African Journal of Biotechnology Vol. 11(7), pp. 1724-1728, 24 January, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1187
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Biotransformation of dihydro-epi-deoxyarteannuin B by suspension-cultured cells of *Averrhoa carambola*

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Accepted 7 July, 2011

The biotransformation of dihydro-epi-deoxyarteannuin B (compound 1) was investigated by using suspension-cultured cells of *Averrhoa carambola*. One novel sesquiterpene, 7α-hydroxy-dihydro-epi-deoxyarteannuin B (compound 2, 29.02%), and one known sesquiterpene, 3-α-hydroxy-dihydro-epi-deoxyarteannuin B (compound 3, 23.26%), were obtained upon the addition of the substrate (compound 1). All the biotransformation products were obtained for the first time by using suspension-cultured cells of *A. carambola* as a new biocatalytic system, and their structures were identified based on nuclear magnetic resonance and mass spectral analyses. The results indicate that the cultured cells of *A. carambola* have the abilities to hydroxylate sesquiterpene compounds in a regio- and stereo-selective manner. Moreover, antitumor activities of compounds 2 and 3 were investigated against K562 and HeLa cell lines. These data demonstrate that 7-hydroxyl product (compound 2) exhibited stronger antitumor activity than the 3-hydroxyl product (compound 3) against the K562 and HeLa cell lines.

Key words: *Averrhoa carambola*, biotransformation, dihydro-epi-deoxyarteannuin B, regio- and stereo-selective hydroxylation.

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 possesses very good antihepatitis and anticancer activities in addition to its antimalarial activity (Romero et al., 2005; Efferth et al., 2001). Dihydro-epi-deoxyarteannuin B (compound 1) was proposed to be one of the intermediates in biosynthesis pathway leading to artemisinin in *Artemisia annua* (Sy et al., 2001). Compound 1 has similar chemical structure (cadinane-type sesquiterpene) with that of artemisinin. Therefore, the utilization of compound 1 as starting material is of practical importance to the synthesis of artemisinin analogs.

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Over the past few decades, biotransformation has been extensively studied because it is considered as an important method for converting inexpensive and plentiful organic compounds into the more expensive and scarce ones (Liu et al., 2010; Gren et al., 2010). The essence of biotransformation is enzyme reaction. The availability of the enzymatic system, its mild reactions, the ease of work-up and safety system are some of its advantages. Using plant-derived enzymes is important from the view-point of the green chemistry. Recently, plant cell cultures as an important biotransformation system have been used widely because of their biochemical potential to produce specific secondary metabolites that could confer such useful elements as flavors, pigments and agrochemicals (Suga and Hirata, 1990; Ishihara et al., 2003).

As of now, there is still no report about the biotransformation of dihydro-epi-deoxyarteannuin B. The suspension culture system of *Averrhoa carambola* L. was established by our research group. In this study, dihydro-

Table 1. ^{13}C NMR (100 MHz) data for compounds 1 to 3 (δ ppm).

Carbon	δC	δC	δC
	1	2	3
1	46.6	43.4	41.6
2	21.0	21.0	32.0
3	30.8	30.9	67.2
4	142.2	145.6	131.1
5	121.8	118.1	125.0
6	83.2	84.4	82.7
7	42.8	76.2	42.8
8	23.4	40.9	24.3
9	32.4	29.8	32.0
10	29.6	29.6	31.3
11	39.7	45.6	39.7
12	179.4	176.3	179.1
13	9.4	6.9	9.6
14	19.6	19.4	19.6
15	23.8	23.8	24.3

epi-deoxyarteannuin B, 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 study the bioconversion capability of this new cultured system on important sesquiterpene compounds.

