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Specific methylation and epoxidation of sinenxan A by *Mucor genevensis* and the multi-drug resistant tumor reversal activities of the metabolites

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Abstract

Mucor genevensis were used to bioconvert sinenxan A [2α,5α,10β,14β-tetraacetoxy-taxa-4(20),11-diene], a taxoid isolated from callus tissue cultures of *Taxus* spp., and 10 metabolites were obtained. On the basis of chemical and spectroscopic data analyses, their structures were determined as 10β-methoxy-2α,5α,14β-triacetoxy-taxa-4(20),11-diene (**2**), 10β-hydroxy-2α,5α,14β-triacetoxy-taxa-4(20),11-diene (**3**), 2α,5α,10β,14β-tetraacetoxy-4β,20-epoxy-taxa-11(12)-ene (**4**), 6α-hydroxy-2α,5α,10β,14β-tetraacetoxy-taxa-4(20),11-diene (**5**), 9α-hydroxy-2α,5α,10β,14β-tetraacetoxy-taxa-4(20),11-diene (**6**), 10β-hydroxy-2α,5α,14β-triacetoxy-4β,20-epoxy-taxa-11(12)-ene (**7**), 6α,10β-dihydroxy-2α,5α,14β-triacetoxy-taxa-4(20),11-diene (**8**), 6α-hydroxy-2α,5α,10β,14β-tetraacetoxy-4β,20-epoxy-taxa-11(12)-ene (**9**), and 9α,10β-dihydroxy-2α,5α,14β-triacetoxy-taxa-4(20),11-diene (**10**), and 9α,10β-*O*-(propane-2,2-diyl)-2α,5α,14β-triacetoxy-taxa-4(20),11-diene (**11**). Among them, metabolites **2**, **4**, and **9** were three new compounds. The three major metabolites **2**, **3**, and **4** along with **1** were pharmacologically evaluated for their multi-drug resistance (MDR) reversal activities towards taxol-resistant A549 tumor cells, and the results showed that **4** possessed about two-fold activity as verapamil, while **2**, and **3** possessed lower activity than verapamil and **1**.

Keywords: Taxanes; Biotransformation; *Mucor genevensis*; Tumor MDR reversal activity

1. Introduction

The treatment of cancer with chemotherapeutic drugs is frequently impaired or ineffective as a result of either de novo or acquired resistance of tumor cells. In both cases, tumor can be refractory to a variety of antineoplastic drugs with different structures and mechanisms of action. This phenomenon is termed multi-drug resistance (MDR). Although there are several different mechanisms associated with the development of MDR, a common cause is believed to be overexpression of a *Mr* 170,000 plasma membrane glycoprotein (P-gp) of transporter proteins, and it acts as energy-dependent drug efflux pump, preventing adequate intracellular accumulation of a broad range of cytotoxic drugs including anthracyclines, *Vinca* alkoloids, epipodophyllotoxins, and taxanes for cell kill [1]. A broad range of compounds (verapamil, cyclosporin A, etc.), have been reported to reverse MDR in vitro and in vivo. These reversing agents competitively inhibit the binding of antitumor drugs to P-gp in MDR tumor cells, and increase the intracellular accumulation of antitumor drugs and overcome MDR. However, the clinical use of these MDR reversing agents has been hampered by the toxic side effects that occur when the non-physiological doses, which are required to achieve a siginificant reversal of MDR, are used [2]. Therefore, the search for novel and more potent MDR reversing agents without side effects is of major importance.

Sinenxan A, 2α,5α,10β,14β-tetraacetoxy-taxa-4(20),11-diene (Fig. 1), is a taxoid isolated from the callus cultures of *Taxus* sp. in high yield (ca. 1–2% of dry weight) [3]. The abundant resources and its taxane-skeleton endow it valuable potential for the semisynthesis of paclitaxel or other structurally related bioactive compounds (such as tumor MDR reversing

