VOL. 56 NO. 1, JAN. 2003 THE JOURNAL OF ANTIBIOTICS pp. 16 — 23

Generation of Novel Epothilone Analogs with Cytotoxic Activity by Biotransformation

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(Received for publication September 24, 2002)

The epothilones represent a new class of bacterial natural products with broad spectrum of antiproliferative activity against various types of human tumors and tumor cell lines. The attractive preclinical profile of epothilones has made them promising lead compounds for novel anticancer agents and has spurred a strong interest in obtaining different derivatives to fully evaluate their therapeutic potentials. We have generated a number of novel epothilone D and 10,11-dehydroepothilone D (Epo490) analogs *via* biotransformation using *Amycolata autotrophica* to alter the oxidation state of the parental compounds. The bioconverted compounds displayed different degrees of potency in cytotoxicity assays against a panel of human tumor cell lines, with 11-hydroxyepothilone D, 14-hydroxyepothilone D, and 21-hydroxyepothilone D showing comparable activity to that of epothilone D, and 21-hydroxy Epo490 being comparable to Epo490. The addition of hydroxyl group(s) seems to cause a decrease in cytotoxic activity against multiple drug resistant cell lines (with overexpressed P-glycoprotein). The compounds generated by biotransformation exert differential effects on tubulin polymerization, which correlate with their biological activities.

Epothilones, a new class of macrolactones originally isolated from the soil bacterium *Sorangium cellulosum*, represent a new class of naturally occurring microtubule depolymerization inhibitors[2,3]. Epothilones A and B were first identified. The 12,13-desoxy counterparts of epothilones A and B, also known as epothilones C and D, have been chemically synthesized *de novo* but also observed in fermentation extracts of *S. cellulosum* along with a variety of other epothilone-like structures produced as minor constituents[1,4,5]. Epothilone D is reported to exhibit the highest therapeutic index. Epothilones have great potential for the treatment of cancer. Although structurally dissimilar, the mechanism of action of the epothilone family is similar to the cancer drug paclitaxel (Taxol), involving induction of tubulin polymerization and stabilization of microtubule assembly[6,7]. These compounds exhibit potent cytotoxic activity against various cancer cell lines. In particular, they display dramatically improved potency against multiple drug resistant (MDR) tumor cell lines that are highly resistant to Taxol and other anticancer drugs[8,9]. Epothilone D and 21-hydroxyepothilone D have been demonstrated to have curative effects against human tumor xenografts in nude mice[9,10]. Epothilone B, BMS-247550[11] (a derivative of epothilone B) and epothilone D are currently undergoing clinical trials.

We recently reported the sequence of the epothilone gene cluster and its heterologous expression in *Streptomyces coelicolor*[12] and *Myxococcus xanthus*[13]. In both hosts, either epothilones A and B or epothilones C and D were produced as major compounds, depending on whether the epoxidase that converts epothilones C and D to A and B, respectively, was coexpressed with the PKS genes. 10,11-Dehydroepothilone (Epo490) was isolated as a minor fermentation component from the epothilone D producing strain *M. xanthus* K111-72[13,14]. We also constructed a mutant *M. xanthus* strain, K165-78, from *M. xanthus* K111-72 to produce Epo490 as a major component[14].

The great potential of epothilones in cancer therapy has generated strong interest in novel epothilone analogs. In this study, we used the microorganism *Amycolata autotrophica* to alter the oxidation state of epothilone D and Epo490 to generate analogs. The analogs include novel compounds, some with potent cytotoxic activity.

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Experimental

Chemicals, Microorganisms and Tumor Cell Lines

Epothilone D and Epo490 were purified from the recombinant *M. xanthus* strains K111-72 and K165-78, respectively[13,14]. The *A. autotrophica* strain was purchased from the American Type Culture Collection (ATCC35203). The following cell lines were obtained from the National Cancer Institute: MCF-7 and NCI/ADR-Res human breast carcinoma cells, NCI-H460 human non-small lung carcinoma cells, and SF-268 human glioma cells. NCI/ADR-Res is a MDR cell line that overexpresses P-glycoprotein.

Biotransformation of Epothilone D and Epo490

A. autotrophica was inoculated into 100 ml of a transformation medium (10 g dextrose, 5 g polypeptone, 5 g yeast extract, 5 g malt extract in 1 liter of deionized water) in a 500 ml flask, and allowed to grow at 28°C and 250 rpm for 24 hours. An aliquot of 25 ml was transferred into a 250 ml flask and 10 mg of epothilone D or Epo490 (in ethanol) was added. The culture was then grown for another two days. The conversion of epothilones in the culture was monitored by LC/MS (Perkin-Elmer Sciex API 100 LC) to determine the maximum conversion and harvest time.

