.7). These data suggested that the upfield shift of the C-15 signal was caused by a γ-effect due to hydroxylation of **1** at C-3. Correlations were observed in the HMBC spectrum between the proton signal at δ 4.37 (Glc-1-H) and the carbon resonance at δ 77.7 (C-3), suggesting that glucose was connected to C_3-OH. To elucidate the stereochemistry of C_3-OH in **3**, the NOESY spectrum was determined. NOEs were observed between H-3 (δ 3.86) and Me-15 (δ 1.76), H-3 and H-2α (δ 1.66). However, no NOE was observed between H-3 and H-10 (δ 2.44). These data could only be accounted for by the assignment that the configuration of H-3 in **3** was α (β-OH) type. To further confirm the configuration of H-3, acid hydrolysis of **3** was done. TLC showed that the aglycone of **3** had the same R_f value as that of **2**, indicating that the aglycone of **3** is 3-β-hydroxyartemisinic acid. All the information indicated that the β-D-glucopyranosyl residue was attached to 3-β-OH of **1**. Thus, **3** was identified as artemisinic acid 3-β-O-β-D-glucopyranoside, which was a new compound.

The structures of **2** and **4** were determined to be 3-β-hydroxyartemisinic acid (**2**) and 3-β-hydroxyartemisinic acid β-D-glucopyranosyl ester (**4**), respectively, according to the spectra of ESI–MS, ^{1}H-NMR and ^{13}C-NMR and by comparing the data with those from the literature (Zhu et al., 2010).

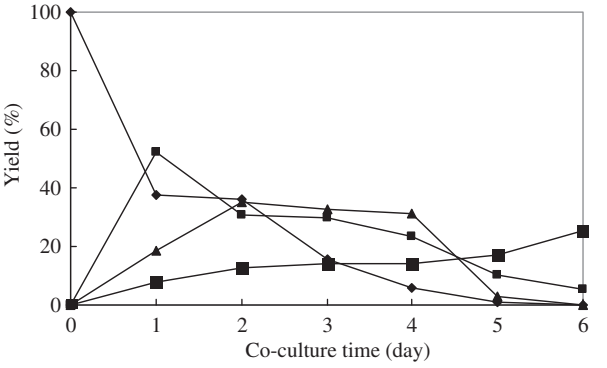

Figure 1. Time-course of biotransformation of artemisinic acid (**1**) by the cultured cells of *A. carambola*. Yields of **1** (◆), **2** (■), **3** (▲) and **4** (■) are plotted.

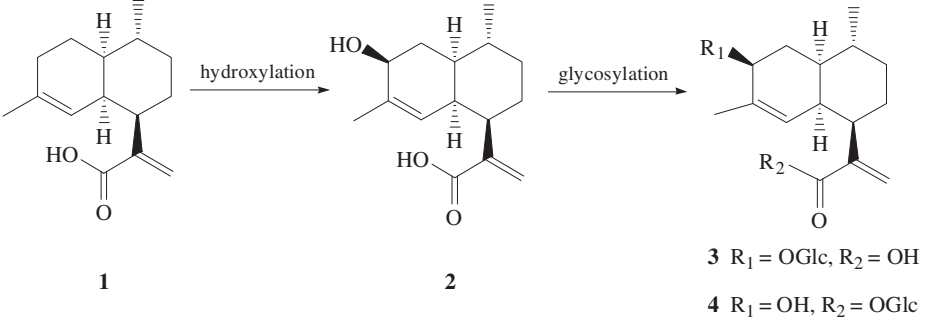

Figure 2. Biotransformation of artemisinic acid (**1**) by the suspension-cultured cells of *A. carambola*.

To investigate the biotransformation pathway, a time course in the conversion of **1** was followed. As shown in Figure 1, the concentration of substrate **1** decreases sharply in 1 day (100% → 38%), indicating that transformation happened on the 1st day. At the same time, compound **2** reached its maximum transformation rate (36.69%); after its climax, the concentration of **2** decreased gradually, indicating that **2** may act as one of the intermediates. Compound **3** reached its maximum transformation rate (31.24%) on the 2nd day, and then remained stable until the 4th day. The amount of **4** increased tenuously, suggesting that **4** may be the end product of this process. All products were found in the cultures, and no product was detected in the medium.