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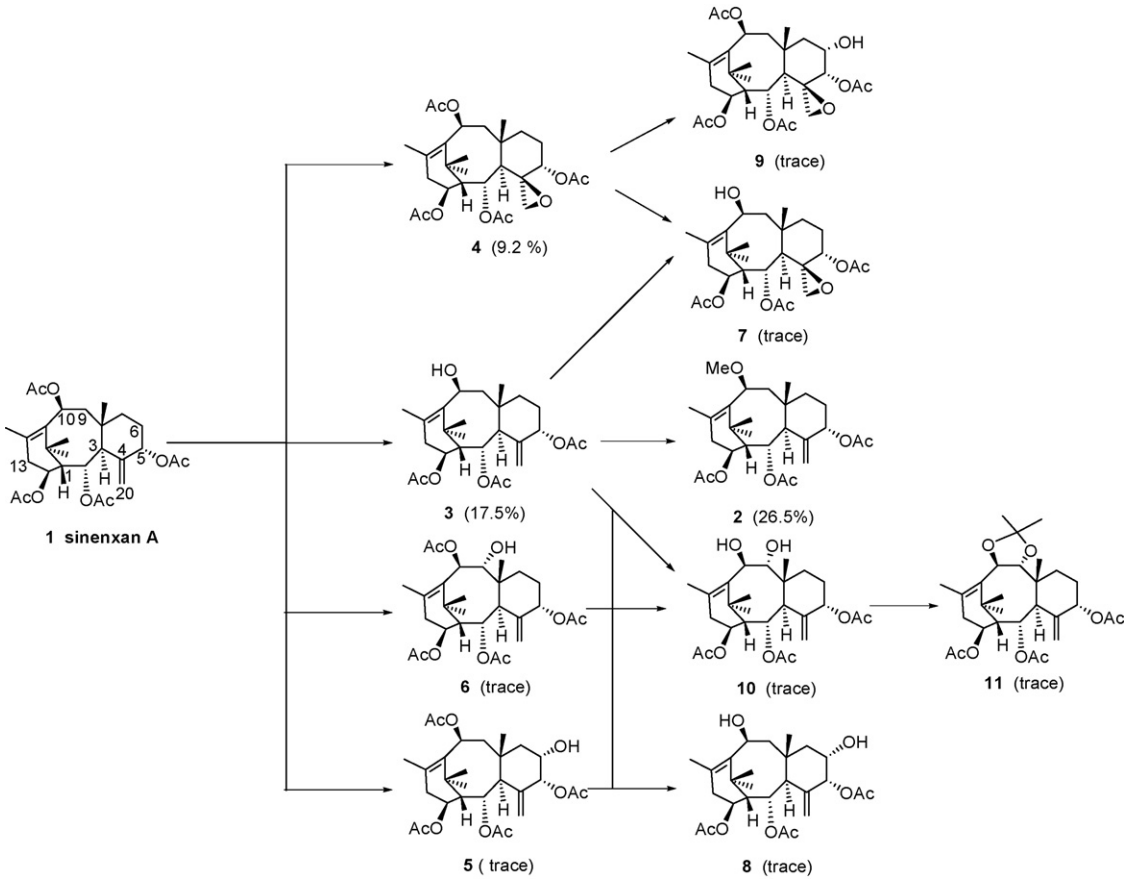

Fig. 1. The structures of metabolites and the plausible biotransformtion pathway of sinenxan A by *Mucor genevensis*.

agents). A number of studies on its structural modification by chemical and biocatalytic approaches were reported, and many derivatives have been evaluated for the MDR reversal activity, as a result, a lot of intriguing results have been achieved [4–11]. As a part of our ongoing studies, in order to diversify these taxanes for direct evaluation of MDR reversal activity and for further chemical derivatization, herein, microbial conversion of sinenxan A by a fungus, *Mucor genevensis* was carried out, and the MDR reversal activities of the three major products towards a paclitaxel-resistant human non-small cell lung cancer (NSCLC)-lung adenocarcinoma cell line, A549/taxol, were evaluated.

2. Experimental

2.1. General

Melting points were determined on an XT-4 micro melting point apparatus and are uncorrected. Optical rotations were obtained using a Horiba SEPA-200 polarimeter. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded with a Varian Unity-PS instrument using $CDCl_3$ as solvent and internal standard reference. ^{1}H NMR and ^{13}C NMR assignments were determined by ^{1}H-^{1}H COSY, DEPT, HMQC, and HMBC experiments. HREIMS spectra were measured on a JEOL-HX 110 instrument. IR spectra were taken on a Hitachi 270-30 spectrometer in $CHCl_3$. Semi-preparative HPLC was performed on a Hitachi L-6200 HPLC instrument with an Inertsil Prep-sil (GL Science, 25 cm × 10 mm i.d.) stainless steel column and an YRU-883 RI/UV bi-detector, the flow rate was 5.0 mL/min. Analytic HPLC was recorded on the same instruments with an Inertsil Sil (GL Science, 25 cm × 4.6 mm i.d.) stainless steel column eluting with ethyl acetate at the flow rate of 1.0 mL/min, and detected at 227 nm. Silica gel (230–300 mesh) was employed for flash column chromatography, analytical TLC plates (silica gel 60 F_{254}, Merck) were visualized by spraying 5% H_2SO_4 (in EtOH) followed by heating.