Purification of Bioconverted Products

The biotransformation culture was vigorously mixed with 2 volumes of ethyl acetate and the mixture was filtered through a bed of Celite 521 (Aldrich Chemicals Co.). The filtrate was transferred to a separating funnel, and the upper organic phase was concentrated to dryness on a rotary evaporator to give a crude extract. The crude extract was then subjected to column chromatography, eluting with 10%, 20%, 30%, 40% and 50% of acetone in hexane. After TLC analysis (50% acetone in hexane), five pools were collected, evaporated and subjected to HPLC using a C-18 reverse phase column (250×10 mm ODS-3 5 μ Inertsil, MetaChem), eluting with a gradient of 30~100% acetonitrile/water over 45 minutes at a flow rate of 4 ml/minute with UV detection at 250 nm.

Structural Elucidation of the Compounds

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data were recorded in $CDCl_3$ solution at 300 K with a Bruker DRX 400 spectrometer equipped with a QNP z-axis gradient probe head. Chemical shifts in $CDCl_3$ solution were referred to δ 7.26 and 77.0 for ^{1}H and ^{13}C spectra, respectively. HRMS spectra were obtained by FIA with manual peak-matching on an Applied Biosystems Mariner TOF spectrometer with a turbo-ionspray source in positive ion mode.

The structure of 11-hydroxyepothilone D (**1**) was confirmed by ^{1}H, ^{13}C NMR spectroscopy and high-resolution MS (HRMS). The structures of 14-hydroxy-epothilone D (**2**), 9-hydroxyepothilone D (**3**), epothilone B, 21-hydroxyepothilone D (**4**), 26-hydroxyepothilone D (**5**), 21,26-dihydroxyepothilone D (**6**), 21-hydroxy Epo490 (**7**), 26-hydroxy Epo490 (**8**) were confirmed by ^{1}H NMR spectroscopy and HRMS (see below). The following compounds were used as standards to confirm the structures of the hydroxylated epothilone analogs: Epothilone B was purified from the engineered epothilone A and B producer *M. xanthus* K111-32-25[13]; 9-hydroxyepothilone D was semi-synthesized from 9-oxoepothilone D which was purified from an engineered *M. xanthus* strain (TANG & LI unpublished data); 21-hydroxyepothilone D, made by chemical total synthesis, was a gift from SAMUEL DANISHEFSKY.

11-Hydroxyepothilone D (**1**): HRESITOFMS *m/z* 508.2703; calcd for $C_{27}H_{42}NO_6S$ $[M+H]^+$, 508.2727. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.97 (s, H-19), 6.56 (s, H-17), 5.28 (t, J=8 Hz, H-13), 5.22 (dd, J=6.0, 3.0 Hz, H-15), 4.74 (t, J=8.0 Hz, H-11), 4.42 (dd, J=11.0, 2.0 Hz, H-3), 3.75 (dd, J=6.5, 1.0 Hz, H-7), 3.24 (qd, J=7.0, 1.0 Hz, H-6), 2.69 (s, H-21), 2.52 (2H, m, H_2-14), 2.44 (dd, J=14.0, 11.0, H-2a), 2.19 (dd, J=14.0, 2.0, H-2b), 2.01 (d, J=1.0 Hz, H-27), 1.90 (m, H-8), 1.72 (m, H-10a), 1.70 (s, H-26), 1.58 (H-10b), 1.55 (m, H-9a), 1.37 (s, H-23), 1.29 (m, H-9b), 1.14 (d, J=7.0, H-24), 1.04 (s, H-22), 1.03 (d, J=7.0, H-25). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 221.1 (C-5), 170.1 (C-1), 165.3 (C-20), 151.5 (C-18), 140.7 (C-12), 138.6 (C-16), 120.9 (C-13), 118.4 (C-17), 115.1 (C-19), 77.7 (C-15), 74.0 (C-7), 71.4 (C-3), 69.8 (C-11), 54.4 (C-4), 40.7 (C-6), 39.9 (C-2), 36.5 (C-8), 31.5 (C-10), 30.6 (C-14), 26.6 (C-9), 22.8 (C-23), 18.8 (C-21), 17.8 (C-26), 16.4 (C-27), 16.3 (C-22), 14.6 (C-25), 11.8 (C-24).

