# Large-Scale Isolation and Crystallization of Epothilone D From *Myxococcus xanthus* Cultures

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*Received September 5, 2001*

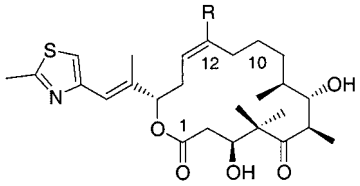
The introduction of the epothilone polyketide synthase (PKS) into *Myxococcus xanthus* has enabled the heterologous production of epothilone D (**1**) on a large scale. To isolate this valuable product from the fermentation medium, an economical, scalable, and high-yielding purification process was developed. With the crystallization of **1** from a binary solvent system that consisted of ethanol and water, the product was recovered as white crystals with a final purity of ≥97% (w/w). This is the first reported crystallization of **1**.

Epothilones are polyketides that were originally isolated from the myxobacterium *Sorangium cellulosum*.[1] They are cytotoxic compounds that share a mechanism of action similar to that of Taxol in their ability to stabilize microtubule assembly.[2] Unlike Taxol, these compounds are found to be effective against tumor cell lines that are multidrug resistant.[3] Initial in vitro testing revealed epothilone B (**2**) to be the most potent of the naturally occurring epothilones in its cytotoxic activity.[3] However, the use of this compound has led to severe toxic side-effects in a subsequent in vivo mouse study.[4] Furthermore, **2** was not as effective as Taxol in reducing tumor size. It was observed in this study that **1**, the least abundant of the four major epothilones, performed better than both Taxol and **2**.[4] Moreover, **1** did not exhibit the same acute toxic effects as **2**.

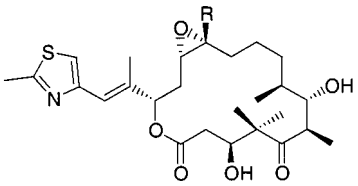
Epothilone D (**1**) is an intermediate in the biosynthetic pathway of **2**.[5,6] It is produced at a low titer of <1 mg/L by the wild-type *S. cellulosum* strain.[7] Production strains of **1** and **3** have been obtained by random mutation using UV light.[8] Epothilone D has been obtained in good yield from **2** using a single-step chemical transformation.[9] The total chemical synthesis of **1** has been accomplished.[10] However, there remains a need for a cost-effective method for producing **1** on a large scale.

The epothilone gene cluster has been cloned from the natural epothilone-producing *S. cellulosum* strain[5,11] and expressed in *Myxococcus xanthus*, a heterologous host that is more amenable to genetic manipulation.[12] The introduction of epothilone polyketide synthase (PKS) into *M. xanthus* and the construction of a deletion mutant with an inactive EpoK P450 epoxidase resulted in a recombinant strain that is incapable of converting **1** and epothilone C (**3**) into **2** and epothilone A (**4**), respectively, and produces **1** and **3** as the main fermentation products at a ratio of 4:1.[12] With the identification of methyl oleate as a suitable carbon source for the *M. xanthus* strain and the development of other improvements in the fermentation process, the production of **1** currently exceeds 20 mg/L.[7]

In addition to **1**−**4**, more than 30 other epothilones have been isolated from fermentations of strains of *S. cellulosum* by Reichenbach, Höfle, and co-workers.[1,13] The methods that were used to recover these products are not amenable to scale-up. The key steps that lead to a cost-effective and high-yielding large-scale purification process for **1** are discussed here.




Epothilone D (**1**): R = Me
Epothilone C (**3**): R = H




Epothilone B (**2**): R = Me
Epothilone A (**4**): R = H

A purification process was developed to recover **1** from 1000 L fermentations at high yield and purity (Figure 1, Table 1). To minimize product degradation and facilitate product recovery, XAD-16 resin was used in the fermentations to capture the epothilone compounds that were secreted by the cells.[7] Addition of this hydrophobic resin to the medium resulted in the binding of the epothilones as well as the methyl oleate that was used as a carbon source in the fermentations. Furthermore, it was found that elution of the XAD-16 resin with a strong solvent such as 100% methanol led to the concomitant release of both the bound methyl oleate and epothilones. Epothilone products were effectively removed from the resin, while most of the methyl oleate remained bound when using 78:22 methanol−water as an elution solvent.

Following the initial solid-phase extraction, a defatting step was performed with HP-20SS, a polystyrene−divinylbenzene resin, to remove residual methyl oleate that eluted with the epothilones. The product pool from the solid-phase extraction was loaded onto the column in 57:43 methanol−water, and the epothilones were eluted from the column with 78:22 methanol−water. This purification step reduced the methyl oleate concentration in the eluant and enriched the concentration of **1** from <4% (w/w) to ca. 10% (w/w).

