Studies on the Biosynthesis of Epothilones: The PKS and Epothilone C/D Monooxygenase

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Nonproducer mutants support the assumption that epothilones A and B are synthesized by the same polyketide synthase (PKS). The endproducts of the PKS, epothilones C and D, compete for the active site of a constitutively synthesized monooxygenase which is regulated by product inhibition. The postulated C-13 hydroxy-epothilones as direct precursors of epothilones C and D were not detected.

With the aim to find producers of new epothilones or better producers of epothilone A and B1) (Fig. 5), thousands of myxobacteria were screened for epothilone production, but this ability seems to be restricted to strains of Sorangium cellulosum. Even within this species epothilone biosynthesis is a rare feature. Among 1600 strains, we found 39 positive ones which came from soil samples from Europe, Asia, Africa and the Americas. Interestingly these strains differed with respect to their growth and production behavior, which could be used for production optimization. Epothilone A is usually the main and B the minor component, but in a few cases only epothilone A was produced. Very often spirangiens2) or icumazoles3) are simultaneously synthesized and compete with epothilones for common precursors, like acetate or propionate.

The studies on the biosynthesis of epothilones we report in this paper were prerequisites for a directed strain and process improvement which was required for the production of sufficient amounts of epothilones for chemical and clinical research.

Materials and Methods

Strain and Culture Conditions
Sorangium cellulosum strain So ce90, the producer of epothilone which we used for the experiments, and the culture conditions have been described in a previous paper4).

For cultivation in liquid culture, medium E with the following composition was used: Skim milk powder (Sprühmagermilch, Kurhessische Molkereizentrale, Kassel) 0.4% (w/v); soybean meal (Soyamine 50T, Lucas Meyer, Hamburg) 0.4% (w/v); starch (Cerestar SF 12618, Cerestar Deutschland, Krefeld) 1% (w/v); yeast extract (Marcor) 0.2%; glycerol 86% (Ph.Eur., Roth, Karlsruhe) 0.5%; CaCl2·2H2O (Merck, Darmstadt) 0.1% (w/v); MgSO4·7H2O (Merck, Darmstadt) 0.1% (w/v); ethylenediamine tetraacetic acid, iron(III)-sodium salt (Fluka, Buchs, Switzerland) 8 mg/liter; HEPES (Serva, Heidelberg) 1.2%; XAD-16 (Rohm und Haas, Frankfurt/M) 2% (w/v). The medium was adjusted to pH 7.6 with KOH and autoclaved for 30 minutes at 121°C.

Mutants were obtained from S. cellulosum So ce90 by treatment with mutagens to a mortality of 95%.

UV treatment: 10 ml of a culture with 5×10^5 cells per ml were treated with UV for one and a half minutes. Then the culture was incubated overnight in the dark before plating.

NTG treatment: NTG (Fluka) was added to a culture with 5×10^5 cells per ml (final concentration 1 mg/ml). After incubation for 10 minutes the culture was diluted 1:100 and again incubated overnight before plating.

Mutant B2: an overproducer of epothilones A and B isolated from a culture after treatment with UV.

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Mutant C2: a nonproducer mutant isolated from a culture treated with NTG.

Mutant D48: produces epothilones C and D as main compounds. This mutant was isolated from a culture after treatment with UV.

Measurement of de novo Biosynthesis

Samples (10 ml) taken from 4 days old precultures of mutant B2 grown in the presence of XAD-16 were incubated overnight at 30°C in the absence of resin but with 10 µCi 1-14C-propionic acid sodium salt (Sigma, 1-10 mCi per mmol) and epothilones as indicated (Fig. 1). In order to harvest the epothilones, the cultures were incubated for 1 hour in the presence of 2% resin. After separation from the cells by sieving, the resin was washed with water and extracted with methanol. The eluates were evaporated to dryness and dissolved in 0.5 ml methanol. The epothilones were then purified by HPLC. Aliquots of the epothilones A or B fractions were pipetted into scintillation vials. After addition of 5 ml of liquid scintillator (Ready Safe™, Beckman) the radioactivity was determined in a liquid scintillation counter (Beckman LS6500).

Epothilone C Binding Studies

To differentiate between active transport and passive binding processes two parallel cultures of mutant C2 were gassed with oxygen or nitrogen for 15 minutes before 100 mg/liter of epothilone C was added. Epothilone C was determined by HPLC after centrifugation of samples by direct injection of 5 µl of supernatant. To determine the concentration at time zero, epothilone C was given to medium without cells and measured after 5 minutes of incubation.

In Vivo Assay of Epothilone C/D Monooxygenase

To avoid interference with epothilone C/D biosynthesis, the experiments were performed with the non-producer mutant C2 grown for 4 days in medium E in the presence of XAD-16. To get rid of the resin, the supernatant was decanted and the culture centrifuged at 15,000 r.p.m. If required, protein biosynthesis was inhibited by preincubation of the culture without resin for 1 hour in the presence of 20 µg/ml tetracycline prior to centrifugation. The pellet was resuspended in 1/5 volume of the supernatant. Tests were performed with 5 ml cell suspension in 100 ml Erlenmeyer flasks and the added compounds. For analysis, twice 0.5 ml cultures were pipetted into Eppendorf tubes with 0.05 ml resin and incubated for 1 hour. Then the resin was harvested, the epothilones extracted with 0.5 ml methanol and analyzed by HPLC.