14-Hydroxyepothilone D (**2**): HRESITOFMS *m/z* 508.2728; calcd for $C_{27}H_{42}NO_6S$ $[M+H]^+$, 508.2727. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.02 (s, 1H), 6.67 (s, 1H), 5.22 (d, J=9.4 Hz, 1H), 4.98 (d, J=9.4 Hz, 1H), 4.57 (t, J=9.2 Hz, 1H), 4.26 (*bro* d, J=10.1 Hz, 1H), 3.70 (m, 1H), 3.14 (qd, J=6.7, 1.7 Hz, 1H), 2.70 (s, 3H), 2.45 (m, 1H), 2.44 (dd, J=14.3, 11.2 Hz, 1H), 2.22 (*bro* d, J=14.3 Hz, 1H), 2.17 (s, 3H), 1.96 (m, 1H), 1.76 (m, 1H), 1.73 (m, 1H), 1.72 (s, 3H), 1.29 (m, 3H), 1.34 (s, 3H), 1.20 (d, J=6.8 Hz, 3H), 1.06 (s, 3H), 1.03 (d, J=7.0, 3H).

9-Hydroxyepothilone D (**3**): HRESITOFMS *m/z* 508.2728; calcd for $C_{27}H_{42}NO_6S$ $[M+H]^+$, 508.2727. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.96 (s, 1H), 6.58 (s, 1H), 5.22

(dd, J=9.1, 2.8 Hz, 1H), 4.40 (m, 1H), 4.01 (m, 1H), 3.76 (d, J=6.2 Hz, 1H), 3.61 (m, 1H), 3.30 (qd, J=7.0, 1.8 Hz, 1H), 2.68 (s, 3H), 2.62 (m, 1H), 2.41 (m, 4H), 2.18 (m, 1H), 2.06 (d, J=2.1 Hz, 3H), 1.85 (m, 2H), 1.75 (s, 3H), 1.72 (m, 1H), 1.35 (s, 3H), 1.19 (d, J=6.9 Hz, 3H), 1.05 (s, 3H), 0.99 (d, J=7.2 Hz, 3H).

21-Hydroxyepothilone D (**4**): HRESITOFMS *m/z* 508.2713; calcd for $C_{27}H_{42}NO_6S$ $[M+H]^+$, 508.2727. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.01 (s, 1H), 6.59 (s, 1H), 5.34 (m, 1H), 5.24 (d, J=8.6 Hz, 1H), 5.13 (dd, J=10.0, 4.9 Hz, 1H), 4.90 (s, 2H), 4.30 (dd, J=11.2, 2.7 Hz, 1 H), 3.70 (dd, J=4.0, 2.4 Hz, 1 H), 3.14 (qd, J=6.8, 2.1 Hz, 1H), 2.62 (dt, J=15.0, 9.8 Hz, 1H), 2.47 (dd, J=14.7, 11.1 Hz, 1H), 2.30 (overlap, 1H), 2.27 (dd, J=14.7, 2.7 Hz, 1H), 2.06 (s, 3H), 2.00 (m, 1H), 1.89 (m, 1H), 1.75 (m, 1H), 1.68 (m, 1H), 1.66 (s, 3H), 1.33 (s, 3H), 1.26 (overlap, 3H), 1.19 (d, J=6.9 Hz, 3H), 1.05 (s, 3H), 1.01 (d, J=7.0 Hz, 3H).

26-Hydroxyepothilone D (**5**): HRESITOFMS *m/z* 508.2723; calcd for $C_{27}H_{42}NO_6S$ $[M+H]^+$, 508.2727. ^1H NMR ($CDCl_3$, 400 MHz) δ 6.98 (s, 1H), 6.66 (s, 1H), 5.45 (dd, J=9.4, 5.8 Hz, 1H), 5.24 (dd, J=8.8, 2.0 Hz, 1H), 4.33 (dd, J=11.2, 2.4 Hz, 1H), 4.09 (d, J=13.2 Hz, 1H), 4.01 (d, J=13.2 Hz, 1H), 3.69 (dd, J=4.4, 2.0 Hz, 1H), 3.18 (qd, J=6.8, 2.4 Hz, 1H), 2.71 (s, 3H), 2.63 (dt, J=15.2, 9.2 Hz, 1H), 2.46 (dd, J=14.8, 11.2 Hz, 1H), 2.37 (m, 1H), 2.25 (overlap, 1H), 2.24 (dd, J=14.8, 2.4 Hz, 1H), 2.12 (m, 1H), 2.05 (d, J=1.2 Hz, 3H), 1.76 (m, 1H), 1.67 (m, 1H), 1.36 (s, 3H), 1.34 (m, 3H), 1.18 (d, J=6.8 Hz, 3H), 1.05 (s, 3H), 0.98 (d, J=9.6 Hz, 3H).

