A New Cytotoxic Epothilone from Modified Polyketide Synthases Heterologously Expressed in *Myxococcus xanthus*

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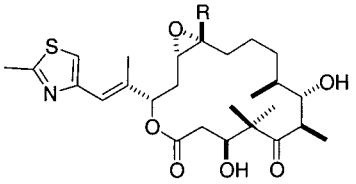
A new epothilone, 10,11-didehydroepothilone D (**5**), was isolated from a strain of the heterologous host *Myxococcus xanthus* genetically engineered to produce epothilone D (**4**). The structure of **5** was determined from NMR and MS data. The epothilone polyketide synthase was further modified in a recombinant *M. xanthus* strain to produce **5** as the major epothilone-related metabolite. The cytotoxicity of **5** against a panel of tumor cell lines, including several with multidrug resistance, and its effect on tubulin polymerization were comparable to epothilone D (**4**).

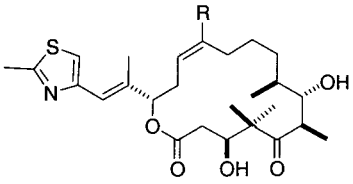
Epothilones are secondary metabolites originally isolated from the Gram-negative myxobacterium *Sorangium cellulosum*.[1] Epothilones A (**1**) and B (**2**) are the major cytotoxic compounds produced by the natural host, and recently an additional 37 epothilone-related metabolites produced in minor amounts have been reported from various strains of this organism.[2] Included among these minor metabolites are epothilones C (**3**) and D (**4**), which are biosynthetically converted to epothilones A and B, respectively, by a P450 epoxidase.[3] Epothilones A−D (**1**−**4**) have great potential for the treatment of cancer because, as with paclitaxel, their mode of action involves induction of tubulin polymerization and microtubule assembly, and they are highly cytotoxic against tumor cell lines resistant to paclitaxel and other anticancer drugs. Of epothilones A−D, epothilone D (**4**) has the greatest therapeutic index,[4] but it is produced in the lowest amount in *S. cellulosum*. We recently reported the sequence of the epothilone gene cluster and its heterologous expression in *Streptomyces coelicolor*.[3] Subsequently we have incorporated the epothilone PKS into another myxobacterium, *Myxococcus xanthus*.[5,6] *M. xanthus* has a shorter doubling time than *S. cellulosum* and is more amenable to genetic manipulations. To create a host capable of producing epothilone D (**4**) as the major epothilone, we constructed a deletion mutant, K111-40.1, to inactivate the P450 epoxidase responsible for the conversion of **3** and **4** to **1** and **2**, respectively, encoded by the *epoK* gene.[3]

Compound **5** was isolated as a minor metabolite from the production of **4** by strain K111-40.1. A crystallization step in the purification of **4** afforded a mother liquor that contained approximately 5% of **5** relative to epothilones C and D.[7] Cocrystallization of **5** and **4** followed by extensive reversed-phase chromatography provided **5**.

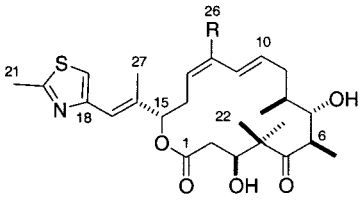
The molecular formula of $C_{27}H_{39}O_5NS$ for 10,11-didehydroepothilone D (**5**), established from HRESIMS and ^{13}C NMR data, differed from **4** by an additional double-bond equivalent. The ^1H and ^{13}C NMR data revealed that **5** did possess an additional carbon−carbon double bond, which was determined to be of the *E*-configuration on the basis of a coupling constant of $^3J_{H-H}$ of 16.0 Hz for two protons resonating at δ 6.52 and 5.76. The ^1H NMR spectrum of **5** displayed five methyl singlets (δ 2.69, 2.09, 1.79, 1.29, 1.03) and two methyl doublets (δ 1.11, J = 7.0 Hz; 1.03, J = 7.0 Hz), analogous to that of epothilone D. Multiplicity-edited HSQC data were used to determine $^1J_{C-H}$ connectivities and indicated that three methylene groups were present in **5**. Pure-absorption TOCSY,[8] COSY-60, and constant time HMBC[9] data established the spin system H_3-26, H-13, H_2-14, and H-15. HMBC correlations from H_3-26 to three olefinic carbon signals at δ 129.1 (C-11), 135.7 (C-12), and 123.1 (C-13), as well as from δ 5.76 (H-10) to C-12 and δ 6.52 (H-11) to C-12 and C-13, placed the additional double bond at the 10−11 position. Additional 2D NMR data were entirely consistent with C-1 through C-9 being the same as found in **4**. The configuration of the 12,13-double bond was determined to be *Z* on the basis of chemical shift calculations and comparison of the carbon shift for C-26 of