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10.1021/np010438s CCC: $22.00 
Published on Web 02/23/2002

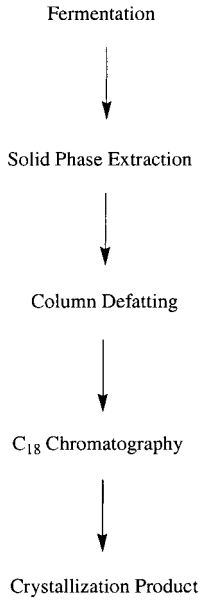


**Figure 1.** Epothilone D (**1**) purification process.

**Table 1.** Yield and Purity of **1** in the Described Purification Process

| unit operation | yield of **1** | purity of **1** (% w/w) |
|---|---|---|
| solid-phase extraction (%) | 100 | 2 |
| column defatting (%) | 96 | 10 |
| $C_{18}$ chromatography (%) | 89 | 70 |
| solvent exchange column (%) | 99 | 70 |
| crystallization (%) | 80 | 97 |
| overall yield of **1** (%) | 67 | N/A |
| final product (g) | 63 | N/A |

**Table 2.** Comparison of $C_{18}$ Sorbents

| sorbent | resolution factor ($R_s$)[a] | yield of **1** (%) |
|---|---|---|
| Bakerbond | 0.6 | 98 |
| Uetikon C-gel | 0.5 | 84 |
| Lichroprep | 0.4 | 58 |

[a] $R_s$ for **1** relative to **3**.

A $C_{18}$ chromatographic step was incorporated in the purification process to remove the major epothilone contaminant, **3**, from the product pool. The separation of **1** and **3** using $C_{18}$ chromatography has been published.[13,14] Column loads of 1 g/L (g of **1**/L of $C_{18}$ sorbent) have been previously reported. In this work we detail the successful operation of $C_{18}$ production columns using column loads of 4 g/L. Indeed, most recently in one small-scale experiment, a column load of 10 g/L resulted in a 90% yield of **1**. With chromatography being one of the most expensive unit operations, the ability to operate at 4−10 times normal loading is economically attractive. In an effort to maximize column efficiency, three $C_{18}$ sorbents (Bakerbond, Lichroprep, and Uetikon) were evaluated on a small-scale. Of the three sorbents tested, the Bakerbond $C_{18}$ sorbent was the most effective in resolving **1** from **3** and led to the highest yield of the desired product (Table 2). Moble-phase optimization experiments were conducted in order to maximize product yield (Table 3). At 4 g/L loading it was found that yields of >90% could be obtained by using 67:33 methanol−water. With the identification of a suitable sorbent and optimal elution conditions, the large-scale $C_{18}$ chromatography step enabled the removal of 97% of the contaminating **3**.

Crystallization is commonly used as a method of purification to obtain compounds of high purity. The crystallizations of **2** and **4** from single and binary solvent systems have been published.[6,14,15] Attempts to crystallize **1** under these conditions were unsuccessful. After testing various other solvents, we found that the crystallization of **1** was promoted by the controlled addition of water to an ethanol solution. Activated carbon was used to remove trace impurities from the crystallization feedstock. With the development of this crystallization procedure, white crystals of **1** with a purity of ca. 97% (w/w) were obtained.

**Table 3.** Effect of Solvent Composition on $C_{18}$ Chromatography

| solvent composition (methanol−water) | retention volume of **1** (column volume) | yield of **1** (%) |
|---|---|---|
| 69:31 | 10 | 82 |
| 67:33 | 13 | 91 |
| 65:35 | 20 | 94 |

This process was used to produce six 10 g lots of **1** as a white crystalline material. The overall yield of **1** from the 1000 L fermentations of the recombinant *M. xanthus* strain was 67%. The average final product purity was 97% (w/w) (Table 1). The final product contained two major epothilone impurities, which based on HPLC accounted for <2% of **1**.

## Experimental Section

**General Experimental Procedures.** HPLC analysis was performed on either a Hitachi D-7000 HPLC system (consisting of a D-7100 pump, a D-7200 autosampler, and a L-4500A diode array detector) or a Hitachi D-6000 system (consisting of a L-6200A pump, a AS-2000 autosampler, and a L-4500A diode array detector). Solution delivery was carried out using a pressurized gas system or an air-driven gear pump (Micropump). A Thermoquest UV detector with an adjustable pathlength and preparative flow cell was used for on-line monitoring.