The proposed biosynthesis pathways of **2**, **3** and **4** are shown in Figure 2. Artemisinic acid was first regio- and stereoselectively hydroxylated in the allyl group to yield product **2** and then was glycosylated to yield products **3** and **4**, respectively.

From Figure 1, we can see that hydroxylated product (**2**) and glycosylated product (**3**) were the prominent products, both of which achieve their maximum transformation rate in a short reaction time (36.69%, 1 d; 31.24%, 2 d), and compound **4** was obtained in a lower yield. This result indicated that plant culture cells of *A. carambola* have the abilities to regio- and stereoselectively hydroxylate and glycosylate sesquiterpenes. Compound **4** was reported to be the product of artemisinic acid when the cultured cells of *A. annua* were used as biocatalysis (Kawamoto et al. 1998). However, there are no reports on the

biotransformation of artemisinic acid (**1**) to clarify the biotransformation pathways of **1**–**3**. For the first time, this article describes the biosynthesis pathway of compound **3**.

The anti-tumour activity of compounds **3** and **4** was investigated against K562 and HeLa cell lines. The IC_{50} values of **3**, **4** and cisplatin as a positive control against HeLa cell line were 2.25 ± 0.13, 0.56 ± 0.01 and $5.46 \pm 0.37\,\mu mol\,L^{-1}$, and those of **3**, **4** and adriamycin (ADR) as a positive control against K562 cell line were 4.10 ± 0.61, 1.30 ± 0.50 and $0.27 \pm 0.12\,\mu mol\,L^{-1}$ against K562 cell line, respectively. These data demonstrated that β-D-glucopyranosyl ester (**4**) exhibited stronger anti-tumour activity than 3-β-O-β-D-glucopyranoside (**3**) against the K562 and HeLa cell lines, and the HeLa cell line was more sensitive to those products than the K562 cell line.

3. Experimental

3.1. *General*

The melting points were measured on a X-5 melting point apparatus. Optical rotations were obtained on a Perkin-Elmer 241 polarimeter. 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 4000 Q TRAP LC/MS/MS by direct inlet using MeOH as solvent. HR–ESI–MS spectrum was obtained on a Micromass Q-TOF and an Agilent 6210 LC/MSD TOF mass spectrometers. HPLC was performed on a Agilent 1200 liquid chromatograph system (Palo Alto, CA, USA), equipped with vacuum degasser, quaternary gradient pump, autosampler and DAD, connected to a Agilent ChemStation software. A Pheomenex synergi C_{18} column (5 μm, $\varphi 4.6 \times 250\,mm^2$) and a Pheomenex ODS C_{18} guard column ($4.6 \times 12.5\,mm^2$, μm) were used. Silica gel (100–200 and 200–300 meshes) used for column chromatography and silica GF_{254} (10–40 μm) for TLC were supplied by the Qingdao Marine Chemical Factory, China, and ODS from YMC Co. Ltd., Japan.

3.2. *Substrate*

Artemisinic acid (**1**) was extracted and isolated from *A. annua* by our research group. The structure of **1** was determined by MS and NMR, and its purity was estimated to be greater than 98% by the HPLC analysis.

3.3. *Plant cell cultures*

Culture cells of *A. carambola* were sub-cultured on MS liquid medium (Murashige and Skoog medium containing 3% sucrose without agar) supplemented with $2.0\,mg\,L^{-1}$ 6-benzylaminopurine (6-BA) and $2.0\,mg\,L^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) at 25°C in the dark at 2-week intervals.

3.4. *Biotransformation of artemisinic acid (1)*

The substrate (**1**, 100 mg) was administered to 20 flasks which contained suspension-culture cells of *A. carambola*. Co-culture proceeded for 2 days at 25°C on a rotary shaker (110 rpm) in the dark. After incubation, the cultures and medium were separated by filtration with suction. The dried cultures were extracted with methanol for four times by ultrasound-assisted extraction. Each of MeOH fractions was concentrated and partitioned between H_2O (30 mL) and EtOAc (40 mL × 3). EtOAc fractions were combined and evaporated to dryness *in vacuo*. The residue was chromatographed on silica gel columns using petroleum ether–ethyl acetate as solvent systems. The eluting fractions were further

separated by silica gel columns using chloroform–methanol as solvent systems to afford products **2**, **3** and **4**.

