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Studies on the Biosynthesis of Epothilones: Hydroxylation of Epo A and B to Epothilones E and F

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When *Sorangium cellulosum* So ce90 is grown without XAD adsorber resin there is a steady state of epothilone A and B biosynthesis and hydroxylation of these products to epothilones E and F. This biotransformation at position C-21 of the thiazole ring is not restricted to producers of epothilones. It is carried out by a substrate induced monooxygenase. Epothilones E and F are further degraded by opening of the lactone ring by an esterase. Steps of degradation of different strains of *S. cellulosum* are compared.

In preceding papers on the biosynthesis of the novel antitumor compound epothilone by *S. cellulosum* So ce90 we reported on the biosynthetic origin of epothilones[1] and the epoxidation of epothilones C and D to epothilones A and B by a post polyketide modifying monooxygenase[2]. From large scale fermentations the new epothilones E and F were isolated as two of 35 trace components[3]. Both compounds, which show a cytotoxicity comparable to that of A and B[3], and are interesting starting materials for further chemical derivatisation[4]. Here we report on the biological hydroxylation reaction and subsequent steps of degradation by strains of *S. cellulosum*.

Materials and Methods

Strain and Culture Conditions

The culture conditions for *Sorangium cellulosum* strain So ce90 have been described in a previous paper[1]. Mutants of So ce90 used for these experiments are B2, a high producer strain, A6, a producer of epothilone A only , and C2, a epothilone-nonproducer mutant.

Monitoring of the Hydroxylation

Strain So ce90 was grown for 4 days with or without adsorber resin, XAD-16. If present, the resin was removed by sieving before the addition of 20 $\mu g\,ml^{-1}$ of tetracycline and incubation for 1 hour at 30°C on a rotary shaker. Then the cells were centrifuged down at 10000 rpm in a Sorval cold centrifuge. The pellet was resuspended in 1/5 of the supernatant and incubated in the presence of added epothilone A or B (200 $mg\,l^{-1}$). At the various times samples of 0.1 ml were withdrawn, the cells were centrifuged down in an Eppendorf centrifuge and the supernatant analyzed directly by HPLC.

Induction of the Epothilone A/B Monooxygenase

A culture of So ce90 (200 ml in a 500 ml Erlenmeyer flask) was grown for 4 days in the presence of XAD-16 (Rohm und Haas, Frankfurt/M). The resin was separated by sieving, and 50 ml portions of the culture were pipetted into sterile 250 ml flasks with 5 mg of epothilone A or B added. The cultures were incubated over night. Then the cells were centrifuged down, washed with the same volume of fresh medium and then resuspended in 5 ml medium E.

Biotransformation of Epothilone A to E

Mutant B6, a producer of epothilone A only, was used for the biotransformation of epothilone A to E. Precultures, 14×100 ml in 250 ml Erlenmeyer flasks with medium E

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without resin, were grown for 3 days at 30°C. Then the cultures were transfered into 14×400 ml medium without resin in 1 liter Erlenmeyer flasks and incubated an additional 3 days before epothilone A (0.5 g l^{-1}) was added.

Analysis of the biotransformation was done by taking samples of 0.5 ml which were incubated in Eppendorf tubes for 1 hour in the presence of XAD resin. Then cells and resin were centrifuged down, the supernatant was discarded and the pellet resuspended in 1 ml of methanol. After 1 hour the samples were centrifuged again and the supernatant was analyzed by HPLC. After 45 hours the pH of the cultures was adjusted to pH 4, 3% of XAD-16 resin was added and the cultures shaken for two hours in order to bind the epothilones.

Biotransformation of Epothilone A by Different *S. cellulosum* Strains

Sorangium strains were grown in 250 ml medium E without XAD resin in 1 liter Erlenmeyer flasks for 3 days. Then the cells were centrifuged down, the pellet was resuspended in 50 ml of the supernatant which was poured into a sterile 250 ml Erlenmeyer flask containing 20 mg of epothilone A.

After two days of incubation 4% (v/v) of XAD-16 resin was added and the culture was incubated for 1 hour. Then the resin was harvested by sieving and eluted with methanol. The concentrated eluate was analyzed by HPLC.

Test of Esterase Activity

Strain *S. cellulosum* So ce90 B2 was used for the assay of an esterase activity in the culture supernatant. An aliquot of 10 ml of a 7 day old culture grown in the presence of XAD resin was centrifuged and 10 ml of the supernatant pipetted into a sterile 100 ml Erlenmeyer flask. After the addition of epothilone A (200 mg l^{-1}) the supernatant was analyzed directly by HPLC during the following 3 days.

