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Structural characterization of 1β,11α-dihydroxycanrenone biotrans-formed from canrenone by *Aspergillus ochraceus* SIT34205

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Abstract

Canrenone (**1**) was biotransformed into 11α-hydroxycanrenone (**2**) and a main byproduct (**3**) by *Aspergillus ochraceus* SIT34205. Compound **3** was separated and purified using silica gel column chromatography, and its structure was characterized *via* MS and NMR methods. These results indicated that **3** was 1β,11α-dihydroxycanrenone and the product of further hydroxylation of **2**. Thus, investigating the structure and synthesis of **3** may be a promising method to improve the efficiency and purity of **2**.

Keywords: *Aspergillus ochraceus*; Biotransformation; Canrenone; 1β,11α-Dihydroxycanrenone

11α-Hydroxycanrenone (**2**) is used mainly as a pharmaceutical intermediate for synthesizing eplerenone, which has cardiovascular protective effects [1,2]. In the semi-chemical synthesis of eplerenone from canrenone (**1**), 11α-hydroxylation of **1** by microbial transformation is the first step and one of the most critical steps [3].

Reactions involved in microbial transformation for the production of steroid agents typically include hydroxylation, dehydrogenation, and sterol side-chain cleavage, among others [4,5]. The most important reaction directed to microbial transformation of steroid compounds is the hydroxylation reaction. A number of microorganisms in nature are capable of transformation *via* 11α-hydroxylation of steroids, and most of them are molds [6]. *Aspergillus ochraceus* is used extensively to convert **1** into **2**. In the middle and late stages of the biotransformation, a main byproduct (**3**) with a polarity that was very close to that of **2** was previously found. Compound **3** caused inconvenience to the purification of **2**, which, in turn, was disadvantageous to the chemical synthesis of highly pure eplerenone. The production and the preparative processes of **2** have been reported previously [7,8]. However, to the best of our knowledge, no reports on **3** have been published. In the present study, we aim to identify the structure of **3** in order to clarify the process of microbial conversion of **2** from **1** by *A. ochraceus* SIT34205.

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1. Experimental

Strain and culture media: *A. ochraceus* SIT34205 was obtained and screened by our laboratory. The composition of the slant culture medium (g/L) was as follows: Corn steep liquor 10, tryptone 10, and agar 25. The composition of seed culture medium (g/L) was as follows: Glucose 20, yeast extract 15, fish peptone 5, corn starch 5, corn steep liquor 0.5, and KH_2PO_4 0.5. The composition of biotransformation medium (g/L) was as follows: glucose 25, yeast extract 15, peptone 5, and KH_2PO_4 0.5.

Biotransformation of **2** from **1**: Strain SIT34205 was cultured in slant medium at 28 °C for 5–7 days, and transferred into sterile physiological saline to obtain a spore suspension at a concentration of 1×10^7 spores/mL. The suspension was inoculated into a 100 mL seed culture medium in a 500 mL shake flask, cultured at 28 °C and 160 rpm/min for 20 h. The seed culture was inoculated into the biotransformation medium in an amount of 4% and cultured for 20 h, followed by the addition of 10 g/L of **1**. Incubation was continued for 50 h under the same conditions.

HPLC assay of **2** and **3**: A RP C18 column (Dikma Diamonsil C_{18}, 250 mm × 4.6 mm, 5 μm) was used as chromatographic column. The wavelength for detection was 280 nm, mobile phase was methanol–water (7:3, v/v), injection volume was 10 μL, column temperature was 25 °C, and flow rate was 1 mL/min.