3.4.1. *Artemisinic acid (**1**)*

Colourless square crystal, m.p.: 130–131°C. ESI–MS: 233 $[M - H]^-$, 257 $[M + Na]^+$. ^{1}H-NMR (400 MHz, chloroform-d): δ 0.89 (3H, d, $J = 6$ Hz, Me-14), 1.59 (3H, s, Me-15), 2.6 (1H, br s, H-6), 2.71 (1H, m, 7-H), 4.98 (1H, s, H-5), 5.55 (1H, s, H-13α) and 6.45 (1H, s, H-13β); ^{13}C-NMR (100 MHz, chloroform-d): δ 172.6 (C-12), 142.6 (C-11), 134.9 (C-4), 126.5 (C-13), 120.1 (C-5), 42.0 (C-7), 41.3 (C-1), 37.8 (C-6), 35.2 (C-9), 27.5 (C-10), 26.3 (C-3), 25.9 (C-8), 25.5 (C-2), 23.6 (C-15) and 19.7 (C-14).

3.4.2. *3-β-Hydroxyartemisinic acid (**2**)*

Colourless needles ($CHCl_3$); ESI–MS *m/z*: 249 $[M - H]^-$ and 273 $[M + Na]^+$; ^{1}H-NMR (400 MHz, acetone-d_6): δ 0.97 (3H, d, $J = 6.4$ Hz, Me-14), 1.74 (3H, s, Me-15), 2.27 (1H, dd, $J = 14$, 2.0 Hz, H-2), 3.94 (1H, d, $J = 5.6$ Hz, H-3α), 5.16 (1H, s, H-5), 5.54 (1H, s, H-13α) and 6.34 (1H, s, H-13β); ^{13}C-NMR (100 MHz, acetone-d_6): δ 168.3 (C-12), 144.5 (C-11), 137.2 (C-4), 124.5 (C-13), 123.6 (C-5), 67.2 (C-3), 43.1 (C-7), 41.9 (C-1), 39.5 (C-6), 36.5 (C-9), 35.5 (C-2), 29.4 (C-10), 26.8 (C-8), 21.1 (C-15) and 20.6 (C-14).

3.4.3. *Artemisinic acid 3-β-O-β- D-glucopyranoside (**3**)*

Amorphous solid; m.p. 231–232°C; $[\alpha]_D^{15} = +7.0°$ ($C = 0.27$, MeOH); HR–ESI–MS *m/z*: 435.19874 $[M + Na]^+$ (Calcd for $C_{21}H_{32}Na_{O8}$, 435.19894); ^{1}H-NMR (400 MHz, methanol-d_4): δ 0.97 (3H, d, $J = 13.0$, 13.0, 13.0 and 3.0 Hz, H-9α), 1.09 (3H, d, $J = 6.5$ Hz, Me-14), 1.38 (1H, m, H-1), 1.66 (1H, m, H-2α), 1.76 (3H, s, Me-15), 2.44 (1H, m, H-10), 3.1–3.4 (6H, m, H-2′, 3′, 4′, 5′ and 6′), 3.86 (1H, d, $J = 5.6$ Hz, H-3α), 4.37 (1H, d, $J = 8.0$ Hz, H-1′), 5.18 (1H, s, H-5), 5.45 (1H, s, H-13α) and 6.25 (1H, s, H-13β); ^{13}C-NMR(100 MHz, methanol-d_4): δ 171.6 (C-12), 144.8 (C-11), 136.3 (C-4), 125.5 (C-13), 124.4 (C-5), 107.0 (C-1′), 78.6 (C-5′), 78.1 (C-3′), 77.7 (C-3), 75.8 (C-2′), 72.1 (C-4′), 63.3 (C-6′), 43.5 (C-7), 42.3 (C-1), 39.9 (C-6), 36.9 (C-9), 34.8 (C-10), 29.5 (C-8), 27.3 (C-2), 21.2 (C-15) and 20.6 (C-14).