HPLC Analysis of Epothilones

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, Duren) was used. Temperature was 40°C, flow rate 0.5 ml/min, solvent water-acetonitrile (60:40) isocratic for 6 minutes and a gradient to 100% acetonitrile from 6 to 7 minutes, detection at 250 nm.

Results and Discussion

Mutants of S. cellulosum So ce90

In the course of strain improvement 24,000 clones were screened by HPLC analysis after UV or NTG treatment. More than 50 non-producing mutants which are probably defective in the PKS or the regulatory region, have been isolated. One of which, named C2, was used for further studies. The fact that all these mutants had lost their potential of epothilone A and B synthesis simultaneously supports the hypothesis that both are synthesized by the same PKS (Fig. 5) that means, the responsible keto acyl synthase is unspecific for malonyl-CoA or methylmalonyl-CoA binding.

Regulation of Epothilone Biosynthesis

The effect of epothilones A and B on de novo synthesis in strain So ce90 B2 was investigated by measuring the incorporation of radioactive propionate into epothilones. Results are shown for epothilone A only (Fig. 1). Fig. 1 as the effects of epothilone B were the same. While the radioactivity of isolated epothilones A and B still increased a little when 100 mg/liter epothilone A was added, concentrations of 500 mg/liter were inhibitory and reduced yields of de-novo synthesis to 60-70 percent of the control cultures. At the same time the production of epothilones C and D, which were detected in the control culture only in traces, increased dramatically.

We assume that epothilones A and B are endproduct inhibitors of their own synthesis, and that therefore the direct precursors, epothilones C and D with a double bond between C-12 and C-13 (Fig. 5), accumulated. To detect C-13 hydroxy-epothilones (Fig. 5) which were postulated as endproducts of the PKS and precursors of epothilones C/D by Molnar et al. and Tang et al., we also studied the de novo synthesis of epothilones with mutant D48 the
Fig. 1. The effect of epothilone A on de-novo synthesis of epothilones.

Epothilones A and B are measured by the incorporation of radioactive 1-14C-propionate and shown as percentage of the control culture without epothilone A. The effect on epothilone C and D synthesis was determined from the area of absorption in the HPLC and is shown in % mAU as percentage of the control culture. The results are mean values of two parallels.

producer of epothilones C/D. In analogy to the feed back inhibition of the monooxygenase described above, we hoped to detect C-13 hydroxy-epothilones as precursors of C/D in the presence of 500 mg/liter of the endproduct epothilone C. Radioactivity from 1-14C propionate was found only incorporated into epothilones C/D. This suggests that epothilones C and D are the true endproducts of the PKS and are later modified to epothilones A and B by a post-PKS modifying enzyme (Fig. 5).

The Epothilone C/D Monooxygenase

When epothilones C or D were fed to cultures of S. cellulosum mutant C2 we had troubles with their recovery from the supernatant. In contrast to epothilones A/B, only a small percentage of added epothilones C/D was found in the supernatant. Rather, the compounds had to be isolated from the bacterial cells. The compounds were bound very fast, almost independently from temperature, even under anaerobic conditions, and proportional to the cell mass. Within 5 minutes of shaking after the addition, the equilibrium was established.

Similar effects were seen, when we used cells of Sorangium strains that were not epothilone producers. We assume that unspecific hydrophobic interactions between epothilones C/D, which are more lipophilic than A/B, and the cell surface are responsible for this adsorption.

Epothilone A is a feedback inhibitor of epothilone B formation, and vice versa (Fig. 3). When epothilones C/D were added simultaneously, both substrates competed for the same binding site on the enzyme (Fig. 4). An increase in epothilone D concentration reduced epothilone A production and simultaneously increased the concentration of epothilone B in the supernatant. If equal amounts of epothilones C and D were added, more epothilone A was produced than B. This can be explained
Fig. 3. Effect of increasing concentrations of epothilone A on the epoxidation of 100 mg/liter epothilone D to epothilone B by mutant C2.

Parallel samples were taken and epothilones harvested by adsorption on XAD-16 resin added 1 hour after start of the reaction.

Fig. 4. Epoxidation of epothilone C and D by the non-producer mutant C2.

Circles: Epo A₁, Epo B₁ = epothilones A and B produced when 200 mg/liter epothilone C and 100 mg/liter D were added simultaneously; triangles: Epo A₂, Epo B₂ = epothilones A and B produced when epothilones C and D were added 200 mg/liter each; The values are mean values of two parallel samples.

Fig. 5. Steps of epothilone biosynthesis.

Numbering of atoms is given for epothilone C. Carbon C-13 hydroxy compounds are shown as intermediary products of the PKS only. Bonds printed in bold type show the incorporation of an acetate or propionate unit in the growing polyketide which results in epothilone C or D formation.

by differences in the affinity to the monooxygenase. Even small structural changes may cause significant changes in the affinity. For example, 6-demethyl epothilone C, one of the trace variants isolated from large scale fermentations⁸, was not epoxidised. When we investigated the epoxidation of epothilone C to A without and in the presence of 6-demethyl epothilone C we found neither an effect on epothilone C epoxidation nor the formation of 6-demethyl
epothilone A. Loss of the methyl group on C-6, which is far away from C-12 and C-13, prevents epoxidation. In contrast to mutant C2, which can not produce epothilones at all, but has the monoxygenase for the epoxidation of C/D, mutant D48 is a block mutant with a defective monoxygenase and is of interest as producer of epothilones C and D.

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References