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BIOTRANSFORMATION OF ARTEMISINIC ACID BY CULTURED CELLS OF *ARTEMISIA ANNUA*

HIROSHI KAWAMOTO, YOSHIHISA ASADA,† HIROSHI SEKINE* and TSUTOMU FURUYA‡

Noda Institute for Scientific Research, 399 Noda, Noda-shi, Chiba 278-0037, Japan; † School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo 108, Japan; ‡ Faculty of Science, Okayama University of Science, Ridai-cho, Okayama 700, Japan

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Key Word Index—*Artemisia annua*; Compositae; cell culture; biotransformation; sesquiterpene; cadinane; artemisinic acid; artemisinic acid β-D-glucopyranosyl ester; 9-β-hydroxyartemisinic acid β-D-glucopyranosyl ester; 3-β-hydroxyartemisinic acid β-D-glucopyranosyl.

Abstract—Three new biotransformation products, artemisinic acid β-D-glucopyranosyl ester, 9-β-hydroxyartemisinic acid β-D-glucopyranosyl ester and 3-β-hydroxyartemisinic acid β-D-glucopyranosyl ester, were isolated from a cell culture of *Artemisia annua* following the administration of artemisinic acid.

INTRODUCTION

Artemisinin (**1**), a cadinane-type sesquiterpene lactone containing an endoperoxide group, has been established as an antimalarial component in the plant *Artemisia annua* L. [1]. Artemisinin and its semisynthetic derivatives, artemether, arteether and artesunate, have shown great promise against multidrug resistant strains of *Plasmodium* (the malarial parasite) [2]. However, **1** remains expensive and is not available on a global scale [3]. Total chemical syntheses of **1** have been achieved, but they are too complicated to have a commercial value [3, 4]. Therefore, several groups have directed their investigations toward production of **1** by tissue cultures of *A. annua* [3]. However, little or none of this compound is synthesized *de novo* by undifferentiated cell cultures of *A. annua* [3]. Artemisinic acid (**2**) is a putative biogenetic precursor for the synthesis of **1** [5–7]. Though **2** has no antimalarial activity, the utilization of **2** as a starting material for the synthesis of **1** has a practical importance, because it has a related chemical structure (cadinane-type sesquiterpene) to that of **1**. Moreover, **2** has been reported to be more abundant than **1** in the leaves of *A. annua* [8, 9] and our unpublished data. Microbiological transformations of **2** by *Mucor mucedo* and *Aspergillus flavipes* were reported to yield epimeric 3-hydroxy artemisinic acids [10]. Methyl artemisinate was converted to methyl 3-oxoartemisinate by plant suspension cell culture of *Mentha piperita* L. [11]. There has been no report on the transformation of **2** by cultured cells of *A. annua*. In this paper, the fates of **2** were studied after its administration to a cell culture of *A. annua*.

RESULTS AND DISCUSSION

Calli of *A. annua* were induced from young stems. The initial calli were heterogeneous, consisting of green and compact parts, and white and friable parts. However, after subculturing only green and compact parts (or only white and friable parts) selectively for several times, two apparently almost homogeneous cell lines (green and compact, and white and friable) were established. Compounds **1** and **2** were not detected in either cell line. For the biotransformation experiment, the white and friable cell line was used because of its good growth. The cells were inoculated into a mini jar fermentor immediately followed by the administration of **2**. After two days of culture, the cells and medium were harvested and extracted as described in the Experimental. Approximately 60% of the administered **2** had been transformed, while **2** was not degraded for two days without the cells. These results show that **2** was transformed to some compounds by the cells, though **1** was detected in neither the cells nor the medium.

As described in the Experimental, product **3** was isolated from the medium, while products **4** and **5** were isolated from the cells (Fig. 1). Products **3**–**5** were not detected on HPLC analyses of the culture to which no substrate was added.

The chemical structures of **3**–**5** were determined as

* Author to whom correspondence should be addressed.