**Solid-Phase Extraction.** Twenty liters of XAD-16 resin (Rohm & Haas) were collected from a 1000 L fermentation of the recombinant *M. xanthus* strain[7] with a wire-mesh filter-basket. The resin was washed with 2 volumes of water and transferred to an Amicon VA-250 process column. The column was then washed with 4 column volumes of water (2 L/min) and eluted with 5 column volumes of 78:22 methanol−water (1 L/min). This eluant was collected as a single fraction. Two additional 0.5 column volumes fractions were subsequently collected, and the three fractions were analyzed by HPLC. Fractions containing **1** were combined as the solid-phase extraction product pool.

**Column Defatting.** An Amicon VA-180 process column was packed with 5.5 L of HP-20SS resin (Mitsubishi) and equilibrated with 5 column volumes of 57:43 methanol−water (1 L/min). The product pool from the solid-phase extraction was diluted with water to a final methanol concentration of 57%. The resulting suspension was loaded onto the column at a flow rate of 1 L/min. The column was washed with 3 column volumes of 57:43 methanol−water (1 L/min). Column elution was carried out under isocratic conditions using 18 column volumes of 78:22 methanol−water (380 mL/min) and was monitored using UV detection at 250 nm. The first 5 column volumes were collected as a single fraction, and the remaining eluant was collected as a second fraction until elution of the epothilone peak was complete.

**$C_{18}$ Chromatography.** An Amicon VA-130 process column was flow-packed with 2.7 kg (4 L) of $C_{18}$ chromatographic sorbent (Bakerbond, 40 $\mu$m) and equilibrated with 4 column volumes of 50:50 methanol−water (630 mL/min). The product pool from the column defatting step was diluted with water to a final methanol concentration of 50% and loaded onto the column at 475 mL/min. The column was then washed with 2 column volumes of 50:50 methanol−water (475 mL/min). Column elution was carried out using 21 column volumes of 67:33 methanol−water (310 mL/min) and was monitored using UV detection at 250 nm. The initial eluant was collected as a

single fraction until the peak maximum for **3** was reached. Fractions of 0.25 column volumes (1 L) were then collected until the peak corresponding to **1** was at its maximum. When this peak maximum was reached, a single fraction was collected until the elution of **1** was complete. HPLC analysis was carried out on individual fractions. Fractions were combined such that the final concentration of **3** was <0.5%. The remaining fractions of **1** that contained **3** were rechromatographed using a second $C_{18}$ column. The second $C_{18}$ chromatography was preformed in the same manner as the first. The two $C_{18}$ product pools were subsequently combined.

**Solvent-Exchange.** An Amicon VA-130 column was packed with 0.4 kg (600 mL) of $C_{18}$ sorbent (Bakerbond $C_{18}$, 40 $\mu$m) and equilibrated with 4 L of 40:60 methanol−water (4 L/min). The product pool from the $C_{18}$ chromatography step was diluted with water to a final methanol concentration of 40%, and the resulting solution was loaded onto the column at 1 L/min. The column was allowed to run dry and was eluted with ethanol at 250 mL/min (Gold Shield, 200 proof). Three fractions (100 mL, 500 mL, 300 mL) were collected and analyzed by HPLC. All fractions containing **1** were combined.

**Activated Carbon Treatment.** Activated carbon (J. T. Baker) was added to the eluant of the solvent-exchange column (1 g carbon/1 g **1**). The mixture was stirred for 15 min and filtered through Whatman No. 2 filter paper under vacuum. The filtrate was pumped through a 0.2 $\mu$m filtration capsule (Millipore). The capsule was rinsed with three 20 mL aliquots of ethanol, and these rinses were combined with the filtrate and evaporated to an oil on a 2 L rotary evaporator (Buchi 40 °C). The oil was dried in a vacuum oven (40 °C, 12 mbar) for 4−8 h.

**Crystallization.** Ethanol was added to the vacuum-dried oil (24 mL ethanol/1 g **1**) from the charcoal step. The resulting solution was transferred to a 2 L heavy-walled beaker. While stirring, water was added (WFI, 29 mL $H_2O$/1 g **1**) to the solution at ambient temperature at a rate of 10 mL/min. When the solution became cloudy, water addition was stopped, and seed crystals of **1** (2−5 mg) were added. Vigorous stirring was maintained for 15 min, after which the initial stir rate was restored. Crystal formation was typically observed within 5 min. Water addition was then resumed until all of the remaining water was added. The solution was stirred at room temperature for an additional 15−30 min and maintained at 4 °C for 12 h. The resulting solids were vacuum-filtered using a Buchner funnel with a Whatman No. 2 filter paper. The vacuum was maintained for approximately 10 min. Crystals were transferred to a crystallization dish and vacuum-dried for approximately 12 h at 40 °C and 12 mbar. The crystalline **1** was stored at 4 °C.