3.4.4. *3-β-Hydroxyartemisinic acid β-D-glucopyranosyl ester (**4**)*

Amorphous solid; ESI–MS *m/z*: 411 $[M–H]^-$ and 435 $[M + Na]^+$; ^{1}H-NMR (400 MHz, pyridine-d_5): δ 0.97 (1H, dddd, $J = 13.0$, 13.0, 13.0 and 3.0 Hz, H-9α), 1.06 (3H, d, $J = 6.5$ Hz, Me-14), 1.35 (1H, m, H-1), 1.46 (1H, dddd, $J = 13.0$, 13.0, 13.0 and 3.0 Hz, H-8β), 1.64 (1H, m, H-2α), 1.72 (1H, dd, $J = 13.0$ and 2.0 Hz, H-9β), 1.89 (3H, s, Me-15), 2.25 (1H, m, H-10), 2.33 (1H, dd, $J = 14.0$ and 2.5 Hz, H-2β), 2.87 (1H, br s, H-6), 2.99 (1H, ddd, $J = 13.0$, 3.0 and 3.0 Hz, H-7), 4.03 (1H, ddd, $J = 9.5$, 4.0 and 2.5 Hz, H-5′), 4.07 (1H, dd, $J = 6.5$ and 5.5 Hz, H-3), 4.30–4.5 (5H, m, H-2′, 3′, 4′and 6′), 5.29 (1H, s, H-5), 5.55 (1H, s, H-13α), 6.45 (1H, d, $J = 8.0$ Hz, H-1′) and 6.62 (1H, s, H-13β); ^{13}C-NMR (100 MHz, pyridine-d_5): δ 167.2 (C-12), 144.5 (C-11), 138.1 (C-4), 126.6 (C-13), 123.8 (C-5), 97.3 (C-1′), 80.4 (C-5′), 79.5 (C-3′), 75.3 (C-2′), 71.9 (C-4′), 67.7 (C-3), 63.0 (C-6′), 43.7 (C-7), 42.3 (C-1), 40.1 (C-6), 36.9 (C-9), 36.2 (C-2), 29.8 (C-10), 27.4 (C-8), 22.4 (C-15) and 21.5 (C-14).

3.5. *Hydrolisation of compound 3*

The solution of **3** (1 mg) in 0.5 mL of HCl (1 mol L^{-1}) was heated at 100°C for 2 h and then evaporated. Compared with standard glucose and compound **2**, the reaction mixture was detected using TLC methods.

3.6. *Time-course of biotransformation*

The cultures of *A. carambola* (10 g) were transferred to a 500-mL Erlenmeyer flask containing 200 mL medium and cultured by continuous shaking for 11 days at 25°C. Substrate **1** (5 mg per flask) was added to the cultures and incubated at 25°C in a rotary shaker (110 rpm). At 1-day interval, three of the flasks were taken out and the cultures and medium were separated by filtration. The extraction and analytic procedures were the same as those described above. The yield of the products was determined on the basis of the peak area from HPLC and expressed as a relative percentage to the total amount of whole reaction products. A binary gradient elution system consisted of water (A) and methanol (B), and separation was achieved using the following gradient programme: 0–5 min 40–65% B; 5–10 min 65–70% B; 10–15 min 70–85% B; 15–20 min 85–100% B; 20–25 min 100% B, and finally, reconditioning the column with 40% B isocratic for 5 min. The flow rate was 0.8 mL min^{-1} and the system operated at 30°C. The detection wavelength was set at 230 nm.

3.7. *MTT cell proliferation assay*

The inhibitory effect of products **3** and **4** on the growth of K562 and HeLa cells was evaluated *in vitro* by MTT[3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium-bromide] assay as described in the literature (Sabo et al., 2005). Cisplatin and adriamycin (ADR) were used as positive controls. The concentrations of hydroxyl products and positive controls on the selected cell lines were in the range 0.0041–1.0000 μmol mL^{-1}. (The values of IC50 are given as means ± standard deviation of three independent experiments.)

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