21,26-Dihydroxyepothilone D (**6**): HRESITOFMS *m/z* 524.2699; calcd for $C_{27}H_{42}NO_7S$ $[M+H]^+$, 524.2677. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.12 (s, 1H), 6.62 (s, 1H), 5.46 (dd, J=9.2, 5.6 Hz, 1H), 5.28 (d, J=6.8 Hz, 1H), 4.91 (s, 2H), 4.28 (dd, J=10.8, 2.4 Hz, 1H), 4.09 (d, J=13.2 Hz, 1H), 4.01 (d, J=13.2 Hz, 1H), 3.68 (dd, J=4.0, 2.4 Hz, 1H), 3.15 (qd, J=6.8, 2.4 Hz, 1H), 2.68 (overlap, 1H), 2.65 (dt, J=15.2, 9.2 Hz, 1H), 2.47 (dd, J=14.8, 11.2 Hz, 1H), 2.38 (m, 1H), 2.26 (dd, J=14.8, 2.4 Hz, 1H), 2.23 (overlap. 1H), 2.12 (m, 2H), 2.06 (s, 3H), 1.75 (m, 2H), 1.38 (m, 1H), 1.32 (s, 3H), 1.17 (d, J=6.8 Hz, 3H), 1.05 (s, 3H), 1.00 (d, J=7.2 Hz, 3H).

21-Hydroxy Epo490 (**7**): HRESITOFMS *m/z* 506.2562; calcd for $C_{27}H_{40}NO_6S$ $[M+H]^+$, 506.2571. ^1H NMR ($CDCl_3$, 400 MHz) δ 7.12 (s, 1H), 6.59 (s, 1H), 6.53 (d, J=15.4 Hz, 1H), 5.77 (m, 1H), 5.31 (overlap. 1H), 5.27 (overlap. 1H), 4.94 (m, 2H), 4.20 (dd, J=9.4, 3.7 Hz, 1H), 3.72 (d, J=6.8 Hz, 1H), 3.25 (qd, J=7.0, 2.0 Hz, 1H), 2.87 (overlap. 1H), 2.82 (dt, J=14.2, 10.5 Hz, 1H), 2.70 (m. 1H), 2.56~2.25 (m, 3H), 2.12 (d, J=1.0 Hz, 3H), 2.11~1.95 (m, 2H), 1.80 (s, 3H), 1.31 (s, 3H), 1.12 (d, J=6.8 Hz, 3H), 1.07 (d, J=6.8 Hz, 3H), 1.04 (s, 3H).

26-Hydroxy Epo490 (**8**): HRESITOFMS *m/z* 506.2573; calcd for $C_{27}H_{40}NO_6S$ $[M+H]^+$, 506.2571. ^1H NMR ($CDCl_3$, 400 MHz) δ 6.96 (s, 1H), 6.59 (s, 1H), 6.33 (d, J=15.9 Hz, 1H), 5.95 (td, J=15.9, 7.1 Hz, 1H), 5.52 (dd, J=9.9, 6.9 Hz, 1H), 5.29 (dd, J=9.2, 1.9 Hz, 1H), 4.25 (d, J=12.2 Hz, 1H), 4.25 (overlap, 1H), 4.16 (d, J=12.7 Hz, 1 H), 3.70 (dd, J=8.1, 1.8 Hz, 1H), 3.25 (qd, J=6.9, 1.8 Hz, 1H), 2.77 (dt, J=14.2, 9.7 Hz, 1H), 2.69 (s, 3H), 2.64 (m, 1H), 2.45 (m, 1H), 2.43 (dd, J=15.0, 10.7 Hz, 1H), 2.34 (dd, J=15.0, 2.6 Hz, 1H), 2.08 (d, J=1.2 Hz, 3H), 2.07 (overlap, 1H), 1.96 (m, 1H), 1.32 (s, 3H), 1.08 (d, J=6.7 Hz, 3H), 1.05 (d, J=7.2 Hz, 3H), 1.04 (s, 3H).