epothilone A (**1**) R = H
epothilone B (**2**) R = Me



epothilone C (**3**) R = H
epothilone D (**4**) R = Me



10,11-didehydroepothilone D (**5**) R = Me
epothilone C6 (**6**) R = H

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Table 1. Cytotoxic Activity of Epothilone D (**4**) and 10,11-Didehydroepothilone D (**5**)

		IC_{50} (nM), mean ± SD (*n*)	
cell line	tumor origin	**4** (*n* = 2−3)	**5** (*n* = 2)
MCF-7	breast	8 ± 1	28 ± 8
NCI/ADR-Res	breast, MDR[a]	30 ± 6	35 ± 9
SF-268	glioma	14 ± 6	40 ± 5
NCI-H460	lung	8 ± 2	34 ± 1
HL60	promyelocytic leukemia	8 ± 4	25 ± 6
HL60/MX2	promyelocytic leukemia, MDR	10 ± 1	18 ± 12
CCRF-CEM	T-cell leukemia	22 ± 3	23 ± 7
CCRF-CEM/C2	T-cell leukemia, MDR	15 ± 8	11 ± 6

[a] Multidrug resistant.

4. After the completion of this work, the epothilone C congener of **5**, epothilone C6 (**6**), was described, and the chemical shifts of **5** are also consistent with those reported for that compound.[2]

The cytotoxicity of **5** was assessed in a panel of human tumor cell lines. Three of the cell lines in the panel are multidrug resistant. Its activity was found to be comparable to epothilone D (Table 1). Compound **5** also induced tubulin polymerization in the MCF-breast cancer cell line with similar kinetics and potency relative to **4** (data not shown).

With the structure and biological activity of **5** established, we engineered the epothilone D-producing strain to produce **5** as the major epothilone-related metabolite. The methylene moiety at C-11 of epothilone results from the full reduction of the keto group that is formed during biosynthesis of the polyketide backbone. The reduction is carried out by three domains, a ketoreductase (KR), a dehydratase (DH), and an enoyl reductase (ER), located in module 5 of the epo PKS. During the process of reduction, the carbonyl function is reduced to a hydroxyl group by the KR domain; the DH domain removes the OH and an H atom from the adjacent carbon, leaving the corresponding double bond; the ER domain reduces the unsaturated carbons, resulting in the adjacent dimethylene structure.

The ER domain of module 5 was inactivated by replacement of two adjacent amino acids, GG, with the dipeptide moiety AS in the NADPH binding site. The resulting PKS bypassed the enoyl reduction step, leaving a 10,11-*trans* double bond in the completed epothilone polyketide backbone and encoded the production of **5** (Figure 1). With subsequent process development we are able to produce amounts of **5** suitable for further evaluation of its biological properties and its use as a synthetic scaffold for new epothilone derivatives.

