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NOTE

Biotransformation of 14-deoxy-14-methylenetriptolide into a novel hydroxylation product by *Neurospora crassa*

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The biotransformation of 14-deoxy-14-methylenetriptolide by *Neurospora crassa* CGMCC AS 3.1604 to produce a new hydroxylation derivative was studied. The structure of this novel compound was determined using spectral data. This biotransformation using whole cells conditioned for 4 days transformed approximately 65% (mol ratio) of the substrate into the compound (5R)-5-hydroxy-14-deoxy-14-methylenetriptolide.

[**Key words:** Biotransformation; *Neurospora crassa*; Hydroxylation; Triptonide; 14-Deoxy-14-methylenetriptolide; (5R)-5-Hydroxy-14-deoxy-14-methylenetriptolide]

Triptolide, the major active ingredient isolated from the Chinese herbal plant *Tripterygium wilfordii* Hook. f. by Kupcham in 1972 (1), has been shown to be effective in the treatment of autoimmune diseases (2) with potent anti-leukemic and antitumor activities (3,4). Triptonide, as another major active ingredient of *T. wilfordii*, has effective anti-inflammatory and antiproliferative activities (5). The compound 14-deoxy-14-methylenetriptolide (Fig. 1) can be obtained from triptonide using a two-step chemical reaction process. A pharmacodynamic evaluation of this compound suggested significant inhibition of human ovarian cancer (SK-OV-3), human breast cancer (MDA-MB-468) and human prostate cancer (PC-3) cell growth, as described previously (Li, Y., Li, Z., Zhou, Z., Lin, L., Miu, Z., Ren, J., Li, C., Gan, Y., Ding, J., and Feng, H.: Chinese patent 200810035891.9, 2008). As a potent anticancer drug, 14-deoxy-14-methylenetriptolide has been limited by significant toxicity. To produce a highly efficient biologically active substance with low toxicity, structural modifications of 14-deoxy-14-methylenetriptolide and its analogues have been performed in recent years (6). With an ability to catalyze novel reactions, biotransformation is an alternative tool for the chemical modification of natural product analogues, producing compounds with differences in the regio- and stereo-selectivity as compared with chemical synthesis (7,8). *Neurospora crassa*, a filamental fungus belonging to the Sordariaceae family, produces numerous enzymes, including xylitol dehydrogenase (9), catalase-1 (10), polyphenol oxidase and laccase (11), without generating toxic secondary metabolites. Several reports have suggested that *N. crassa* can be used in the hydroxylation of steroidal structures, such as estradiol (12), desoxycorticosterone (13) and androst-4-en-3,17-dione (14). The bioconversion of 14-deoxy-14-methylenetriptolide and its analogues by this fungus has not, to the best of our knowledge, been reported in the literature. In this study, we report on the biotransformation of 14-deoxy-14-methylenetriptolide by *N. crassa* CGMCC AS 3.1604 to produce (5R)-5-hydroxy-14-deoxy-14-methylenetriptolide (Fig. 1).

For this study, a screening-scale run was performed in 250 mL Erlenmeyer flasks containing 30 mL of a potato dextrose broth medium (PDB medium, 20 g dextrose, 200 g potato extract and 1000 mL water). Microorganisms were transferred into the flasks from the agar plates of the medium consisting of potato dextrose agar medium (PDA medium, 20 g dextrose, 20 g agar, 200 g potato extract and 1000 mL water). The cultures were cultivated on a rotary shaker at 250 rpm at 25°C. The substrate 14-deoxy-14-methylenetriptolide was dissolved in ethanol–DMSO (v/v = 4:1) and diluted to 10 mg/mL before use. After 48 h, 200 μL of this substrate solution was added into the culture. The incubation was continued for 72 h under the same conditions as described above. Fermentation in which microorganisms were grown without substrate but with the same amount of ethanol–DMSO (v/v = 4:1) solution served as a culture control. Sterile PDB medium containing the same amount of substrate and incubated under the same conditions served as a substrate control in this study. Each fermentation broth was extracted twice with 50 mL of ethyl acetate. After the ethyl acetate phases were evaporated to dryness under vacuum, the residues were dissolved in 1.5 mL acetone. The samples were analyzed by HPLC on an ODS C18 reverse phase column (Dikma Technologies Diamonsil C18 (2), 5 μm, 250 × 4.6 mm, serial number 6006167) at 218 nm and eluted under isocratic conditions with acetonitrile–water (v/v = 1:1) for 30 min

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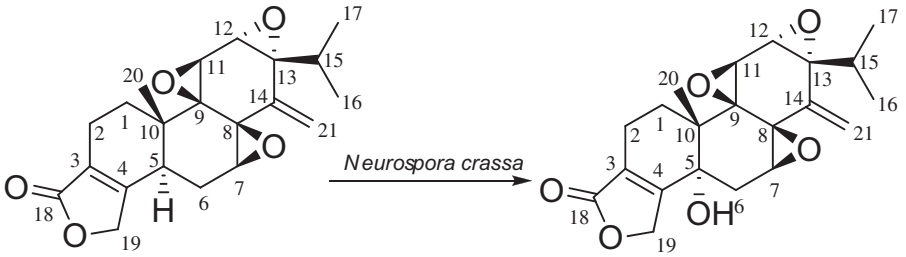

FIG. 1. Bioconversion of 14-deoxy-14-methylenetriptolide into a novel (5R)-5-hydroxylated derivative by *N. crassa* AS 3.1604.

at a flow rate of 0.8 mL/min. The HPLC trace revealed a highly polar product appearing at a peak of 11.7 min with the substrate at a less polar peak of 21.9 min (Fig. 2A). The product exhibited a UV absorption spectrum similar to that of the substrate, with a maximum at 208 nm. No transformation products were found in the controls. The broth extract was also analyzed on an Agilent 1100 LC–MS instrument (LCQ Fleet Ion Trap tandem mass spectrometer, Thermo Fisher Scientific). In the positive mode, the ESI mass spectrum ($[M + H]^+$, $[M + NH_4]^+$ and $[M + Na]^+$ ions) of the substrate was observed at *m/z* 357.43, 374.18 and 379.17 (Fig. 2B). The mass spectrum of the product had a similar pattern to that of the substrate. There was a 16-Da increase in the molecular weight between the substrate and the product, indicating an addition of one oxygen atom to the substrate.