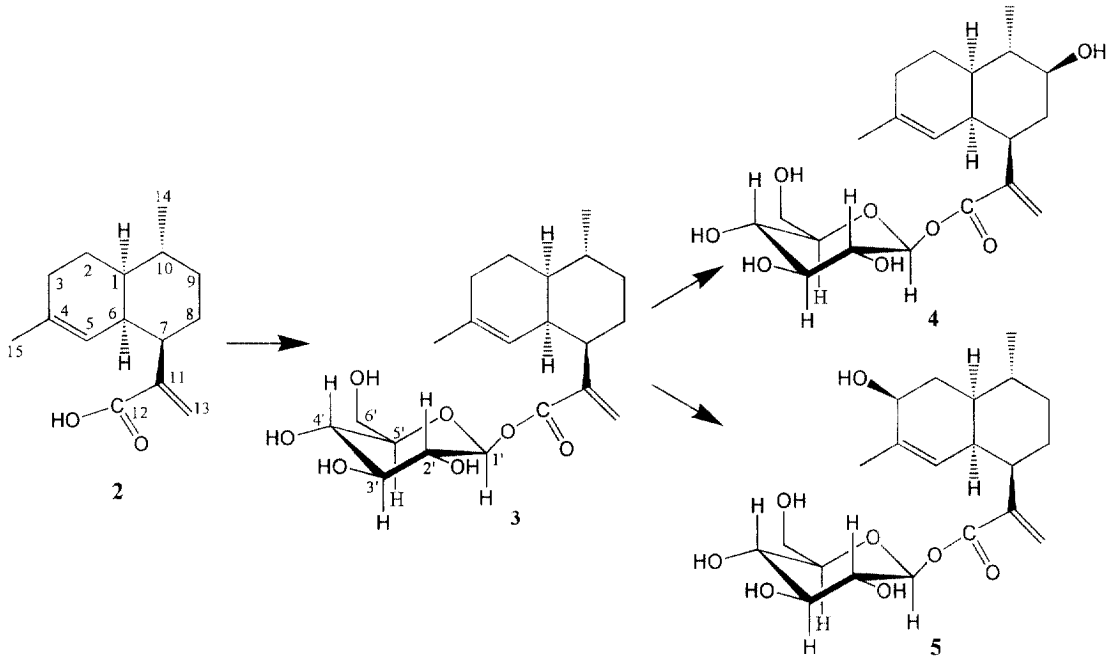

Fig. 1. Proposed scheme for the biotransformation of artemisinic acid (**2**) by cultured cells of *A. annua*.

follows. The assignments of the ^{13}C NMR spectrum [12] and ^{1}H NMR spectrum [13] of **2** have been reported.

Product **3** was assigned a M_r of 396 (SIMS), i.e. 162 more than **2**. Its ^{1}H NMR spectrum showed an anomeric proton signal at δ 5.58 (1H, *d*, J = 7.6 Hz), indicating β-configuration. In addition, the proton signal due to the carboxyl group of **2** [δ 10.6 (1H, *br s*, COOH)] was absent. In the ^{13}C NMR spectrum (Table 1), 21 carbon signals, i.e. six more than **2**, were observed. A comparison of ^{13}C NMR spectrum of **3** with that of **2** showed that carboxyl carbon signal was shifted by 2.4 ppm, suggesting that **3** was an ester. From the patterns of the carbon and proton signals due to the sugar moiety, the component sugar in **3** was indicated to be β-D-glucose. Therefore, **3** was assigned as artemisinic acid β-D-glucopyranosyl ester.

Product **4** was assigned a M_r of 412 (SIMS), i.e. 16 more than **3**. An anomeric proton signal was observed at δ 6.50 (1H, *d*, J = 8.0 Hz) in the ^{1}H NMR spectrum of **4**. On comparison of ^{13}C NMR spectrum (Table 1) of **4** with that of **3**, it was found that they were similar to each other, except that the carbon signal at C-14 in **4** was shifted upfield by 4.2 ppm, and one carbon signal (δ 75.5) was shifted substantially downfield. From these data, it was concluded that the upfield shift of the C-14 signal was caused by a γ-effect due to hydroxylation of **3** at either C-9 or C-1. Since the carbon signal (δ 75.5) shifted downfield was an oxymethine carbon in the DEPT spectrum, **4** was shown to be a C-9 hydroxylated derivative of **3**. To elucidate the stereochemistry of the OH group in **4**, the NOESY spectrum was measured. A NOE correlation was observed between H-9 (δ 3.48) and H-7 (δ 3.08). This data can only be accounted for if configuration of H-9 in **4** is α (β OH), in which H-9 lies in close proximity to H-7. In this conformation, the coupling pattern of H-9 [δ 3.48 (1H, *dddd*, J = 10.0, 10.0, 5.5, 4.5 Hz)]