Isolation and identification of **3**: The biotransformation solution and the mycelia were extracted three times with ethyl acetate. The organic phases were pooled, and concentrated under reduced pressure to obtain slurry. The slurry was mixed with silica gel to homogeneity, dried completely, and then subjected to column chromatography. Gradient elution was performed with dichloromethane–methanol (100:1; 50:1; 25:1) at 2 mL/min to obtain **3**. The structure of **3** was identified by MS, ^{1}H NMR, ^{13}C NMR, DEPT135, and ^{1}H–^{1}H NOESY spectroscopy. Compound **3** was crystallized from methanol; $[\alpha]_D^{30}$ −47 (*c* 0.11, CH_3OH), ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.18 (d, 1H, J = 10 Hz), 6.09 (d, 1H, J = 10 Hz), 5.77 (s, 1H), 4.18 (dd, 1H, J = 10, 20 Hz), 4.18–4.13 (m, 1H), 3.49 (s, 1H), 2.70–2.52 (m, 4H), 2.43–2.30 (m, 3H), 2.18–2.00 (m, 1H), 1.98–1.90 (m, 3H), 1.59–1.56 (m, 1H), 1.48–1.42 (m, 3H), 1.22 (s, 3H), 0.88 (s, 3H). ESI-MS (*m/z*): 373.9 $[M+H]^+$, 395.9 $[M+Na]^+$.

2. Results and discussion

Compound **1** was biotransformed into **2** using *A. ochraceus* SIT34205 with **3** produced during the middle and late stages of the biotransformation. The results of the TLC analysis of the sample after microbial transformation indicated a small amounts of **1** with R_f = 0.73, **2** with R_f = 0.20, and **3** with R_f = 0.09 (data not shown). Therefore, the polarity of **3** was greater than that of **2** (Fig. 1).

The molecular weight of **2** was 356 [9], and that of **3** was *m/z* 373.9 in the ESI-MS result which appeared as a $[M+H]^+$ ion peak (Figure not shown), indicating that **3** had an additional hydroxyl group relative to **2**. Thus, **3** was speculated to be a dihydroxycanrenone. Compound **3** was structurally characterized using NMR (^{1}H, ^{13}C NMR, DEPT135 and NOESY) according to the methods described previously [10,11].

The ^{1}H NMR data of **3** revealed many similarities with those of **2** (Table not shown). The major differences were as follows. For chemical shifts δ 3.49, δ 4.17, and δ 4.28 in the low-field region, the strong peak at δ 3.49 disappeared after deuterium oxide exchange, suggesting the presence of a reactive hydrogen atom. Moreover, as demonstrated by

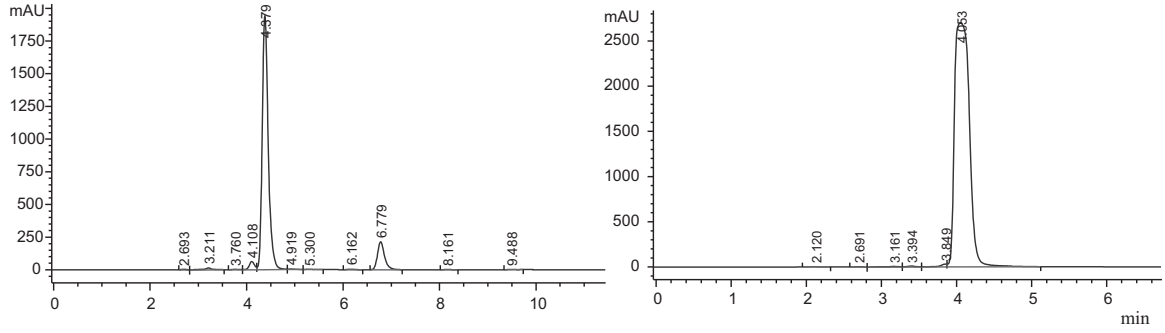

Fig. 1. HPLC chromatogram of the biotransformation sample (left panel) and the purified compound **3** (right panel). In the left panel, the retention time of 4.108, 4.379, and 6.779 min were correspond to compounds **3**, **2** and **1**, respectively.

Table 1
^{13}C NMR data for compounds **2** and **3**.

C at various positions	**2**[a]	**3**[a]
C-1	35.43	71.19
C-2	35.43	41.93
C-3	199.75	198.06
C-4	124.83	124.94
C-5	162.68	161.45
C-6	128.84	128.66
C-7	137.90	138.89
C-8	37.73	36.57
C-9	55.75	56.45
C-10	36.28	42.15
C-11	67.96	66.04
C-12	35.85	35.37
C-13	43.92	43.36
C-14	46.58	46.19
C-15	22.51	22.69
C-16	34.17	35.37
C-17	94.70	94.97
C-18	15.61	15.28
C-19	17.18	11.93
C-20	31.08	30.97
C-21	29.09	29.11
C-22	176.28	176.64

[a] Data were obtained at 125 MHz in $CDCl_3$ using TMS as internal standard (chemical shift δ (ppm)).

integration, the number of hydrogen atom corresponding to either of the chemical shifts was one, suggesting the presence of oxygen atoms individually bonded to a methenyl group. Therefore, compound **3** can be identified as a dihydroxyl compound.