2.2. Substrates

Compound **1** was isolated from callus cultures (Ts-19 strain) of *T. chinensis* and identified by chemical and spectroscopic methods. Their purities were more than 95% by HPLC analysis. The substrate was dissolved in EtOH before use, and the final concentration added was 10 mg/flask (66.7 mg/L).

2.3. Organism and cultural condition

The fungus, *M. genevensis* JCM 10585 was purchased from Japan Collection of Microorganisms (JCM), The Institute of Physical and Chemical Research, Japan, and kept on solid medium (PDA) containing potato (200 g/L), dextrose (20 g/L)

and agar (20 g/L) at 4 °C. The seed cultures were prepared in 500 mL flask with 150 mL of liquid medium and incubated for 2 days. Five millilitre of the seed cultures was added to one 500 mL flask and cultivated on a rotary shaker at 110 rpm and (25 ± 2) °C in the dark for the use of biotransformation.

2.4. Biotransformation of *1* with M. genevensis

1 (500 mg) was dissolved in EtOH (10.0 mL), distributed among fifty flasks of cultures and incubated for 7 days, after which time the cultures were filtered, the filtrate was saturated with NaCl and extracted five times with ethyl acetate. All the extracts were pooled, dried with anhydrous Na_2SO_4, and concentrated under vacuum at 40 °C to give 700 mg of residue. The dried cell cultures were extracted thrice by sonication with EtOAc, the given extracts were pooled and concentrated under vacuum at 40 °C to afford 483 mg of residue. Both the extracts were combined and separated by combination of open silica gel chromatography and normal phase semi-preparative HPLC to afford substrate, **1** (166.5 mg, ca. 33.3%; t_R = 5.90 min; mobile phase: hexane/ethyl acetate = 80/20, v/v); analyzed by TLC and ^{1}H NMR), **2** (112.7 mg, ca. 26.5%; t_R = 13.50 min; mobile phase: hexane/ethyl acetate = 80/20, v/v), **3** (87.5 mg, ca. 17.5%; t_R = 10.79 min; mobile phase: hexane/ethyl acetate = 60/40, v/v), **4** (46.2 mg, ca. 9.2%; t_R = 6.98 min; mobile phase: hexane/ethyl acetate = 75/25, v/v), **5** (5.8 mg, ca. 1.2%; t_R = 30.11 min; mobile phase: hexane/ethyl acetate = 60/40, v/v), **6** (4.5 mg, ca. 0.9%; t_R = 10.0 min; mobile phase: hexane/ethyl acetate = 60/40, v/v), **7** (4.0 mg, ca. 0.8%; t_R = 30.06 min; mobile phase: hexane/ethyl acetate = 67.5/22.5, v/v),**8** (3.3 mg, ca. 0.7%; t_R = 32.64 min; mobile phase: hexane/ethyl acetate = 1/2, v/v), **9** (0.7 mg, t_R = 43.02 min; mobile phase: hexane/ethyl acetate = 65/35, v/v), **10** (2.0 mg, t_R = 21.00 min; mobile phase: hexane/ethyl acetate = 65/35), **11** (1.7 mg, t_R = 6.70 min; mobile phase: hexane/ethyl acetate = 65/35, v/v) (Fig. 1). Among these products, **2**, **4**, and **9** were three new compounds, their physical and chemical data were shown as follows.