MATERIALS AND METHODS

General

^{1}H and ^{13}C nuclear magnetic resonance (NMR) and two dimensional nuclear magnetic resonance (2D NMR) spectra were recorded on a Bruker DRX-400 spectrometer, the chemical shifts (δ) were given in ppm relative to tetramethylsilane (TMS) as an internal standard, and coupling constants were given in Hz. Electrospray ionization-mass spectrum (ESI-MS) data were obtained with a 4000 Q TRAP LC/MS/MS system by direct inlet using methanol as solvent. High performance liquid chromatography (HPLC) analyses were performed on an Agilent 1200 liquid chromatograph system (Palo Alto, CA, USA), equipped with quaternary gradient pump and diode array detection (DAD), connected to an Agilent ChemStation software. A Pheomenex synergi C_{18} column (5 µm, φ4.6 mm × 250 mm) and a Pheomenex octadecyl silane (ODS) C_{18} guard column (4.6 × 12.5 mm, µm) were used. Silica gel (100 to 200 mesh and 200 to 300 mesh) used for column chromatography (CC) and Silica GF_{254} (10 to 40 µ) for thin layer chromatography (TLC) were supplied by the Qingdao Marine Chemical Factory, China, and ODS from YMC Co., Ltd, Japan.

Substrate

Dihydro-epi-deoxyarteannuin B was extracted and isolated from *A. annua* by our research group according to established protocols (Sy and Brown, 1998). The structure of compound 1 was determined by MS and NMR. The purity was >98% by HPLC analysis.

Plant cell culture

Culture cells of *A. carambola*, which were induced in our laboratory, were sub-cultured on MS liquid medium (Murashige and Skoog media containing 3% sucrose without agar) supplemented with 2.0 mg/l 6-benzylaminopurine (6-BA) and 2.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) at 25°C in the dark at 2-week intervals.

Biotransformation of dihydro-epi-deoxyarteannuin B and purification of products

Suspension-cultured cells of *A. carambola* were maintained in 250-ml flasks, each containing 100 ml MS liquid medium, and grown in the dark at 25°C under agitation (110 rpm). Substrate (100 mg) was administered to 20 flasks. After incubation, the cultures and medium were separated by filtration with suction. The dried cultures were extracted with methanol 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 system to afford products compounds 2 and 3.

Dihydro-epi-deoxyarteannuin B (compound 1)

Oil; ESI-MS: 233 $[M–H]^-$, 257 $[M+Na]^+$; ^{1}H NMR [400 MHz, (acetone-d_6)]: δ 0.93 (3H, d, J = 6.5 Hz, H-14), 1.06 (H-9α), 1.14 (3H, d, J = 7.1 Hz, H-13), 1.18 (H-8β), 1.26 (H-1), 1.42 (H-10), 1.67 (H-9β), 1.69 (H-2β), 1.73 (H-8α), 1.89 (H-2α), 2.06 (H-3β), 2.10 (H-3α), 2.11 (H-7), 3.15 (1H, dq, J =7.1, 7.1 Hz, H-11), 5.63 (1H, d, J = 6.2 Hz). ^{13}C NMR data of compound 1 are shown in Table 1.

7α-hydroxy-dihydro-epi-deoxyarteannuin B (compound 2)