Experimental Section

General Experimental Procedures. The optical rotation was measured at AAI International (Chapel Hill, NC); the UV spectrum was acquired by diode-array-detected HPLC; ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded in $CDCl_3$ solution at 300 K with a Bruker DRX 400 spectrometer equipped with a QNP *z*-axis gradient probehead. Chemical shifts were referred to δ 7.26 and 77.0 for ^{1}H and ^{13}C spectra, respectively. HRMS were obtained by FIA with manual peak-matching on an Applied Biosystems Mariner TOF spectrometer with a turbo-ionspray source in positive-ion mode (spray tip potential, 5400 V; spray chamber temperature, 400 °C; nozzle potential, 110 V). The resolution of the measured mass was 6600. HPLC analysis was carried out on a Hitachi L6200 series chromatograph fitted with an L-6200A gradient pump, an AS-2000 auto sampler, and an L-4500A diode array detector. Detection was carried out at 250 nm. A Metachem Inertsil ODS-3 column (5 μm, 4.6 × 150 mm) was used for analyzing both product pools and individual fractions.

Organism. *Myxococcus xanthus* strain K111-40.1 was engineered to produce primarily epothilones C (**3**) and D (**4**) by heterologously expressing the epothilone polyketide synthase and construction of an *epoK* mutant. Details of the genetic engineering of this strain are presented elsewhere.[5,6]

Construction of the 10,11-Didehydroepothilone-Producing Strain. A 2.5 kb *Bbv*CI-*Hin*dIII fragment of *epoD*, encoding a portion of the DH domain, the full ER domain, and a portion of the KR domain of module 5, was cloned in the vector pLitmus (Stratagene). The resulting plasmid, isolated from *E. coli* DH5α, was used as a template for site-directed mutagenesis using the QuikChange kit (Strategene) employing the following PCR primers: 5′-TGATCCATGCTGCGGCCG***CTA***GCGTGGGCATGGCCGC-3′ and 5′-GCGGCCATGCCCACGC***TAG***CGGCCGCAGCATGGATCA-3′. The primers match two DNA strands of the 37 bp segment encoding the NADPH-binding region of ER5 except for the positions highlighted in bold. The three-base change in the bp sequence results in the change of sequence of the corresponding polypeptide from AAGGVG to AAASVG. The PCR employed the entire pLitmus plasmid carrying the inserted *epo* PKS sequence as the template to produce an amplified linear plasmid, designated pLit-ER5*, carrying the mutated sequence. Since template DNA (wild-type) would be present in the mixture of PCR products, the tailoring was done to ensure that the wild-type sequence would not be recovered. The products of the PCR reaction were subjected to treatment with *Dpn*I, which digests only methylated DNA (template, wild-type DNA isolated from *E. coli*) but not the unmethylated, mutated plasmid pLit-ER5*

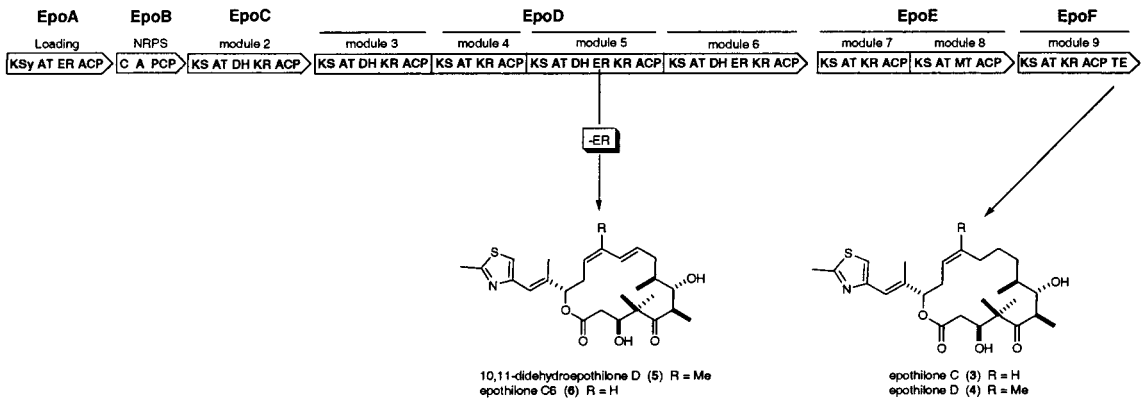

Figure 1. Modular organization of the epothilone polyketide synthase (PKS) and modification to produce **5**. Abbreviations: KSy, β-ketoacyl ACP synthase containing a tyrosine substitution of the active-site cysteine; AT, acyltransferase; ER, enoylreductase; ACP, acyl carrier protein; C, condensation; A, adenylation; PCP, petidyl carrier protein; KS, β-ketoacyl ACP synthase; DH, dehydratase; KR, ketoreductase; MT, methyltransferase; TE, thioesterase.