2.4.1. 10β-Methoxy-2α,5α,14β-triacetoxy-taxa-4(20), 11-diene (*2*)

White powder; mp: 88–91 °C; $[\alpha]_D^{20}$ + 26.0° (*c* 6.1, $CHCl_3$); IR ν_{max} ($CHCl_3$): 2940, 2828, 2472, 1730, 1644, 1438, 1372, 1316, 1176, 1090, 1018 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.88 (1H, d, *J* = 2.2 Hz, H-1), 5.34 (1H, dd, *J* = 2.2, 6.6 Hz, H-2), 2.92 (1H, d, *J* = 6.6 Hz, H-3), 5.27 (1H, t, *J* = 2.7 Hz, H-5), 1.70–1.80 (2H, m, H-6), 1.89 (1H, m, overlapped, H-7β), 1.20 (1H, dt, *J* = 3.2, 12.0 Hz, H-7α), 2.28 (1H, dd, *J* = 12.0, 15.0 Hz, H-9β), 1.66 (1H, dd, *J* = 5.6, 15.0 Hz, H-9α), 4.60 (1H, dd, *J* = 5.6, 12.0 Hz, H-10), 2.82 (1H, dd, *J* = 9.0, 19.0 Hz, H-13β), 2.44 (1H, dd, *J* = 4.9, 19.0 Hz, H-13α), 4.99 (1H, dd, *J* = 4.9, 9.0 Hz, H-14), 1.63 (3H, s, H-16), 1.17 (3H, s, H-17), 1.99 (3H, brs, H-18), 0.82 (3H, s, H-19), 5.25 (1H, s, H-20a), 4.84 (1H, s, H-20b), 2.17, 2.04, 2.02 [3H each, s, OAc (CH_3)], 3.28 (3H, 10-OMe); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 59.01 (d, C-1), 70.69 (d, C-2), 41.19 (d, C-3), 142.45 (s, C-4), 78.39 (d, C-5), 24.94 (t, C-6), 33.94 (t, C-7), 39.50 (s, C-8), 45.22 (t, C-9), 75.88 (d, C-10), 137.06 (s, C-11), 134.46 (s, C-12), 39.74 (t, C-13), 70.75 (d, C-14), 37.34(s, C-15), 28.93 (q, C-16), 31.54 (q, C-17), 20.99 (q, C-18), 22.54 (q, C-19), 116.77 (t, C-20), 21.85, 21.49, and 21.44 [q, 3 × OAc (CH_3)], 170.02, 169.97, and 169.79 [s, 3 × OAc (CO)], 55.27 (q, 10-OMe); HREIMS *m/z* 476.2770 $[M]^+$ (calcd. 476.2774 for $C_{27}H_{40}O_7$).

2.4.2. 2α,5α,10β,14β-Tetraacetoxy-4β, 20-epoxy-taxa-11(12)-ene (*4*)

White powder; mp: 87–89 °C; $[\alpha]_D^{20}$ + 67.9° (*c* 2.5, $CHCl_3$); IR ν_{max} ($CHCl_3$): 3032, 2936, 1732, 1438, 1370, 1236, 1214, 1180, 1156, 1106, 1020 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.68 (1H, d, *J* = 1.2 Hz, H-1), 5.33 (1H, dd, *J* = 1.2, 3.7 Hz, H-2), 2.55 (1H, d, *J* = 3.7 Hz, H-3), 4.20 (1H, brs, H-5), 2.03 (1H, m, H-6a), 1.67 (1H, m, H-6b), 1.92 (1H, m, H-7β), 1.31 (1H, m, H-7α), 2.35 (1H, dd, *J* = 12.2, 14.6 Hz, H-9β), 1.54 (1H, dd, *J* = 5.5, 14.9 Hz, H-9α), 6.04 (1H, dd, *J* = 5.6, 12.2 Hz, H-10), 2.66 (1H, dd, *J* = 9.0, 19.0 Hz, H-13β), 2.54 (1H, dd, *J* = 4.9, 19.0 Hz, H-13α), 5.15 (1H, dd, *J* = 4.9, 9.0 Hz, H-14), 1.60 (3H, s, H-16), 1.12 (3H, s, H-17), 2.10 (3H, brs, H-18), 1.06 (3H, s, H-19), 3.67 (1H, d, *J* = 5.1 Hz, H-20a), 2.26 (1H, d, *J* = 5.1 Hz, H-20b), 2.14, 2.06, 2.05, and 1.98 [3H each, s, 4 × OAc (CH_3)]; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 58.82 (d, C-1), 69.97 (d, C-2), 37.05 (d, C-3), 59.45 (s, C-4), 78.10 (d, C-5), 24.34 (t, C-6), 32.86 (t, C-7), 38.49 (s, C-8), 44.23 (t, C-9), 69.88 (d, C-10), 136.03 (s, C-11), 135.63 (s, C-12), 39.54 (t, C-13), 69.57 (d, C-14), 38.15 (s, C-15), 25.45 (q, C-16), 31.41 (q, C-17), 20.97 (q, C-18), 22.65 (q, C-19), 50.02 (t, C-20), 21.71, 21.51, 21.41, and 21.27 [q, 4 × OAc (CH_3)], 170.22, 170.08, 2 × 169.20 [s, 4 × OAc (CO)]; HREIMS *m/z* 520.2668 $[M]^+$ (calcd. 520.2672 for $C_{28}H_{40}O_9$).