Oil; ESI-MS: 249 $[M–H]^-$ and 273 $[M+Na]^+$; ^{1}H NMR [400 MHz,

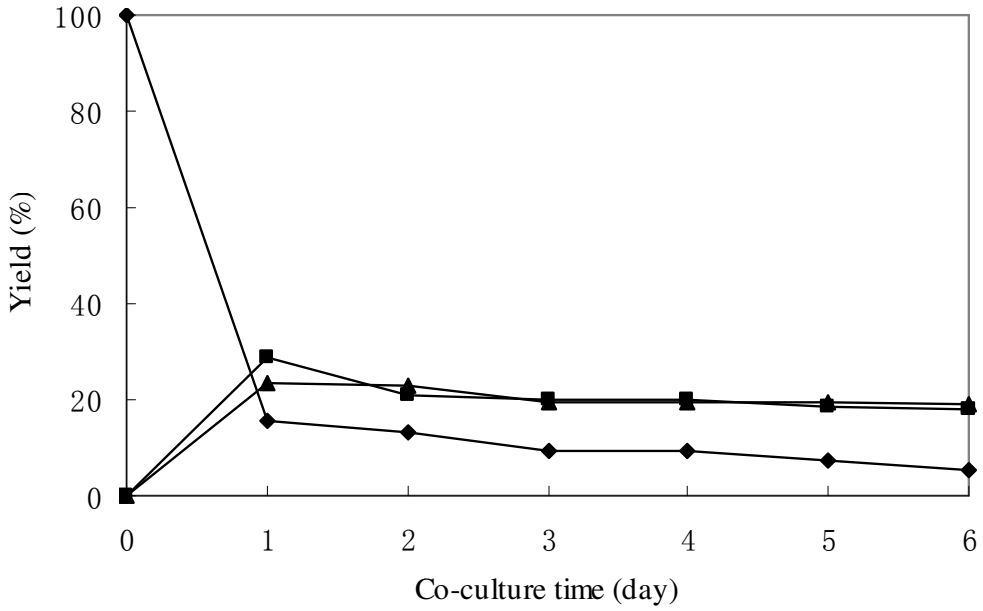

Figure 1. Time-course of biotransformation of dihydro-epi-deoxyarteannuin B (1) by the suspension-cultured cells of *A.carambola*. Yields of 1 (◆), 2 (■), 3 (▲) are plotted.

(acetone-d_6)]: δ 0.90 (3H, d, *J* = 6.2 Hz, Me-14), 1.17 (3H, d, *J* = 8.0 Hz, Me-13), 1.19 (1H, s, H-8β), 1.27 (1H, s, H-1), 2.67 (1H, s, OH), 3.07 (1H, q, H-11) 5.7 (1H, s, H-5). ^{13}C NMR data of compound 2 are shown in Table 1.

3α-hydroxy-dihydro-epi-deoxyarteannuin B (compound 3)

Oil; ESI-MS: 249 $[M–H]^-$ and 273 $[M+Na]^+$; ^{1}H NMR [400 MHz, (acetone-d_6)]: δ 0.94 (3H, d, *J* = 6.5 Hz, Me-14), 1.16 (3H, d, J = 7.0 Hz, Me-13), 3.27 (1H, dq, J = 6.3, 7.0 Hz), 5.84 (1H, d, J = 6.1 Hz, H-5). ^{13}C NMR data of compound 3 are shown in Table 1.

Time-course of biotransformation

The cultured cells 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 (5 mg/flask) was added to the suspension cultures and incubated at 25°C in a rotary shaker (110 rpm). Three of the flasks were taken out from the rotary shaker every day for six days, and the cultures and medium were separated by filtration. The extraction and analytic procedures were the same as those described earlier. The yield of the products was determined on the basis of the peak area from HPLC analyses 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 program: 0 to 5 min 40 to 65% B; 5 to 10 min 65 to 70% B; 10 to 15 min 70 to 85% B; 15 to 20 min 85 to 100% B; 20 to 25 min 100% B, and finally, reconditioning the column with isocratic 40% B for 5 min. The flow rate was 0.8 ml/min and the system was operated at 30ºC. The detection wavelength was set at 230 nm.

MTT cell proliferation assay

The inhibitory effects of compounds 2 and 3 on the growth of K562 and HeLa cells were evaluated *in vitro* by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) assay, which was performed as described in the literature (Sabo et al., 2005). The concentrations of hydroxyl products on the selected cell lines were in the range of 0.0041 to 1.0000 μmol/ml. Cis-platinum and adrimycin (ADR) were chosen as positive controls. The values of IC_{50} were given as means ± standard deviation of three independent experiments.

RESULTS AND DISCUSSION

The results demonstrate that the suspension-cultured cells of *A. carambola* were able to convert compound 1 into the corresponding hydroxylated products.

Compound 2 was assigned the molecular weight of 250 by ESI-MS (*m/z* 249 $[M–H]^-$ and 273 $[M+Na]^+$), 16 more than that of the substrate, indicating that compound 2 might be hydroxyl-dihydro-epi-deoxyarteannuin B. Upon comparison of the ^{13}C NMR data of compounds 1 and 2, the carbon signal at C-13 in compound 2 was shifted upfield (δ 9.4 → 6.9), and carbon signal C-7 in compound 2 was shifted markedly downfield (δ 42.8 → 76.2). These data suggest that the upfield shift of the C-13 signal was caused by a γ-effect due to hydroxylation of compound 2 at C-7. The structure of compound 3 was determined to be 3α-hydroxy- dihydro-epi-deoxyarteannuin B, according to the spectra of ESI-MS, ^{1}H NMR and ^{13}C NMR and by comparing the data with those from the literature (Sy and Brown, 1998; Brown and Sy, 2007).