Cytotoxicity Assays

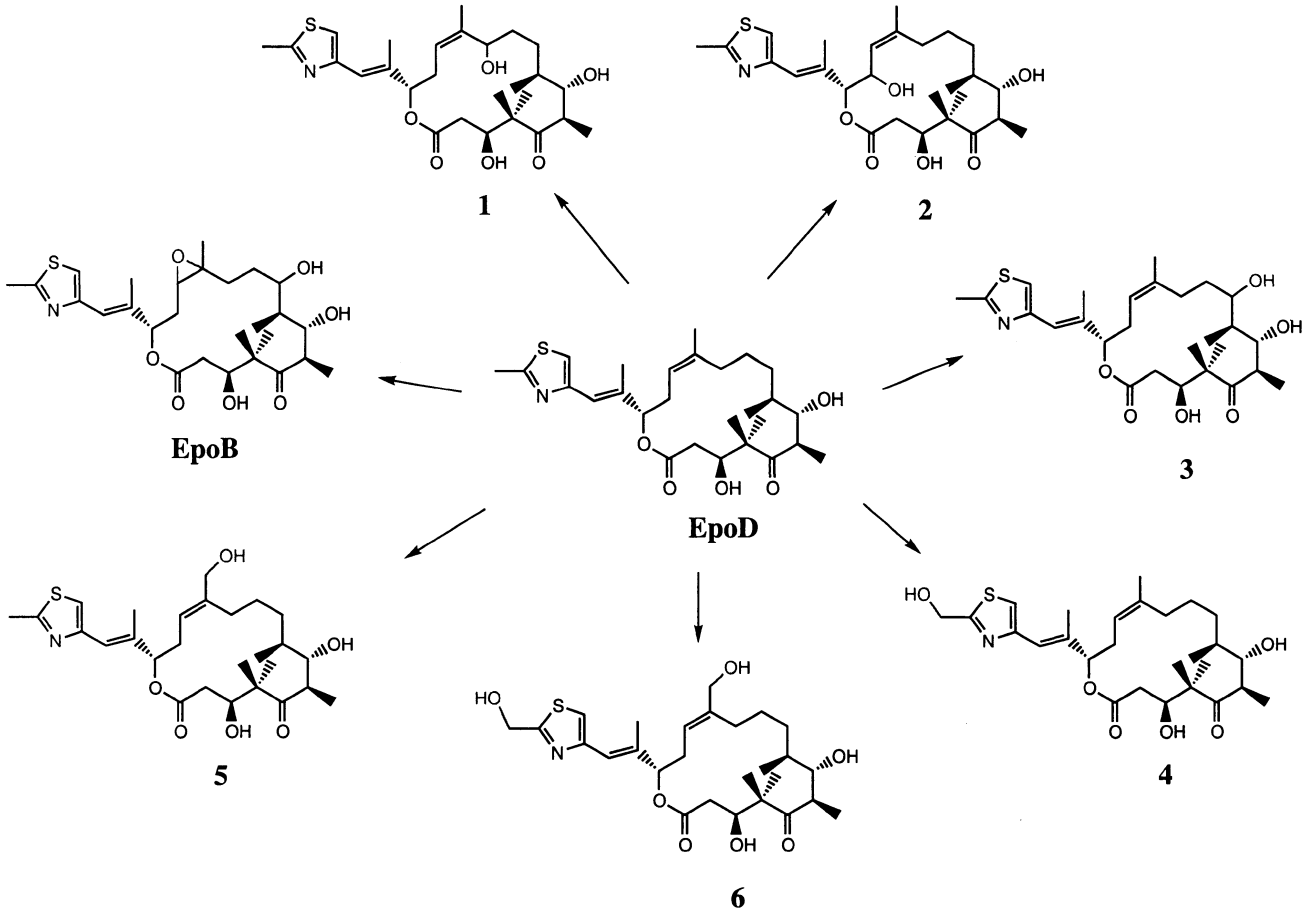
Cells were maintained in a 5% CO_2-humidified atmosphere at 37°C in RPMI 1640 medium (Life Technology) supplemented with 10% fetal bovine serum (Life Technology) and 2 mM L-glutamine (Life Technology).

The cytotoxicity was determined by sulforhodamine B (SRB) assay[15]. Cultured cells were trypsinized, counted, and diluted to suitable concentrations (5000~7500 cells per 100 μl) with growth medium. 96-Well microtiter plates were seeded with 100 μl/well of each cell suspension. At 20 hours 100 μl of compounds, ranging from 2×1000 to 2×0.001 nM diluted in growth medium, were added to each well. After 3 days incubation the cells were fixed with 100 μl of 10% trichloroacetic acid at 4°C for 1 hour and stained with 0.2% SRB/1% acetic acid at room temp for 20 minute. The unbound dye was rinsed away with 1% acetic acid and the bound SRB was extracted by 200 μl of 10 mM Tris base. The bound dye was determined by OD at 515 nm, which correlates with total cellular protein content.

Tubulin Polymerization Assays

The assay was carried out essentially as previously described[16,17]. In brief, MCF-7 cells grown to confluence in 35-mm culture dishes were treated with 1 μM of each compound for 1 hours at 37°C. After washing the cells twice with 2 ml of PBS without Ca and Mg, the cells were lysed at room temp for 5~10 minutes with 300 μl of lysis buffer (20 mM Tris, pH 6.8, 1 mM $MgCl_2$, 2 mM EDTA, 1% Triton X-100, plus protease inhibitors). The lysed cells were scraped and the lysates transferred to 1.5-ml Eppendorf tubes. The lysates were then centrifuged at 18000 g for 12 minutes at room temp. The supernatants containing soluble or unpolymerized (cytosolic) tubulin were separated from pellets containing insoluble or polymerized (cytoskeletal) tubulin and transferred to new tubes. The pellets were then resuspended in 300 μl of lysis

Fig. 1. Biotransformation of epothilone D by *A. autotrophica.*



buffer. Changes in tubulin polymerization in the cell were determined by analyzing the same volume of aliquots of each sample with SDS-PAGE, followed by immunoblotting using an anti-β-tubulin antibody (Sigma). The β-tubulin signals on the blot were then quantified by the NIH Image program.