Table 1. ^{13}C NMR spectral data for biotransformation products **3**–**5**

C	3*	4†	5†
1	42.4	40.4	41.5
2	26.2	25.5	35.5
3	26.9	26.6	67.0
4	135.3	135.2	137.4
5	121.2	120.9	123.4
6	38.7	38.3	39.3
7	43.2	40.4	43.0
8	26.6	36.1	26.7
9	36.0	75.5	36.2
10	28.3	36.5	29.1
11	143.8	142.9	143.7
12	166.3	166.4	166.4
13	125.8	126.0	126.0
14	20.0	15.8	20.8
15	23.8	23.9	21.7
1′	95.8	96.6	96.6
2′	73.7	74.6	74.6
3′	77.9	78.8	78.8
4′	71.0	71.9	71.1
5′	78.4	79.8	79.8
6′	62.3	62.2	62.2

* Measured in $(CD_3)_2CO$ at 50 MHz.
† Measured in pyridine-d_5 at 100 MHz.

was entirely consistent with the dihedral angles (H-9 and H-8β: ca 180°, H-9 and H-10: ca 180°, H-9 and H-8α: ca 60°). Therefore, **4** was shown to be 9-β-hydroxyartemisinic acid β-D-glucopyranosyl ester.

Product **5** was found to have the same M_r as **4**. On comparison of the ^{13}C NMR (Table 1) and ^1H NMR spectra of **5** and **3**, they were found to be similar to each other, but the carbon signal at C-15 in **5** was shifted upfield (δ 23.8 → 21.7, *q*), and one carbon signal was shifted markedly downfield (δ 26.9 → 67.0, *d*). These data suggested that the upfield shift of the C-15 signal was caused by a γ-effect due to hydroxylation of **3** at C-3. To elucidate the stereochemistry of the OH group in **5**, the NOESY spectrum was determined (Fig. 2). NOEs were observed between H-3 (δ 4.12) and Me-15 (δ 1.94), H-3 and H-2β (δ 2.37) and H-3 and H-2α (δ 1.64). The contour plot between H-3 and H-2α was stronger than that between H-3 and H-2β and no NOE was observed between H-3 and H-10. Furthermore, NOEs were observed between OH-3 (δ 6.00) and H-2β and between OH-3 and H-10 (δ 2.32). These data can only be accounted for the assignment that the configuration of H-3 in **5** as α (β OH) with H-3 in close proximity to H-2α, and OH-3 to H-2β and H-10. Therefore, **5** is 3-β-hydroxyartemisinic acid β-D-glucopyranosyl ester.

The scheme for the biotransformations observed in this study is shown in Fig. 1. The carboxyl group of **1** is not esterified with D-glucose but lactonized, and **1** has neither a 9β- nor 3β-hydroxyl group. The potential for esterification with D-glucose and hydroxylation(s) of various exogenous compounds by cultured plant cells have been reported and understood to be a mechanism of detoxification [14–19]. This may be the case here. For the purpose of the transformation of **2** to **1**, the suppression of the esterification and hydroxylations by chemical or genetic regulation will be necessary.

Though the epimeric 3-hydroxylations of **2** by microbes have been reported [10], the 9-β-hydroxylations of a cadinane-type sesquiterpene is new. Since regio- and stereo-selective hydroxylations are difficult to achieve by chemical methods, the possibility of bringing about 9-β- and 3-β-hydroxylations may be useful in the syntheses of various cadinane-type sesquiterpene derivatives.