The ^{13}C NMR and DEPT135 spectra of **3** (Table 1) revealed that one molecule of **3** contains two methyl groups, six methylene groups, eight methenyl groups, and six quaternary carbon atoms. The chemical shifts of the carbon atoms in **3** were essentially similar to those of **2** except for the following differences. The chemical shift of C1 in **2** was at δ 35.43, and this signal was absent in the carbon spectrum of **3**. An additional peak appeared at δ 71.19. The chemical shifts of both C2 and C10 were moved to the low field, with the values of δ 41.93 and δ 42.15, respectively. These data infer that the hydroxyl group is at C1 position. Considering the NOESY spectra results, H-18 and H-19 were correlated with H-11, but not with H-1. The correlation of H-1 with H-9 indicated that the two hydrogen atoms were sterically close and can be considered to be on the same side [12]. H-9 was at α position, and thus H-1 may be at the α position of C1, suggesting that the hydroxyl group might be at β position of C1. In the structural identification of 12β-hydroxymexrenone by Preisig et al. [10], the chemical shift of the adjacent C18 of the compound moved from δ 15.39 to δ 8.54 in comparison with the carbon spectrum of mexrenone. In our study, the chemical shift of C19 of the isolated **3** moved to the high-field, that is, from δ 17.18 to δ 11.93, compared with the carbon spectrum of **2**, which served as further evidence for the above deduction that the hydroxyl group at C1 position is in β configuration.

According to the above analysis, we conclude that **3** is 1β, 11α-dihydroxycanrenone, which is absent in the Reaxys and CA databases (Fig. 2).

Previous studies [10] have shown that **1** may be hydroxylated by microorganisms at positions C2, C7, C9, C11, C14, C15, and C16. In the microbial transformation of steroids by Hu et al. [13], steroids were hydroxylated by microorganisms to several different hydroxysteroids, which indicated the presence of a series of hydroxylases in the cytochrome P450 enzymes. In our study, canrenone was converted to the major product **2** and byproduct **3** by *A. ochraceus* SIT34205. Different steroids focused on hydroxylation at different positions by *A. ochraceus* have been reported. Progesterone was hydroxylated to 11α-hydroxyprogesterone and byproduct 6β,11α-dihydroxyprogester – one by *A. ochraceus* TS [14]. Compound **1** was mainly hydroxylated to 15α-hydroxycanrenone by *A. ochraceus* AS3. 3930 [15]. These results are consistent with the previous reports [16], which revealed that the transformation pattern of steroids can be influenced by substrate structure. Additionally, the result of structural identification indicates that

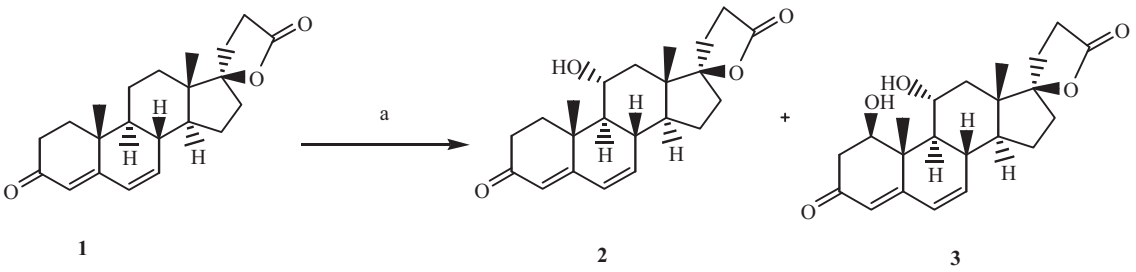

Fig. 2. Schematic of biotransformation of canrenone: (a) *A. ochraceus* SIT34205.

compound **3** is the product of further hydroxylation of **2**. The purification of 11α-hydroxylase and 1β-hydroxylase requires further investigation to inhibit the formation of **3**.

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