2.4.3. 6α-Hydroxy-2α,5α,10β,14β-tetraacetoxy-4β,20-epoxy-taxa-11(12)-ene (*9*)

IR ν_{max} ($CHCl_3$): 3512, 2926, 1732, 1374, 1240, 1106, 1018 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.66 (1H, d, *J* = 1.2 Hz, H-1), 5.33 (1H, dd, *J* = 1.2, 3.7 Hz, H-2), 2.51 (1H, d, *J* = 3.7 Hz, H-3), 4.41 (1H, brd, *J* = 3.7 Hz, H-5), 4.23 (1H,ddd, *J* = 4.6, 4.6, 12.0 Hz, H-6), 1.85 (1H, dd, *J* = 12.1, 15.1 Hz, H-7β), 1.60 (1H, m, overlapped, H-7α), 2.32 (dd, *J* = 12.2, 15.1 Hz, H-9β), 1.60 (1H, m, overlapped, H-9α), 6.03 (1H, dd, *J* = 5.6, 12.2 Hz, H-10), 2.65 (1H, dd, *J* = 9.5, 19.5 Hz, H-13β), 2.36 (1H, dd, *J* = 4.5, 19.0 Hz, H-13α), 5.14 (1H, dd, *J* = 4.9, 9.0 Hz, H-14), 1.60 (3H, s, H-16), 1.13 (3H, s, H-17), 2.11 (3H, brs, H-18), 1.10 (3H, s, H-19), 3.70 (1H, d, *J* = 5.1 Hz, H-20a), 2.31 (1H, d, *J* = 5.1 Hz, H-20b), 1.99, 2.05, 2.06, 2.21 [3H each, s, OAc (CH_3)]; HREIMS *m/z* 536.2610 $[M]^+$ (calcd. 536.2621 for $C_{28}H_{40}O_{10}$).

2.5. Time course of the biotransformation of *1* to *2*, *3*, and *4*

On the day 2, 10 mg/flask (66.7 mg/L, in triplicates) of **1** was added to the culture system, the broth was collected every 1 day and performed as described as in Section 2.4. The organic extract was evaporated to dryness, and the residue was dissolved in 1 mL of ethyl acetate. Samples were filtered through 0.45 μm-pore-size membranes just prior to HPLC analysis. An aliquot of 10 μL was used for each injection.

2.6. Evaluation of MDR reversal activity for compounds **2**, **3**, and **9** in vitro

2.6.1. Cell line and cell culture

The human non-small cell lung cancer (NSCLC)-lung adenocarcinoma cells A549 were maintained in the Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College. The drug-resistant subline of A549/taxol was established by culturing the cells with gradually increasing concentrations of taxol and identified with molecular techniques. The MDR tumor cells were incubated in medium RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were subcultured twice every week by digesting with mixture of 0.25% trypsin and 0.01% EDTA solution.

2.6.2. In vitro proliferation and chemo-sensitivity assays

Cell proliferation was measured by the 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenytetrazolium bromide (MTT) dye reduction method [12]. Briefly, 1×10^4 viable cells (100 μL) were plated into each well of 96-well plates and left to attach to the plate for 24 h, after which time, the medium was changed to one containing or lacking test reversal agents or paclitaxel (dissolved in 10 μL of dimethyl sulphoxide, DMSO). The medium was removed after 72 h of incubation, and 100 μL of fresh serum-free medium with 0.5 mg/mL of MTT and incubated for 4 h. The medium was then removed and 200 μL of DMSO was added to each well to dissolve formazan by shaking in a mini-shaker. Absorbances were measured with a Wellscan MK3 microtitre plate reader (Labsystems Dragon) at test and reference wavelengths of 570 and 450 nm, respectively. The median drug concentration for 50% inhibition (IC_{50}) of tumor cell-growth was determined by plotting the logarithm of the drug concentration against the growth rate (percentage of control) of treated cells.