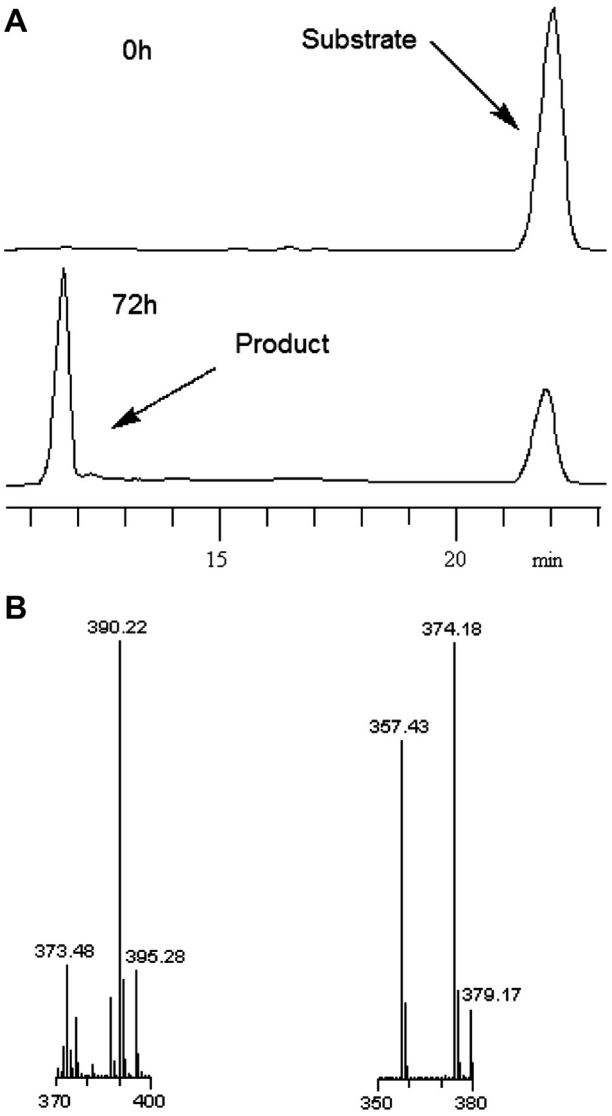

FIG. 2. LC–MS analysis of the biological conversion of 14-deoxy-14-methylenetriptolide into product by *N. crassa* AS 3.1604. (A) HPLC analysis of the biological conversion of the substrate into the product. (B) ESI-MS (+) spectrum of both product and substrate.

For the preparative transformation, aliquots of 12 mL of the substrate solution (total 120 mg of the substrate) were added into six 1000 mL Erlenmeyer flasks containing 300 mL of the PDB medium that had been pre-cultured for 48 h. After 96 h of incubation, the supernatants were extracted with ethyl acetate and the resulting extractions evaporated, as only a little product was present in the mycelium. The residue (550 mg) was subjected to column chromatography over ODS C18 40–60 μm and eluted with a methanol-water gradient [10%, 30%, 50%, 70%, 90% (v/v) and pure methanol]. The 70% aqueous methanol fraction was further separated by reversed-phase HPLC (Agilent Zorbax SB–C18 column, 5 μm, 9.4 × 250 mm) and eluted with 50% aqueous acetonitrile (v/v) to produce 25 mg of the product.

The NMR spectra were determined using a Bruker AV400 spectrometer instrument (400 MHz). High-resolution mass spectra were recorded on a Q-TOF micro mass spectrometer using electrospray ionization (ESI). The biotransformation product was a white powder with a molecular formula determined to be $C_{21}H_{24}O_6$ on the basis of the $[M + Na]^+$ peak at *m/z* 395.1472 (calculated 395.1471) in the HRESIMS. The 5-H signal (δ 2.77) of the substrate was not observed at this position in the ^{1}H NMR spectrum (DMSO-d_6) of the product (Table 1). The other H signals of the substrate were nearly the same as those of the product. This observation was further confirmed by the chemical shift of C-5 in the ^{13}C NMR spectrum data (δ 70.02 in the product vs. δ 40.44 in the substrate), with no change in the other C signals. With only one hydrogen atom in the C-5 position, the structure of the product was determined to be (5R)-5-hydroxy-14-deoxy-14-methylenetriptolide.

To further understand the biotransformation process, three processes using resting cells, resting supernatant and whole-cell biotransformations were tested and compared. *N. crassa* was grown in two 250 mL Erlenmeyer flasks containing 30 mL of a PDB medium. After 48 h, the mycelium in one Erlenmeyer flask was collected and added to a 30 mL phosphate buffer solution (pH 7.4) including 2 mg of the substrate. The resting supernatant was transferred to a sterile empty Erlenmeyer flask, followed by the addition of 2 mg of the substrate. The other control Erlenmeyer flask containing the fermentation broth was prepared by adding 2 mg of the substrate. The incubations at the three experimental conditions were continued for 96 h. With the exception of using ethyl acetate to extract the mycelium and the supernatant, the processing methods described above were used. Approximately