Results

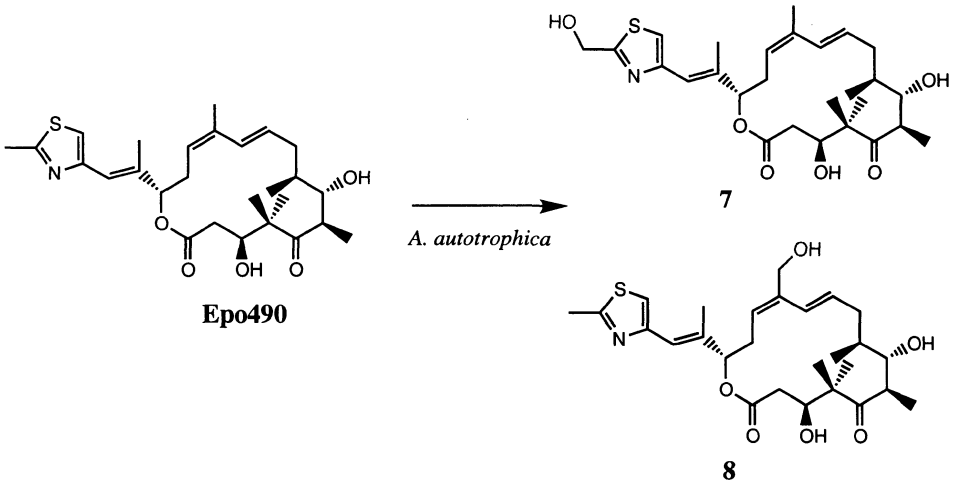
Biotransformation Generates Novel Epothilone Derivatives

LC/MS analysis indicated that biotransformation of epothilone D by *A. autotrophica* resulted in at least 11 different epothilone analogs, including many novel compounds. These compounds include seven derivatives that differ from epothilone D by the addition of a hydroxyl group, two derivatives that differ from epothilone D by the addition of two hydroxyl groups, one derivative that differs from epothilone D by the addition of three hydroxyl groups, and epothilone B, produced from epothilone D by the addition of an epoxide.

Similarly, biotransformation of 10,11-dehydroepothilone D by *A. autotrophica* yielded at least eight different compounds, including five derivatives that differ from the parental compound by the addition of a hydroxyl group and three derivatives that differ by the addition of two hydroxyl groups.

^{1}H NMR spectroscopy analysis and high-resolution MS measurements for the purified compounds identified the following structures from bioconverted epothilone D derivatives (Fig. 1): 11-hydroxyepothilone D (**1**), 14-hydroxyepothilone D (**2**), 9-hydroxyepothilone D (**3**), 21-hydroxyepothilone D (**4**), 26-hydroxyepothilone D (**5**), 21,26-dihydroxyepothilone D (**6**), and epothilone B. The compounds identified from the bioconversion of Epo490 are 21-hydroxy Epo490 (**7**) and 26-hydroxy Epo490 (**8**)

Fig. 2. Biotransformation of Epo490 by *A. autotrophica.*



(Fig. 2). Among the bioconverted products, **5** and **8** are the major derivatives with about 40% conversion; **1**, **4** and **7**, about 10% conversion; others are minor products.

To further characterize 11-hydroxyepothilone D (**1**), high-resolution MS measurements were carried out. The data were consistent with a formula of $C_{27}H_{41}NO_6S$ for a monohydroxylated epothilone D analogue. ^1H and ^{13}C chemical shifts were assigned from multiplicity-edited HSQC, gsCOSY, and gsHMBC data, and these data also confirmed the structure. An HMBC correlation for a methyl singlet at δ 1.70 (H-26) to carbons resonating at δ 120.9 (C-13), 140.7 (C-12), and a secondary alcohol signal at δ 69.8, placed the additional hydroxyl group at the 11 position. Other structures were assigned by the combination of high-resolution MS measurements and comparison of ^1H spectra with that of epothilone D and Epo490. In 14-hydroxyepothilone D, a triplet at δ 4.57 (H-14) appears as a result of coupling with H-13 and H-15. Compared to epothilone D, 21-hydroxyepothilone D was characterized by the disappearance of a methyl singlet at δ 2.69 (H-21) and appearance of a methylene singlet at δ 4.90 (H-21). Similarly, 26-hydroxyepothilone D shows an AB quartet at δ 4.05 (H-26) and the disappearance of the methyl singlet at δ 1.65 (H-26) for epothilone D. Likewise 21,26-dihydroxyepothilone D shows a methylene singlet at δ 4.91 (H-21) and an AB quartet at δ 4.05 (H-26) and the disappearance of methyl singlets at δ 2.69 (H-21) and δ 1.65 (H-26) for epothilone D. Like its epothilone D analog counterpart, 21-hydroxy Epo490 has a methylene singlet at δ 4.94 (H-21) and no methyl singlet at δ 2.69 (H-21) as in Epo490. In the case of 26-hydoxy Epo490, an AB quartet at δ 4.21 (H-26) was observed instead of the methyl singlet at δ 1.79 (H-26) for Epo490. The stereochemistry of the new stereogenic centers was not determined.