3. Results and discussion

3.1. Biotransformation and structural elucidation of new products

After incubation with cell cultures of fungus *M. genevensis* for 7 days, 10 more polar metabolites were obtained from **1** by the combination of open silica gel chromatography and semi-preparative HPLC (Fig. 1). On the basis of the physical and spectroscopic data, their structures were identified as 10β-methoxy-2α,5α,14β-triacetoxy-taxa-4(20),11-diene (**2**, in ca. 26.5% yield), 10β-hydroxy-2α,5α,14β-triacetoxy-taxa-4(20), 11-diene (**3**, in ca. 17.5% yield)[4], 2α,5α,10β,14β-tetraacetoxy-4β,20-epoxy-taxa-11(12)-ene (**4**, in ca. 9.2% yield), 6α-hydroxy-2α,5α,10β,14β-tetraacetoxy-taxa-4(20),11-diene (**5**, trace) [9], 9α-hydroxy-2α,5α,10β,14β-tetraacetoxy-taxa-4(20),11-diene (**6**, trace) [5,6], 10β-hydroxy-2α,5α,14β-triacetoxy-4β,20-epoxy-taxa-11(12)-ene (**7**, trace) [4], 6α,10β-dihydroxy-2α,5α,14β-triacetoxy-taxa-4(20),11-diene (**8**, trace) [4], 6α-hydroxy-2α,5α,10β,14β-tetraacetoxy-4β,20-epoxy-taxa-11(12)-ene (**9**, trace), and 9α,10β-dihydroxy-2α,5α,14β-triacetoxy-taxa-4(20),11-diene (**10**, trace) [5,6], and 9α, 10β-*O*-(propane-2,2-diyl)-2α,5α,14β-triacetoxy-taxa-4(20),11-diene (**11**, trace) [6]. Among them, metabolites **2**, **4**, and **9** were three new compounds. The other products have been obtained from sinenxan A by biotransformation, their physical and chemical data were in good accordance with those reported. Based upon the results observed in the time course and the comparison of the structures of the substrate and metabolites, a plausible biotransformation pathway was proposed (Fig. 1).

The HREIMS, ^1H and ^{13}C NMR spectral data of **2** exhibited an elemental composition of $C_{27}H_{40}O_7$ (see Section 2.4.1). By comparing the ^1H and ^{13}C NMR spectra of **1** and **2**, there existed only three acetoxyl groups in molecule of **2**, and an additional methoxyl group [–OCH3, C (δ 55.27, q), H (δ 3.28,3H, s)] was observed, indicating the replacement of an acetoxyl group by a methoxyl group in **2**. The H-10 signal of **1** shifted upfield from δ 6.06 to 4.60 (in **2**), and C-10 signal of **1** shifted downfield from δ 70.13 to 75.88 (in **2**), which implied that the conversion occurred at C-10 position, and it was confirmed by the HMBC spectrum, in which the correlations between the proton signal of –OMe and 10-C signal, the H-10 signal and the carbon signal of –OMe were observed. Therefore, the structure of **2** was identified to be 10β-methoxy-2α,5α,14β-triacetoxy-taxa-4(20),11-diene, it might form from **1** through acetyl group hydrolysis followed by methylation at C-10 position, which was confirmed by the changes of the yields of **2** and **3** (see Section 3.2). Methylation is a very valuable and common reaction occurred both in the case of microbes and plant cells as biocatalysts [13].