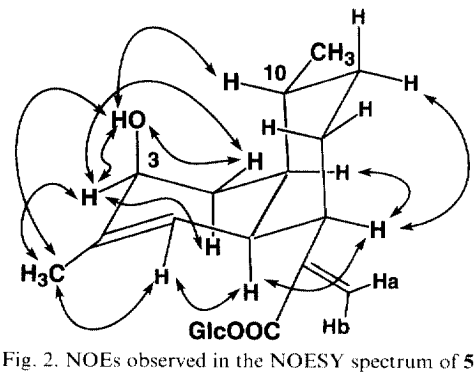

Fig. 2. NOEs observed in the NOESY spectrum of **5**.

EXPERIMENTAL

Materials

An authentic sample of **2** was provided by Dr Nancy Acton. A large quantity of **2**, needed as the substrate for the biotransformation experiment, was prepared from leaves of *A. annua* cultivated in our laboratory according to the methods described in the literature [20, 21].

Chemical analysis

For the analysis of **1** and **2**, cells were freeze dried and extracted with EtOAc (×3). After removal of the solvent, the EtoAc extract was dissolved in MeOH. The medium was extracted with EtOAC (×2) and after evaporation of the solvent the EtOAc extract dissolved in MeOH. The analysis of **1** was carried out according to the method of Zhao and Zeng [22] with partial modifications. The sample soln (0.1 ml) was mixed with 0.2% NaOH soln (0.4 ml), and warmed in a water bath at 45° for 20 min. After being cooled in a cold water bath, the mixture was neutralized and diluted to 1 ml with 0.1 M HOAc in 20% EtOH. This soln was subjected to HPLC, and the absorbance at 260 nm measured. For the analysis of **2**, the MeOH sample soln was subjected directly to HPLC, and the absorbance at 220 nm measured. The conditions used were: Cosmosil 5C18 (4.6 × 150 mm, Nacalai Tasque, Kyoto, Japan); mobile phase 0.01 M Na_2HPO_4–NaH_2PO_4 buffer [H_2O–MeOH (3:2)] for the analysis of **1**, and H_2O–MeCN–TFA (60:40:0.5) for the analysis of **2**.

Spectrometric analyses

SIMS: 70 EV, Hitachi M80B spectrometer with glycerol as matrix; ^1H NMR: 200 and 400 MHz; ^{13}C NMR: 50 and 100 MHz, with TMS as an internal standard.

Callus induction and culture conditions

Seeds of *A. annua* were sterilized by EtOH (70%, 30 s) and NaClO soln (ca 2% w/v, 30 min), and plated on half strength Murashige-Skoog medium (MS medium) [23] supplemented with sucrose (20 g l^{-1}), agar (0.8 g l^{-1}) and MES (2.5 mM). They were incubated at 25° with a 12 h-light (2500 lux) and 12-h dark cycle. Young stems from 3-week-old plantlets were cut into 1 cm segments, and placed on MS medium supplemented with sucrose (30 g l^{-1}), Gellan Gum (2 g l^{-1}, Wako, Japan), MES (5 mM), NAA (0.5 ppm) and 6-benzylaminopurine (0.1 ppm). They were incubated at 25° with a 12 h-light (2500 lux) and 12 h-dark cycle until callus was induced. Callus material was

subcultured every 2–3 weeks under the same culture conditions.

Feeding experiment

Two-week-old statically cultured cells (ca 90 g fr. wt) were inoculated into a 5 l mini jar fermentor (Takasugi Seisakusho, Japan) containing half strength MS medium (4 l) supplemented with sucrose (30 g l^{-1}), MES (2.5 mM), NAA (0.5 ppm) and 6-benzylaminopurine (0.1 ppm). Substrate **2** (120 mg/2.4 ml EtOH) was administered immediately after inoculation with the cells. The cells were cultured for 2 days at 25° in the dark with an aeration ratio of 0.25 VVM and an agitation speed of 50 rpm. The same culture was repeated 11 times.