Bioconverted Epothilone Analogs Display Tumor Cytotoxic Activity

To determine the biological activity of the bioconverted compounds, cytotoxicity assays were carried out on a panel of human cancer cell lines derived from breast, lung and central nervous system tumors. Table 1 summarizes the *in vitro* activity (IC_{50}s) of all the analogs generated in this study. Compounds **1**, **2** and **4** have similar cytotoxic activities that are comparable to or slightly less than epothilone D in drug-sensitive cell lines. Likewise, **7** is comparable to or slightly less active than Epo490 in these cells. The addition of one or more hydroxyl groups decreased the cytotoxic activity in the multiple drug resistant (MDR) cell line NCI/ADR-Res, which overexpresses P-glycoprotein. The 21-hydroxy analogs were more active than the 26-hydroxy analogs, which in turn were more active than the 21,26-dihydroxy analogs.

21-Hydroxyepothilone D (**4**) has been chemically synthesized and reported to have comparable activity to epothilone D in inhibiting the growth of a variety of tumor cell lines, including several MDR lines, and exhibited curative effects against human tumor xenografts in nude

Table 1. Cytotoxicity of epothilone analogs.

Compound	Cytotoxicity (IC50, nM)			
	MCF7 (breast)	NCI/ADR-RES (breast, MDR)	SF-268 (glioma)	NCI-H460 (lung)
EpoD	9	34	17	12
EpoB	0.5	2	0.8	0.7
Epo490	25	61	27	32
11-OH Epo D	21	169	42	29
14-OH Epo D	29	381	35	35
9-OH Epo D	280	>1000	461	382
21-OH Epo D	23	289	30	31
26-OH Epo D	95	800	482	287
21,26 dihydro-Epo D	320	>1000	770	780
21-OH Epo490	35	246	40	37
26-OH Epo490	287	>1000	438	428

IC50 values in nM represent the average of two to three independent 72-h growth inhibition assays. IC50 is defined as the drug concentration that reduce cell protein by 50%.

mice[10]. Our results showed that bioconverted compound **4** was identical to its chemical synthesized counterpart in terms of cytotoxicity in the four-cell line panel (data not shown). Given the comparable IC_{50} profile of compounds **1** and **2** to that of 21-hydroxyepothilone D, it would be of interest to determine their efficacy in animal tumor models.

Bioconverted Epothilone Analogs Show Differential Effects on Tubulin Polymerization

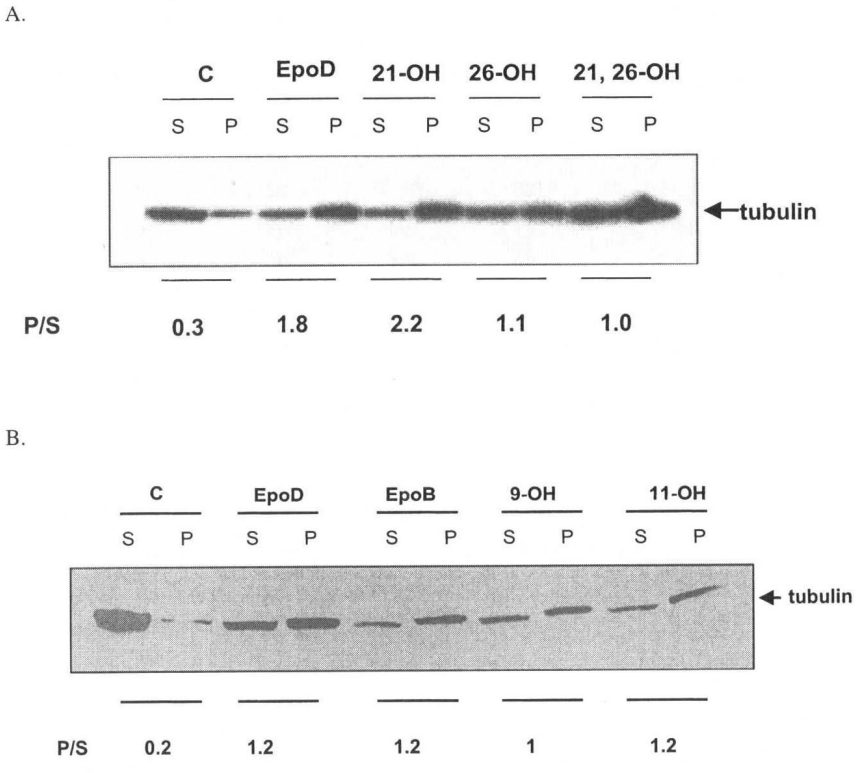
The mechanism of action of epothilone D and Epo490 involves induction of tubulin polymerization and stabilization of microtubule assembly[6,7]. The active bioconverted derivatives were expected to act by the same mechanism. To confirm this, a cell-based tubulin polymerization assay was performed. As expected, all the compounds tested promoted tubulin polymerization within the cell to various degrees (Fig. 3). Epothilone B, and analogs **1**, **2**, **4** and **7** are potent inducers of tubulin polymerization; analogs **5**, **8** and **3** are moderate inducers. These data confirm the mechanism of action of the bioconverted compounds and indicate that their activity in promoting tubulin polymerization *in vivo* correlates with their potency in inhibiting tumor cell growth.