The HREIMS, ^1H and ^{13}C NMR spectroscopic data of **4** were consistent with the molecular formula $C_{28}H_{40}O_9$ (see Section 2.4.2), with one oxygen atom more than the corresponding value of **1**. The ^1H NMR spectrum displayed the disappearance of the signals responsible for the 4(20) exocyclic methylene proton signals at δ 4.86 and 5.27 in **1** and the appearance of two doublet proton signals at δ 3.67 and 2.26 ($J = 5.1$ Hz) assigned to the C-20 methylene protons of the oxirane bridge, which were supported by the carbon resonances at δ 59.45 (s) and 50.12 (t) assigned to C-4 and C-20, respectively, by the combined analyses of ^{13}C NMR, DEPT, HMQC and HMBC spectroscopic data of **4**. These observations suggested the presence of an epoxide moiety at C-4(20) instead of exocyclic double bond. It was confirmed by which H-5 resonated at unusually high field for an acetoxylated methine of a taxoid (δ 4.20). The β-orientation of the epoxide oxygen of **4** was established by the detection of NOE-effects between H-20a and H-14α, and H-20b and H-5β. The ^1H NMR spectrum showed the downfield (0.22 ppm) of H-19 and the upfield of the H-5β (1.09 ppm) due to their proximities to this β-epoxide moiety further supported the deduced configuration. Furthermore, Hosoyama et al. synthesized a number of 4(20) epoxidized derivatives from taxinine, of which the epoxide orientation was found to be assignable by magnitude of chemical shift differences between the geminal epoxide protons in ^1H NMR spectra, larger chemical shift differences ($\Delta\delta$ 1.21–1.41 ppm) between H-20a and H-20b existing in β-epoxides, while those ($\Delta\delta$ 0.36–0.50 ppm) in α-epoxides.

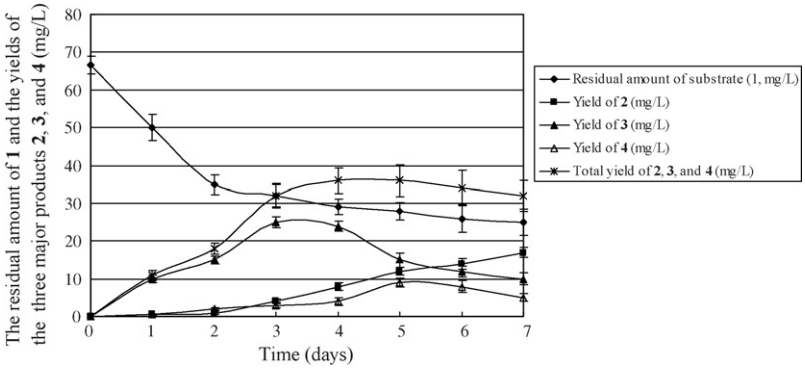

Fig. 2. The time course of biotransformation of sinenxan A (**1**) to **2**, **3**, and **4** by *Mucor genevensis*.

It was proved by X-ray analysis [14]. Therefore, the structure of **4** was identified as 2α,5α,10β,14β-tetraacetoxy-4β,20-epoxy-taxa-11(12)-ene.

The yield of metabolite **9** was very low, so its structure was determined only based on the analyses of the ^{1}H NMR and HREIMS spectroscopic data. The HREIMS spectrum of **9** showed a molecular ion peak at *m/z* 536.2610 $[M]^+$, consistent with the molecular formula of $C_{28}H_{40}O_{10}$, and the ^{1}H NMR spectrum indicated the disappearance of the signals corresponding to the 4(20) exocyclic methylene protons at δ 4.86 and 5.27 in **1** and the appearance of two doublets at δ 3.70 and 2.31 (J = 5.1 Hz), which closely resemled those of **4**. These evidences suggested that an OH group may be introduced in comparison with **4**. Furthermore, ^{1}H NMR spectrum of **9** was similar to that of **4** except that the signals of H-6α (δ 2.03, m) and H-6β (δ 1.67, m) had disappeared, while an additional oxymethine signal at δ 4.23 (1H, ddd, J = 4.6, 4.6, 12.0 Hz) was observed, suggesting that the OH group may be introduced at C-6 position. The stereochemistry of 6-OH was determined to be α-configuration by the NOE difference spectral experiment, in which the integration values of H-5, H-7β and H-19 were enhanced when H-6 was irradiated. Therefore, the structure of **9** was determined as 6α-hydroxy-2α,5α,10β,14β-tetraacetoxy-4β,20-epoxy-taxa-11(12)-ene, maybe formed from **4** via hydroxylating at 6α position.

So far, more than twenty naturally occurring taxoids with 4(20) double bond oxidized to the epoxide are known [15], and all of them have been formulated as β-epoxides. A plausible biogenetic relationship between compounds of this type and oxetane-type taxoids has been proposed [16]. The 4(20)-oxirane in taxane diterpenes was considered to be derived biogenetically from compounds with exocyclic methylene group at C-4 by epoxidation of the double bond. In our experiment, two 4(20)-epoxidized taxoids with natural β-configuration have been obtained. This type of reaction also has been reported in the sinenxan A biotransformation by another fungus, *Cunninghamella echinulata* [4]. These results further suggested microbial transformation could mimic the biosynthetic pathway of the natural taxoids, and also provide a solid evidence for the biogenetic hypothesis of the taxane with a 4(20)-oxirane ring.