generated by the PCR reaction. After the digestion, pLit-ER5* was recovered following transformation in *E. coli*. An approximately 3 kb DNA segment containing the genes *kanR* and *galK*, genes encoding kanamycin resistance and galactokinase, respectively, was inserted in the *Dra*I site of pLit-ER5* to generate the plasmid pKOS165-76. This plasmid was introduced in the epothilone D, producing *M. xanthus* K111-72.14 host by electroporation and selection for kanamycin resistance.[5] Kan^R colonies were checked by PCR for insertion of the plasmid by single reciprocal homologous recombination within the region of *epoD* on the chromosome homologous to the segment carried on the plasmid. Cells that had undergone this event carried a 2.5 kb duplication of *epoD* corresponding to the *epoD* segment on pKOS165-76 and, hence, contained both the wild-type and mutated ER5 sequences. To isolate cells that had eliminated the duplicated region, the cells were grown in liquid culture for many generations in the absence of kanamycin and then plated on media containing 2-deoxygalactose. Cells containing *galK* convert 2-deoxygalactose to 2-deoxygalactose-1-phosphate, which accumulates in the cell and is toxic. Surviving colonies have lost *galK* along with *kanR*, the plasmid sequence, and one of the *epoD* duplications present in the chromosome through a second homologous recombination event, either in the same location (relative to ER5) as the original incoming crossover to leave a cell with a wild-type ER5 domain or in the region of *epoD* opposite the incoming crossover to result in a cell that carried the ER5* domain. Since the two cases could be distinguished on the basis of the compound produced by the respective strains, a number of colonies were picked and grown for 7 days in CTS-XAD medium. Cultures were screened for epothilone production by LC-MS. Approximately half of the cultures produced epothilone D (**4**), while the other half appeared to produce 10,11-didehydroepothilone D (**5**). The compound identified by LC-MS as **5** from this recombinant strain was isolated and confirmed to be **5** by NMR spectroscopy.