Discussion

Epothilones are a class of compounds, including many natural products, with a broad spectrum of cytotoxic activity against various tumor cell lines[4,18]. Of these, epothilone D exhibits the best therapeutic index[8~10], and Epo490 is a novel cytotoxic compound with comparable *in vitro* activity to epothilone D[14]. Therefore epothilone D and Epo490 analogs are highly desired to fully explore the potential of epothilones as anticancer agents. The biotransformation of epothilone D described here represents a new approach to generating epothilone D analogs and provides an avenue to make some analogs that cannot be made by other approaches. In addition, it can generate many different derivatives at the same time, and the hydroxylated derivatives generated are good precursors for further chemical modification.

It was recently reported that *S. cellulosum* was used to convert epothilone B to 21-hydroxyepothilone B (epothilone F)[19]. The total synthesis of 26-hydroxyepothilone B has previously been reported[20]. Our studies have shown that biotransformation by A. autotrophica can result in the addition of one or more hydroxyl groups at positions other than and in addition to C-21 of an epothilone compound. The method can also result in the addition an epoxide across the C-12, 13 *cis*-

Fig. 3. Effect of the bioconverted compounds on tubulin polymerization.



MCF-7 cells were treated with 1 μM or 100 nM of each compound for 1 hour. Polymerized and soluble tubulin in the cell were analyzed by immunoblotting using an anti-β-tubulin antibody. The signals of tubulin were then quantified. C, drug-free control. P, polymerized tubulin, S, soluble tubulin. P/S, the ratio of P to S. **A**. the effects of 21-hydroxyepothilone D, 26-hydroxyepothilone D and 21,26-dihydroxyepothilone D in comparison with epothilone D. **B**. the effects of epothilone B, 11-hydroxyepothilone D and 9-hydroxyepothilone D in comparison with epothilone D.

double bond of epothilone D to form epothilone B. Under our biotransformation conditions, 26-hydroxyepothilone D and 26-hydroxy Epo490 were the major derivatives, with about 40% conversion.

Our studies showed that the addition of a hydroxyl group lowered the cytotoxic activity against NCI/ADR-RES cells with MDR phenotypes that overexpress P-glycoproteins. This observation is consistent with a recent report in which the chemically synthesized 21-hydroxyepothilone D exhibited marked increases in IC_{50}s ranging from about 5 to 34 fold in five of the ten MDR-resistant cell lines examined[10]. It is possible that hydroxylation makes these compounds better substrates of the p-glycoprotein pumps in those cells. In this regard, the cytotoxicity of the hydroxylated compounds should be tested in MDR cells without overexpression of p-glycoprotein. Nevertheless, because compound **4** is very active against some MDR cell lines, it is likely that other hydroxylated compounds, such as compounds **1** and **2**, may also have the same property.

Acknowlegments

We are grateful to PETER LICARI and the Department of Process Sciences at Kosan for providing epothilone D and Epo490, and JOHN CARNEY for determining the structure of 11-hydroxyepothilone D, NINA VISWANATHAN for performing the LC/MS, and SAMUEL DANISHEFSKY for providing the chemically synthesized 21-hydroxyepothilone D. We thank RICHARD HUTCHINSON, DAVID HOPWOOD for advice and ZQ TIAN and GARY LIU for helping on the ^{1}H-NMR data.

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