Isolation of biotransformation products

The cells and the medium (total 1320 mg of **2** administered) were sepd by filtration. The filtrate was passed through a Diaion HP-20 column and then eluted with MeOH. The MeOH eluate was concd under red. pres. The residue suspended in H_2O was extracted with EtOAc (× 2) and the EtOAc layer was concd to obtain M-EtOAc fr. (5 g). The cells were lyophilized (55 g), extracted with EtOAc at room temp. and extracted with *n*-BuOH satd with H_2O. The extract was washed with H_2O, and the *n*-BuOH layer was concd to obtain the C-BuOH fr. (9 g). Both frs were subjected to CC on silica gel, and further purified by HPLC (column: Prep Nova-Pak HR C18, 100 × 25 mm, Waters) with elution with MeCN–H_2O (2:3 for **3**, 1:4 for **4** and **5**). From the M-EtOAc fr., **3** (10 mg) was obtained. From the C-BuOH fr. **4** (58 mg) and **5** (38 mg) were obtained.

Artemisinic acid β-D-glucopyranosyl ester (**3**). Amorphous solid; ^{1}H NMR [200 MHz, $(CD_3)_2CO$]: δ 0.91 (3H, *d*, J = 5.9 Hz, Me-14), 1.33 (1H, *br d*, J = 12.0 Hz, H-8α), 1.59 (3H, *s*, Me-15), 2.63 (1H, *br s*, H-6), 3.4–3.9 (6H, *m*, H-2′, 3′, 4′, 5′ and 6′), 5.04 (1H, *s*, H-5), 5.56 (1H, *s*, H-13a), 5.58 (1H, *d*, J = 7.6 Hz, H-1′), 6.41 (1H, *s*, H-13b); SIMS *m/z* 419 $[M+Na]^+$.

9-β-Hydroxyartemisinic acid β-D-glucopyranosyl ester (**4**). Amorphous solid; ^{1}H NMR (400 MHz, pyridine-d_5): δ 1.24 (3H, *d*, J = 6.5 Hz, Me-14), 1.47 (1H, *m*, H-1), 1.53 (3H, *s*, Me-15), 1.74 (1H, *m*, H-8β), 1.93 (1H, *br d*, J = 12.0 Hz, H-8α), 2.86 (1H, *br s*, H-6), 3.08 (1H, *ddd*, J = 13.5, 3.0 and 3.0 Hz, H-7), 3.48 (1H, *dddd*, J = 10.0, 10.0, 5.5 and 4.5 Hz, H-9), 4.10 (1H, *ddd*, J = 9.5, 4.0 and 2.5 Hz, H-5′), 4.28 (1H, *dd*, J = 8.5 and 8.0 Hz, H-2′), 4.35 (1H, *dd*, J = 9.0 and 8.5 Hz, H-3′), 4.37–4.50 (3H, *m*, H-4′ and 6′), 5.18 (1H, *s*, H-5), 5.57 (1H, *s*, H-13a), 6.10 (1H, *d*, J = 5.5 Hz, OH-9), 6.50 (1H, *d*, J = 8.0 Hz, H-1′), 6.61 (1H, *s*, H-13b); SIMS *m/z* 435 $[M+Na]^+$.

3-β-Hydroxyartemisinic acid β-D-glucopyranosyl ester (**5**). Amorphous solid; ^{1}H NMR (400 MHz, pyridine-d_5): δ 0.99 (1H, *dddd*, J = 13.0, 13.0, 13.0 and 3.0 Hz, H-9α), 1.09 (3H, *d*, J = 6.5 Hz, Me-14), 1.39 (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), 1.68 (1H, *dd*, J = 13.0 and 2.0 Hz, H-9β), 1.94 (3H, *s*, Me-15), 2.32 (1H, *m*, H-10), 2.37 (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), 4.11 (1H, *ddd*, J = 9.5, 4.0 and 2.5 Hz, H-5′), 4.12 (1H, *dd*, J = 6.5 and 5.5 Hz, H-3), 4.30 (1H, *dd*, J = 8.5 and 8.0 Hz, H-2′), 4.36 (1H, *dd*, J = 9.0 and 8.5 Hz, H-3′), 4.38–4.50 (3H, *m*, H-4′ and 6′), 5.34 (1H, *s*, H-5), 5.58 (1H, *s*, H-13a), 6.00 (*d*, J = 5.5 Hz, OH-3), 6.52 (1H, *d*, J = 8.0 Hz, H-1′), 6.66 (1H, *s*, H-13b); SIMS *m/z* 435 $[M+Na]^+$.

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