HPLC Analysis

The quantitative determination of epothilones was performed in a HPLC system 1090 with a diode array detector (Hewlett Packard). A microbore column 125/1/4"2 Nucleosil 120-5C18 (Macherey-Nagel, Düren) was used. The following conditions were used for the quantifying of epothilones A, B, E, F:

Temperature was 40°C, flow 0.5 ml/minute, solvent water/acetonitrile (60 : 40) isocratic for 6 minutes and a gradient to 100% acetonitrile from 6 to 7 minutes, detection at 250 nm, Bw 4; reference measurement at 598 nm, Bw 4; spectrum 200 to 400 nm, step size 2 nm.

For the analysis of the polar degradation products the conditions were as follows:

Solvent 0.2% acetic acid/acetonitrile (90 : 10) for 1 minute; linear increase of acetonitrile to 30% to the 5 th minute; gradient to 50% to the 5.5 th minute which was maintained constant to the 7 th minute; then again an increase to 100% acetonitrile to the 10 th minute. The other conditions were as described above.

Results and Discussion

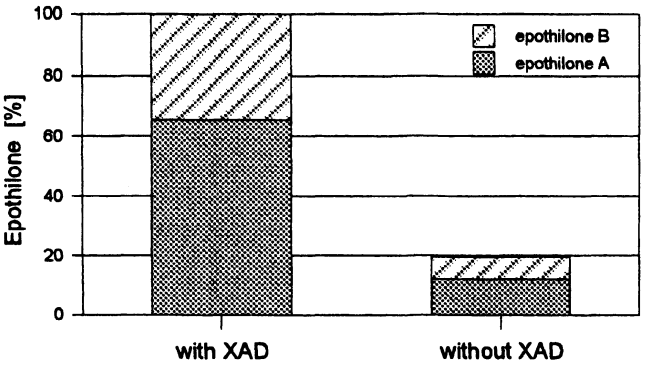
The Effect of Resin on the Yield of Epothilones

The presence of the adsorber resin XAD-16 during cultivation was of great importance for a good yield of epothilones A and B. Without resin only about 20% of epothilones were found after three weeks of cultivation compared with the control culture (Fig. 1). At the same time traces of the epothilones E and F, C-21 hydroxylated analogues of epothilones A and B (Fig. 6), were detected in the culture supernatant. This positive effect of the resin on the overall yield was also described for the production of soraphen[5] by *S. cellulosum* So ce26. It can be understood as being due to prevention of degradation by withdrawing of the excreted endproducts continously from the culture broth as a result of adsorption onto the XAD resin by hydrophobic interactions.

The Epothilones A/B Monooxygenase

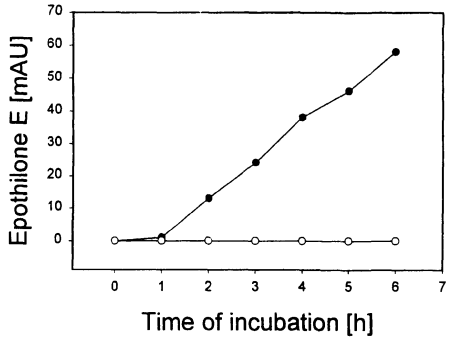
To test the assumption of a degradation of epothilones the fate of added epothilone A was analyzed in dependence of time.

Fig. 1. Effect of the adsorber resin, XAD-16, on the yield of epothilone A and B.



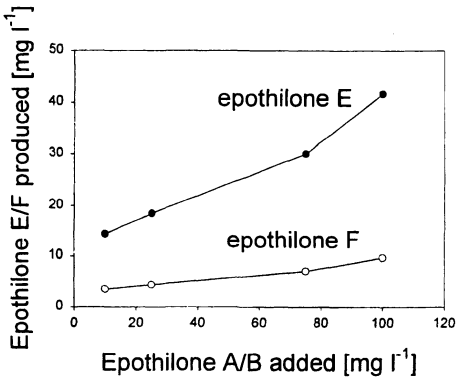
Mutant B2 was cultivated for 3 weeks in 100 ml of medium E in the presence of 2% resin or in its absence. The results are mean values of two parallels.

Fig. 2. Hydroxylation of epothilone A to E by cells of mutant B2 in the presence of tetracycline.