Isolation of 10,11-Didehydroepothilone D (5). Rotary evaporation of the mother liquor from epothilone D produced by mutant K111-40.1 in a 1000 L fermenter gave 3.0 g of solids.[7] HPLC data showed **5** present at approximately 5% with respect to epothilones C (**3**) and D (**4**). The solids were dissolved/suspended in 2 L of MeOH−H_2O (1:1) and pumped at 80 mL/min onto a 4.8 × 25 cm, 500 mL C_{18} column (EM, 40 μm) preequilibrated with MeOH−H_2O (1:1). The column was washed with 500 mL of MeOH−H_2O (1:1) and then eluted with MeOH−H_2O (65:35). A total of 33 250-mL fractions were collected, of which fractions 20−33 were combined and evaporated. The solids were dissolved in 500 mL of EtOH, and to this solution was added 1 g of decolorizing charcoal. The mixture was stirred for 20 min and vacuum filtered, and the filtrate was concentrated to 100 mL on a rotary evaporator. The concentrate was placed into a 250 mL media bottle containing a 0.5 in. magnetic spin bar and fitted with a cap with a feed tube for the slow delivery of the crystallization forcing solvent. The bottle in turn was placed in a 25 °C temperature-controlled alcohol/H_2O bath. With gentle mixing 100 mL of H_2O was added using a positive displacement pump at a flow rate of 2.5 mL/min. Approximately 3 mg of epothilone D seed crystals was added to the turbid mixture. After 5 min of vigorous mixing the stirring speed was reduced. Water addition was resumed until a total of 150 mL had been added. The temperature of the solution was decreased to 0 °C over 30 min and held there for an additional 12 h with slow mixing. The crystals were filtered, then redissolved in 2 L of MeOH. To this solution was added 2 L of H_2O, and the mixture was loaded onto a 4.8 × 25 cm C_{18} (EM 40 μm) column preequilibrated with MeOH−H_2O (1:1). The column was washed with 500 mL of MeOH−H_2O (1:1) and then eluted with MeOH−H_2O (65:35). A total of 36 250-mL fractions were collected. All fractions containing **5** were pooled (F20−F24) and rechromatographed on the same C_{18} column. Following the column load, the column was eluted with 10 column (5 L) volumes of MeOH−H_2O (65:35), which was collected as a single fraction. An additional 33 fractions were collected with each fraction containing 250 mL of eluant. All of the **5**-containing fractions were pooled and rechromatographed on a 2.5 × 30 cm C_{18} (Bakerbond 40 μm) column in MeOH−H_2O (50:50). The column was washed with 130 mL of MeOH−H_2O (1:1) and eluted with 1.8 L of MeOH−H_2O (65:35), followed by 1.8 L of MeOH−H_2O (7:3). A total of 36 100-mL fractions were collected, of which fractions 29−31 were combined. Water was added (70 mL) to adjust the MeOH−H_2O ratio to 4:6. The turbid solution was loaded onto 20 mL of C_{18} silica gel contained in a 30 mL sintered glass funnel. The C_{18} silica gel was eluted with 100 mL of 100% EtOH, and the eluate was concentrated to an oil and dried in a vacuum oven for 12 h. This gave 24 mg of **5** as a colorless oil, which when analyzed by HPLC gave a chromatographic purity of 94%.

10,11-Didehydroepothilone D (5): colorless oil; $[\alpha]_D$ −74° (*c* 0.079, MeOH); UV (DAD, MeCN−H_2O, 7:3) λ_{max} 215, 243 nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.96 (1H, s, H-19), 6.57 (1H, s, H-17), 6.52 (1H, d, J = 16.0 Hz, H-11), 5.76 (1H, ddd, J = 16.0, 9.0, 5.5 Hz, H-10), 5.29 (1H, overlapped, H-15), 5.28 (1H, overlapped, H-13), 4.21 (1H, dd, J = 10.0, 3.5 Hz, H-3), 3.71 (1H, dd, J = 6.5, 2.0 Hz, H-7), 3.25 (1H, qd, J = 7.0, 2.0 Hz, H-6), 2.81 (1H, dt, J = 14.0, 10.5 Hz, H-14_a), 2.69 (3H, s, C-21), 2.52 (1H, dt, J = 14.5, 5.5 Hz, H-9_a), 2.42 (1H, dd, J = 14.5, 10.0 Hz, H-2_a), 2.35 (1H, dd, J = 14.5, 3.5 Hz, H-2_b), 2.28 (1H, dd, J = 14.0, 6.0 Hz, H-14_b), 2.09 (3H, s, H-27), 2.07 (1H, m, H-9_b), 1.98 (1H, m, H-8), 1.79 (3H, s, H-26), 1.29 (3H, s, H-22), 1.11 (3H, d, J = 7.0 Hz, H-24), 1.06 (3H, d, J = 7.0 Hz, H-25), 1.03 (3H, s, H-23); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 220.2 (C-5), 170.2 (C-1), 164.9 (C-20), 152.1 (C-18), 138.1 (C-16), 135.7 (C-12), 129.5 (C-10), 129.1 (C-11), 123.1 (C-13), 119.5 (C-17), 116.1 (C-19), 78.4 (C-15), 71.9 (C-3), 71.6 (C-7), 53.3 (C-4), 41.2 (C-6), 39.4 (C-2), 36.9 (C-8), 36.0 (C-9), 32.1 (C-14), 21.14 (C-23), 21.05 (C-26), 19.2 (C-22), 19.1 (C-21), 16.8 (C-25), 15.6 (C-27), 11.5 (C-24); HRESIMS *m/z* 490.2632; calcd for $C_{27}H_{40}NO_5S$ [M + H]$^+$, 490.2622.