To investigate the biotransformation pathway, the time-course in the conversion of compound 1 was followed. As shown in Figure 1, the concentration of the substrate decreased sharply in one day (100→15.64%), indicating

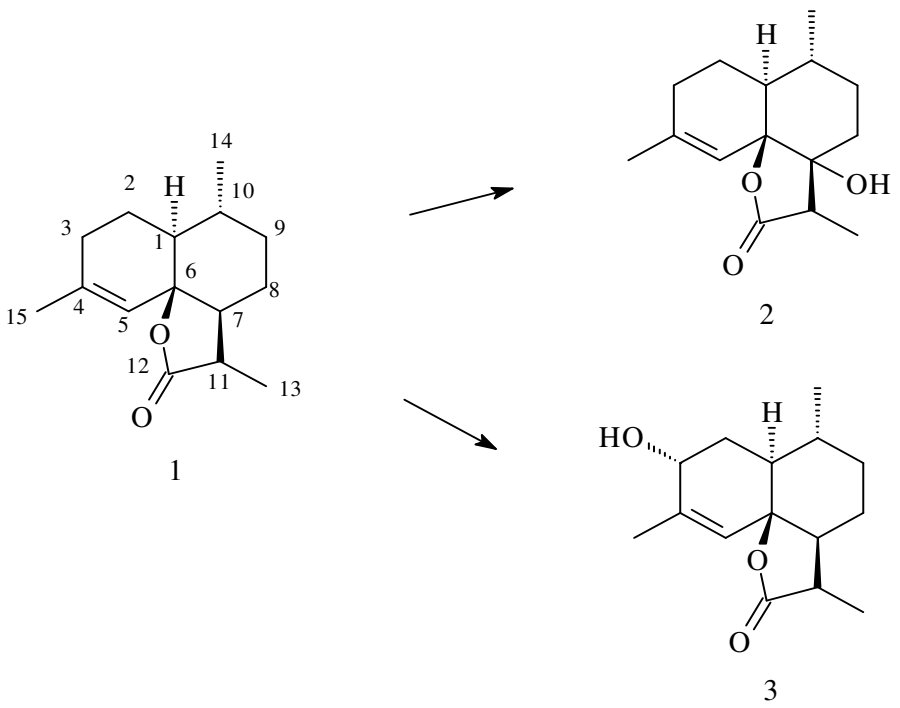

Figure 2. Proposed biosynthesis pathways of dihydro-epi-deoxyarteannuin B (1) by the suspension-cultured cells of *A. carambola*.

Table 2. Inhibitory effects of compounds 2 and 3 (1.0 µmol/ml for each) on proliferation of K562 and HeLa cell lines.

Cell line	Inhibition (%)	
	2	3
K562	59.29 ± 0.99¨	40.63 ± 1.45¨
HeLa	84.04 ± 0.27¨	41.54 ± 0.82¨

Values are given as means ± standard deviation of three separate experiments. ¨As compared to control: p <0.01.

that transformation happened on the 1st day. At the same time, compounds 2 and 3 reached their maximum transformed rates (29.02 and 23.26%, respectively) on the 1st day. Biotransformation products 2 and 3 were detected in the medium. The proposed biosynthesis pathways of compounds 2 and 3 are shown in Figure 2. Dihydro-epi-deoxyarteannuin B was regioselectively hydroxylated to yield products 2 and 3. However, there are no previous reports on the biotransformation pathways of compounds 1 to 2 and 3. For the first time, this study describes the biosynthesis pathways of compounds 2 and 3.

In summary, dihydro-epi-deoxyarteannuin B was converted into two hydroxylated products: 7α-hydroxy-dihydro-epi-deoxyarteannuin B and 3α-hydroxy-dihydro-epi-deoxyarteannuin B by cultured cells of *A. carambola*. The results show that cultured cells of *A. carambola* could be used as biocatalyst to produce regio- and stereo-selective derivatives of cadinane-type sesquiterpenes.