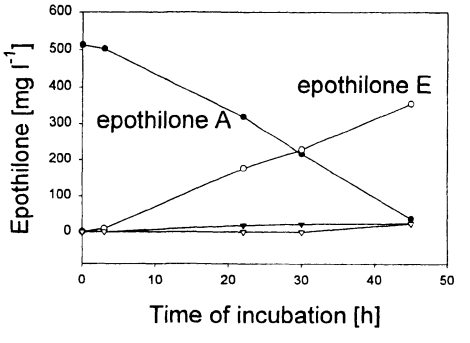
Data are mean values of duplicates. Solid symbols: cells precultured in the absence of XAD. Open symbols: cells precultured in the presence of XAD-16.

Fig. 3. Comparison of epothilone E and F production in dependence of the concentrations of added epothilones A or B, respectively.



The results are mean values of two parallels. Mutant B2, precultured in the presence of resin, was incubated for 24 hours after the addition of epothilones prior to HPLC analysis of the culture supernatant. Protein synthesis was not inhibited.

Fig. 4. Balance of the biotransformation of a total of 3.5 g epothilone A to E.



A three-day old culture of mutant A6 was incubated in the presence of resin for 45 hours. Epothilone A acid (lactone ring opened): solid triangle, and epothilone E acid (lactone ring opened): open triangle.

Mutant So ce90 B2, an epothilone producer strain, was pre-cultivated in the absence of resin and then transferred into epothilone A containing medium. Actually a more polar compound, epothilone E, accumulated in the supernatant, while the concentration of added epothilone A declined. Inhibition of protein synthesis by tetracycline had no effect on this reaction. In contrast, when cells were grown in the presence of resin before start of the experiment, they could not hydroxylate epothilone A (Fig. 2). Nonproducer mutant So ce90 C2 could not convert epothilone to E at all, independent from preculturing with or without XAD resin. When the cells grew however over night in the presence of added epothilone A or B, they became competent too of epothilone E production. Thus apparently the synthesis of an epothilone A/B monooxygenase was induced by the substrates epothilone A or B, respectively, which also occures when cells grow in the absence of resin. Epothilones A/B were produced and simultaneously degraded. The rate of hydroxylation depended on the specific substrate and on substrate concentrations (Fig. 3). Hydroxylation of epothilone A to E was more effective than the hydroxylation of epothilone B to F. In the presence of econazole ($0.5\,mg\,l^{-1}$) the hydroxylation was inhibited by 87%, an indication that the monooxygenase is a P450 containing enzyme[6].

Production of Epothilone E

Epothilones E and F are interesting compounds because hydroxylation of carbon C-21 of the thiazole ring of epothilones A and B had no negative effect on the cytotoxicity and the hydroxyl group opened new strategies for chemical derivatisation[4]. The added epothilone A was converted in all to 93% and from these 74% into epothilone E (Fig. 4). About 16% was degraded completely and could not be detected by HPLC/DAD. Possibly the thiazole ring, which is responsible for the characteristic UV-spectrum, was cleaved. Opening of the lactone ring, a minor side reaction, resulted in epothilone A acid (4%) and E acid (6%) formation (Fig. 6). An esterase activity responsible

for this reaction was detected in the cell-free supernatant of the producer mutant B2 (Fig. 5).

Biotransformation of Epothilone A by *S. cellulosum* Strains

Out of 95 strains of *S. cellulosum*, 30% could not attack epothilone A at all, 35% degraded epothilone completely within two days, and from the culture broth of 35% of the strains we could isolate the typical degradation products which are summarized in Fig. 6. Also these reactions were wide-spread among *S. cellulosum* there were differences with respect to the rate of particular steps. Usually a mixture of products was produced. According to the main products, we can assign *S. cellulosum* strains to 4 groups. So ce90 and 19% of the strains produced epothilone E as main metabolite, *i.e.* the esterase (reactions *b* and *c*) is not very active. The most common reaction, found with 41% of the strains investigated, was the opening of the lactone ring (reaction *b*) to epothilone A acid. Epothilone E-acid was the main product of biotransformation by 18 %. Either epothilone E is here the favoured substrate of the responsible esterase or the hydroxylation reaction from A to E is much faster then the formation of A acid from A. With 22% of the strains compounds **3** and **4** were produced in high and about equal amounts. There is a competition between epothilone A hydroxylation to E and cleavage to A acid. Epothilone E, also a good substrate for the esterase, is then cleaved to E acid (reaction *c*). Reaction *d*), the hydroxylation of epothilone A acid (**3**) to epothilone E acid (**4**) could not be observed.