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 (Hyclone) and 2 mM L-glutamine.

Tumor cell cytotoxicities of **4** and **5** were determined by a sulforhodamine B (SRB) assay.[10] Cultured cells were trypsinized, counted, and diluted to suitable concentrations (5000−7500 cells per 100 μL) with growth medium. Ninety-six-well microtiter plates were seeded with 100 μL/well of each cell suspension. At 20 h 100 μL of either **4** or **5**, ranging from 0.001 to 1000 nM diluted in growth medium, was added to each well. After 3 days the cells were fixed with 100 μL of 10% trichloroacetic acid at 4 °C for 1 h and stained with 0.2% SRB/1% acetic acid at room temperature for 20 min. The unbound dye was rinsed with 1% acetic acid, and the bound SRB was extracted by 200 μL of 10 mM Tris base. The amount of bound dye was determined by absorbance at 515 nm, which correlated with total cellular protein content.

Tubulin Polymerization Assay.[11] MCF-7 human breast carcinoma cells grown to confluency in 35 mm culture dishes were treated with 1 μM of either **4** or **5** for 0, 1, and 2 h at 37 °C. After washing the cells twice with 2 mL of PBS without Ca^{2+} and Mg^{2+}, the cells were lysed at room temperature for 5−10 min 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 cells were scraped and lysates transferred to 1.5 mL Eppendorf tubes. The lysates were then centrifuged at 18000*g* for 12 min at room temperature. 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 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).

References and Notes

(1) (a) Höfle, G.; Bedorf, N.; Steinmetz, H.; Schomburg, D.; Gerth, K.; Reichenbach, H. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1567−1569. (b) Gerth, K.; Bedorf, N.; Höfle, G.; Irachik, H.; Reichenbach, H. *J. Antibiot.* **1996**, *49*, 560−563. (c) Bollag, D. M.; McQeuncy, P. A.; Zhu, J.; Hensens, O.; Koupal, L.; Liesch, J.; Goetz, M.; Lazarides, E.; Woods, C. M. *Cancer Res.* **1995**, *55*, 2325−2333.

(2) Hardt, I. H.; Steinmetz, H.; Gerth, K.; Sasse, F.; Reichenbach, H.; Höfle, G. *J. Nat. Prod.* **2001**, *64*, 847−856.

(3) Tang, L.; Shah, S.; Chung, L.; Carney, J.; Katz, L.; Khosla, C.; Julien, B. *Science* **2000**, *287*, 640−642.

(4) Chou, T. C.; Zhang, X. G.; Balog, A.; Su, D. S.; Meng, D.; Savin, K.; Bertino, J. R.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9642−9647.

(5) Julien, B.; Shah, S. Unpublished results.

(6) Lau, J.; Frykman, S.; Regentin, R.; Ou, S.; Tsuruta, H.; Licari, P. *Biotechnol. Bioeng.*, in press.

(7) Arslanian, R. L.; Parker, C. D.; Wang, R.; McIntire, J. R.; Lau, J.; Starks, C.; Licari, P. J. *J. Nat. Prod.* **2002**, *65*, 570−572.

(8) Parella, T.; Sánchez-Ferrando, F.; Virgili, A. *J. Magn. Reson.* **1997**, *125*, 145−148.

(9) Furihata, K.; Seto, H. *Tetrahedron Lett.* **1998**, *39*, 7337−7340.

(10) Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107−1112.

(11) (a) Giannakou, P.; Sackett, D. L.; Kang, Y. K.; Zhan, Z.; Buters, J. T.; Fojo, T.; Poruchynsky, M. S. *J. Biol. Chem.* **1997**, *272*, 17118−17125. (b) Giannakou, P.; Villalba, L.; Li, H.; Poruchynsky, M.; Fojo, T. *Int. J. Cancer* **1998**, *75*, 57−63.

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