3.2. The time course of biotransformation of *1* to *2*, *3* and *4*

The time course of **1** to the three major metabolites **2**, **3**, and **4** was investigated. The results (Fig. 2) showed that: (1) in the first 2 days of incubation, the amount of the substrate (**1**), decreased sharply, and after which time, decreased very slowly, the final amount in the broth was about 25.0 mg/L; (2) the yield of the metabolite **3**, reached the maximal amount on day 3, about 25.0 mg/L, after which time it decreased, while the yield of the metabolite **2** increased during the incubation, these observation suggested that **2** may be biosynthesized from **1** through the intermediate **3** followed by methylating; (3) the yield of the another metabolite **4**, reached its maximum yield about on day 5; (4) the total yields of the three metabolites increased very sharply in the first 3 days, and reached its maximun yield 36.5 mg/L, on day 4, then slowly decreased, indicating the production of other minor metabolites from these three major metabolites. These results maybe somewhat gave evidences to the plausible biotransformation pathway mentioned previously (Fig. 1).

3.3. Bioassay for the MDR reversal activities of compounds *2*, *3*, and *4* against taxol-resistant A549 tumor cells

The drug-resistant subline of A549/taxol was established by culturing the cells with gradually increasing concentrations of taxol, finally A549/taxol cells were 114.5-fold more resistant to the taxol (Table 1), and also exhibited cross-resistance to VCR (vincristine, 31-fold), VP-16 (etoposide, >100-fold), ADM (adriamycin, 91-fold), and CPT (campothecin, 3.5-fold) [17]. In order to examine the chemosensitizing effects of compounds **1**, **2**, **3**, and **4** against A549/taxol cells preliminarily, the proliferation inhibition of A549/taxol cells by taxol was examined in the presence of compounds **2**, **3**, and **4** at 5 μg/mL. The results were shown in Table 1, where **4** showed about two-fold activity as verapamil, while **2**, and **3** showed lower activity than verapamil and **1**. All of them exhibited weak or no cytotoxicity. Thus, the results showed that compound **4** from biotransformation of sinenxan A might be a promising reversal agents or lead compound against A549/taxol tumor MDR cells.

Table 1
MDR reversal activity of compounds against human NSCLC MDR tumor cells in vitro (IC_{50}, nM, $n = 3$)

Compounds	A549	A549/paclitaxel	RF[a]	SF[b]
Paclitaxel	1.70 ± 0.98[c]	194.5 ± 32.5[c]	114	–
1	16.8 ± 0.3[c]	116.0 ± 18[d]	–	1.67
2	>100[c]	220.0 ± 40.0[d]	–	0.88
3	>100[c]	294.0 ± 98.0[d]	–	0.66
4	79.7 ± 24.6[c]	10.0 ± 2.0[d]	–	19.4
Verapamil	–	24.2 ± 2.5[d]	–	8.1

[a] RF means resistance factor, is the ratio of the IC_{50} of resistant cells to the IC_{50} of sensitive cells.
[b] SF means sensitive fold, is the ratio of the IC_{50} of resistant cells only in the presence of paclitaxel to the IC_{50} of resistant cells only in the presence of both paclitaxel and reversal agents.
[c] The IC_{50} value were examined only in the presence of the single paclitaxel or reversal agents.
[d] The IC_{50} value were examined by co-administering paclitaxel or reversal agents (5 μg/mL).

4. Conclusions

In conclusion, sinenxan A, a readily available taxoid from *Taxus* cell cultures, can be specifically methylated, epoxidized by the fungus *M. genevensis*, and totally 10 products yielded. Bioassays showed that product **4** exhibited more active than verapamil towards taxol-resistant human NSCLC cells. These results suggested that biotransformation is a versatile approach to diversifying bioactive natural products for wider biological evaluation, and searching for more bioactive natural compounds. In addition, microbial transformation can mimic biogenetic pathway of natural products, provide some important intermediates for the biogenetic study and/or solid evidence for the proposed biogenetic hypothesis, as in this report, a conversion of exocyclic methylene to oxirane, which further supports the biogenetic hypothesis of oxetane in naturally occurring taxoids [16].

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