Antitumor activities of compounds 2 and 3 were investigated against K562 and HeLa cell lines. The antitumor activity is summarized in Table 2. The HeLa cell line was more sensitive to those products than the K562 cell line. The IC_{50} values of 2 against HeLa and K562 cell lines were 0.20 ± 0.13 and 0.84 ± 0.02 µmol·mL^{-1}. The IC_{50} value of Cis-platinum against HeLa cell line was 5.46 ± 0.37 µmol·L^{-1}, and that of ADR against K562 cell lines was 0.27 ± 0.12 µmol·L^{-1}. These results demonstrate that compounds 2 and 3 had *in vitro* anti-tumor activities, and that 7-hydroxyl product (compound 2) exhibited stronger antitumor activity than the 3-hydroxyl product (compound 3) against the K562 and HeLa cell lines. In addition, further research is still in progress for screening of broad spectrum antitumor activity of these biotransformation products.

Plant cultured cells possess considerable biochemical ability to transform xenobiotic substrates such as various organic compounds. Therefore, the biotransformation by plant cultured cells is considered to serve as an important tool for structural modification of molecules to give compounds possessing useful properties. It was found that the plant cultured cells of *A. carambola* had high activity for hydroxylation.

ACKNOWLEDGMENTS

This work was supported by a grant from National Natural Science Foundation of China (81073004). The authors thank Dongbo Yu of University of Texas, Southwestern Medical Center, USA, for proof-reading the manuscript.

REFERENCES

Brown GD, Sy LK (2007). *In vivo* transformations of artemisinic acid in *Artemisia annua* plants. Tetrahedron, 63: 9536-9547.

Efferth T, Dunstan H, Sauerbrey A, Miyachi H, Chitambar CR (2001). The anti-malarial artesunate is also active against cancer. Int. J. Oncol., 18: 767-773.

Gren I, Wojcieszynska D, Guzik U, Perkosz M, Hupert-Kocurek K (2010). Enhanced biotransformation of mononitrophenols by *Stenotrophomonas maltophilia* KB2 in the presence of aromatic compounds of plant origin. World J. Microbiol. Biotechnol., 26: 289-295.

Ishihara K, Hiroki H, Toshifumi H, Nobuyoshi N (2003). Biotransformation using plant cultured cells. J. Mol. Catal. B-Enzym., 23: 145-170.

Liu YC, Chen GY, Ge FL, Li W, Zeng LH, Cao WG (2011). Efficient biotransformation of cholesterol to androsta-1,4-diene-3,17-dione by a newly isolated actinomycete *Gordonia neofelifaecis*. World J. Microbiol. Biotechnol., 27: 759-765.

Romero MR, Efferth T, Serrano MA, Castaño B, Macias RIR, Briz O, Marin JJG (2005). Effect of artemisinin/artesunate as inhibitors of hepatitis B virus production in an “in vitro” replicative system. Antiviral Res., 68: 75-83.

Sabo TJ, Dinovic VM, Kaluderovic GN, Stanojkovic TP, Bogdanovic GA, Juranic ZD (2005). Syntheses and activity of some platinum (IV) complexes with n-methyl derivate of glycine and halogeno ligands against HeLa, K562 cell lines and Human PBMC. Inorganica Chimica Acta, 358: 2239-2245.

Suga T, Hirata T (1990). Biotransformation of exogenous substrates by plant cell cultures. Phytochemistry, 29: 2393-2406.

Sy LK, Brown GD (1998). A novel endoperoxide and related sesquiterpenes from *Artemisia annua* which are possibly derived from ailylic hydroperoxides. Tetrahedron, 54: 4345-4356.

Sy LK, Zhu NY, Brown GD (2001). Synthese of dihydroartemisinic acid and dihydro-epi-deoxyarteannuin B incorporating a stable isotope label at the 15-position for studies into the biosynthesis of artemisinin. Tetrahedron, 57: 8495-8510.