Fig. 5. Accumulation of epothilone A-acid by an esterase activity in the culture supernatant of mutant B2, given as mean values of two parallels.

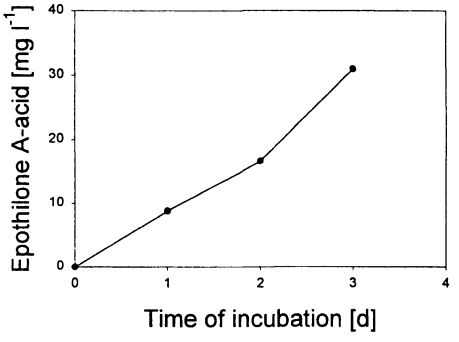
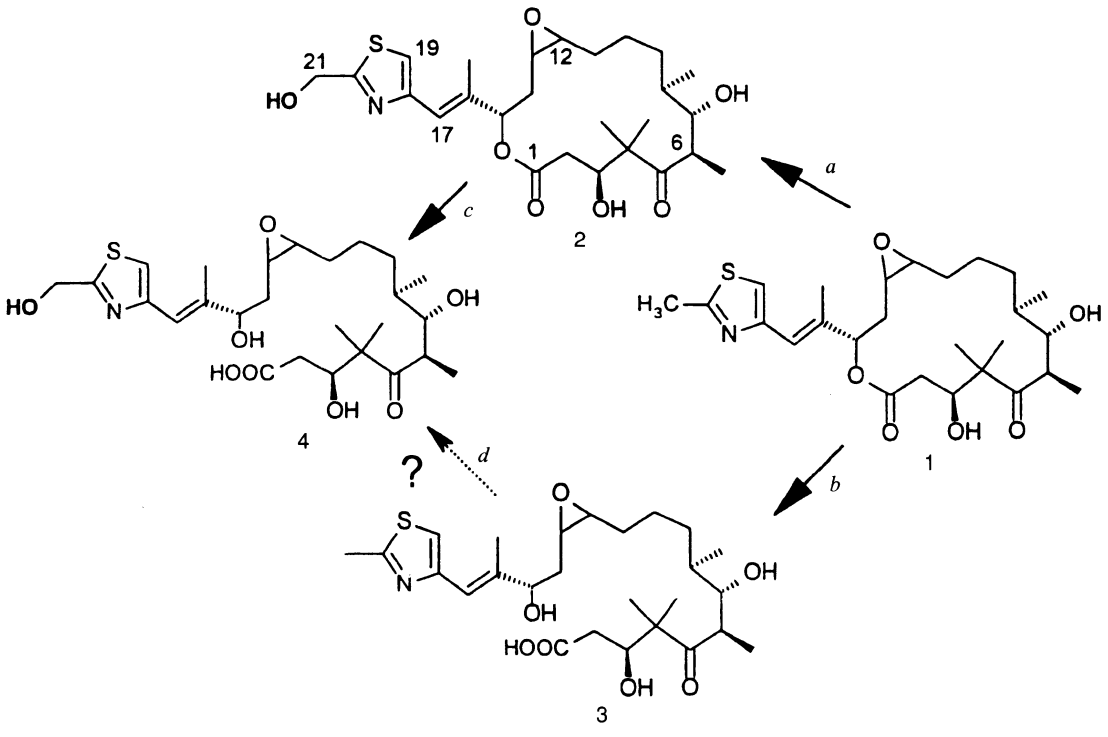


Fig. 6. Flow diagram of steps of epothilone A degradation by strains of *S. cellulosum*.



Compounds: **1**=epothilone A/B; **2**=epothilone E (the epothilone B analog is epothilone F); **3**=epothilone A acid; **4**=epothilone E acid. Reactions: *a* epothilone A/B monooxygenase; *b* epothilone A/B esterase; *c* epothilone E/F esterase; *d* epothilone A/B acid monooxygenase.

The hydroxylation of epothilone A to E is not a reaction specific for epothilone producers, but wide spread among strains of *S. cellulosum*. Epothilones E and F are the first degradation products of epothilone A and B with strain So ce90. Subsequent step of degradation is the opening of the lactone ring (reaction *c*) to carboxylic acid **4**. The general benefit of added adsorber resin for epothilone A and B production is the increase of yield by avoiding endproduct inhibition[2] and degadation.

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