**Epothilone D (1)** was obtained as colorless crystals (EtOH−$H_2O$): mp 120−121 °C; HRESIMS *m/z* 492.2768 ($M + H^+$) (calcd for $C_{27}H_{42}NO_5S$ 492.2778); UV, IR, $^1$H NMR, and $^{13}$C NMR values were consistent with literature values.[13]

**Small-Scale $C_{18}$ Chromatography.** A small-scale chromatographic column (2.5 × 30 cm) was packed to a height of 28 cm with $C_{18}$ sorbent and equilibrated with 4 column volumes of 50:50 methanol−water (25 mL/min). The product pool from the column defatting step was diluted with water to a final methanol concentration of 50% and loaded onto the column at a capacity of 2−4 g of **1** per liter of $C_{18}$ sorbent (25 mL/min). The column was washed with 2 column volumes of 50:50 methanol−water (25 mL/min) and eluted isocratically with methanol−water (12.5 mL/min). Fractions were collected and analyzed by HPLC.

**Acknowledgment.** We thank Dr. J. Carney for generating the NMR and MS spectra and for characterization of the epothilones.

## References and Notes

(1) Gerth, K.; Bedorf, N.; Höfle, G.; Irschik, H.; Reichenbach, H. *J. Antibiot.* **1996**, *49*, 560−563.
(2) Bollag, D. M.; McQuency, P. A.; Zhu, J.; Hensens, O.; Koupal, L.; Liesch, J.; Goetz, M.; Lazarides, E.; Woods, C. M. *Cancer Res.* **1995**, *55*, 2325−2333.
(3) Su, D.; Balog, A.; Meng, D.; Bertinato, P.; Danishefsky, S.; Zheng, Y.; Chou, T.; Lifeng, H.; Horwitz, S. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2093−2096.
(4) Chou, T.; Zhang, X.; Balog, A.; Su, D.; Meng, D.; Savin, K.; Bertino, J.; Danishefsky, S. *Proc. Natl. Acad. Sci. U.S.A.*, **1998**, *95*, 9642−9647.
(5) Tang, L.; Shah, S.; Chung, L.; Carney, J.; Katz, L.; Khosla, C.; Julien, B. *Science* **2000**, *287*, 640−642.
(6) Höfle, G.; Bedorf, N.; Steinmetz, H.; Schomburg, D.; Gerth, K.; Reichenbach, H. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1567−1569.
(7) Lau, J.; Frykman, S.; Regentin, R.; Ou, S.; Tsuruta, H.; Licari, P. *Biotechnol. Bioengin*, submitted.
(8) Gerth, K.; Steinmetz, H.; Höfle, G.; Reichenbach, H. *J. Antibiot.* **2001**, *54*, 144−148.
(9) Johnson, J.; Kim, S.; Bifano, M.; DiMarco, J.; Fairchild, C.; Gougoutas, J.; Lee, F.; Long, B.; Tokarski, J.; Vite, G. *Org. Lett.* **2000**, *2*, 1537−1540.
(10) Harris, C.; Juduk, S.; Balog, A.; Savin, K.; Glunz, P.; Danishefsky, S. *J. Am. Chem. Soc.* **1999**, *12*, 7050−7062.
(11) Molnár, I.; Schupp, T.; Ono, M.; Zirkle, R.; Milnamow, M.; Nowark-Thompson, B.; Engel, N.; Toupet, C.; Stratmann, A.; Cyr, D.; Gorlach, J.; Mayo, J.; Hu, A.; Goff, S.; Schmid, J.; Ligon, J. *Chem. Biol.* **2000**, *7*, 97−109.
(12) Julien, B.; Shah, S. *Proc. Natl. Acad. Sci. U.S.A.*, submitted.
(13) Hardt, I.; Steinmetz, H.; Gerth, K.; Sasse, F.; Reichenbach, H.; Höfle, G. *J. Nat. Prod.* **2001**, *64*, 847−856.
(14) Reichenbach, H.; Höfle, G.; Gerth, K.; Steinmetz, H. (GBF), WO 98/22461; *Chem. Abstr.* **1998**, *129*, 53436.
(15) Hoffman, H.; Mahnke. M.; Memmert, K.; Petersen, F.; Schupp, T.; Küsters, E.; Mutz, M. U.S. Patent 6,194,181, 2001.

NP010438S