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Studies on the Biosynthesis of Epothilones: The Biosynthetic Origin of the Carbon Skeleton

KLAUS GERTH*, HEINRICH STEINMETZ†, GERHARD HÖFLE† and HANS REICHENBACH

GBF, Gesellschaft für Biotechnologische Forschung mbH,
Abteilung Naturstoffbiologie, † Abteilung Naturstoffchemie
Mascheroder Weg 1, D-38124 Braunschweig, Germany

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The biosynthetic origin of the epothilone skeleton was studied by the incorporation of ^{13}C and radioactively labeled precursors by *Sorangium cellulosum* So ce90. The carbon atoms are derived from acetate, propionate, the methyl group of *S*-adenosyl-methionine, and cysteine which also introduces the sulfur and nitrogen atoms. Epothilone biosynthesis starts with the formation of the thiazole part from acetate and cysteine. The incorporation of acetate or propionate units results in the formation of epothilones A and B, respectively. To introduce the epoxide function of epothilones A and B molecular oxygen is used.

The epothilones were originally isolated by us in 1988 from culture extracts of *Sorangium cellulosum* strain So ce90[1] and were patented for their antifungal and cytotoxic activities[2]. The potential of epothilones as anticancer agents became apparent when their tubulin stabilizing activity, comparable with that of paclitaxel (Taxol®), was detected in 1995[3]. In the ensuing race several groups independently worked out total synthesis and used epothilone as a lead compound for the synthesis of a large number of analogs published during the last few years[4].

In contrast, we used our knowledge of the biology of myxobacteria and produced epothilones A and B by large scale fermentations. The epothilones were then modified extensively by chemical means[5~8] as well as by biotransformation.

Besides the main components, epothilones A and B, also the minor components, epothilones C, D, E and F were obtained during large scale fermentations[9,10]. In addition 36 trace components were isolated from fermentation broths and their structures elucidated[11]. Knowledge of the precursors of epothilones were a prerequisite for optimization of media and development of feeding strategies. The base sequences of the biosynthetic gene clusters of two different epothilone producers and the deduced functions of the respective polyketide synthases which have just been claimed[12] and published[13] now allow a comparison with the results of our studies on the biosynthetic origin of the carbon skeleton of epothilones and the direction of biosynthesis.

Materials and Methods

Strain and Culture Conditions

Sorangium cellulosum strain So ce90, the producer of epothilone which we used for the experiments, has been described elsewhere[1]. Mutants derived from this strain by UV treatment are So ce90B2, an overproducer of epothilones and So ce90 A6 which only produces epothilone A. 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% ; $CaCl_2 \cdot 2H_2O$ (Merck, Darmstadt) 0.1% (w/v); $MgSO_4 \cdot 7H_2O$ (Merck, Darmstadt) 0.1% (w/v); ethylendiamine 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. The cultures were incubated at 30°C on a rotary shaker at 180 r.p.m. and

analysed for epothilones after 18 days.

Feeding Experiments

^{13}C-Labeled Precursors

Mutant A6 was grown in 200 ml medium E in 1 liter Erlenmeyer flasks for 2 days. Then the resin-free supernatant was transferred into a sterile 1 liter Erlenmeyer flask, and fresh resin (2%) was added together with the filter-sterilized aquous solution of [^{13}C]-labeled precursors (Cambridge Isotope Laboratories) (Table 1). After 2 days of cultivation the adsorber resin was harvested and extracted exhaustively with methanol. The extracts were evaporated to dryness and dissolved in ethyl acetate to give a refined extract. Isolation of pure epothilones A and B was achieved by preparative HPLC on a Nucleosil C18, 7 μm, column (20×250 mm) with methanol/water (6:4) as eluent. Detection was at 254 nm.

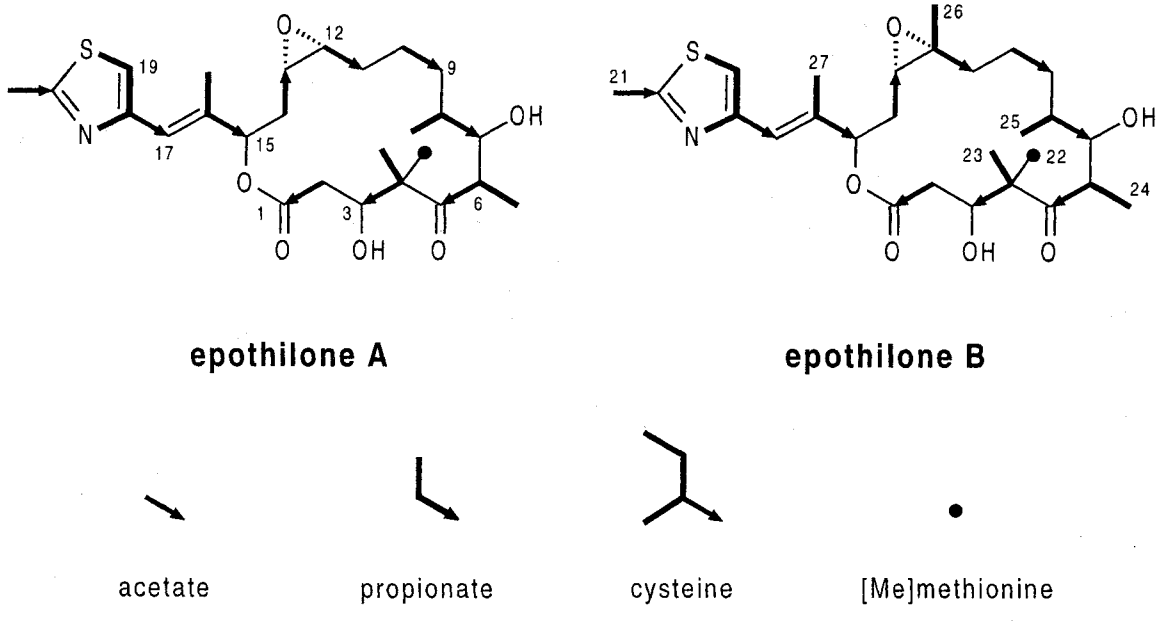
$^{18}O_2$

The conditions were as described above, but after transfer to a fresh flask the culture was gassed with nitrogen for 5 minutes before 50 ml of $^{18}O_2$ gas (Cambridge Isotope Laboratories) diluted with 50% $^{16}O_2$ was added with a syringe. The flask was sealed with parafilm before incubation.

Table 1. Amounts of precursors fed and epothilones A and B isolated.

Precursor	% Labeled	Amount fed	Isolated[mg]	
			Epo. A	Epo. B
Na[1,2-$^{13}C_2$]acetate	99	200 mg	2.3	0.3
Na[1-^{13}C]propionate	97-98	200 mg	5.2	1.5
[$^{13}CH_3$]methionine	99	100 mg	6.4	2.5
Na[2-^{13}C]propionate and $^{18}O_2$	99 95-98	200 mg 50 ml	0.5	0.4
$^{18}O_2$	95-98	50 ml	0.1	0.4

Fig. 1. Biosynthetic origin of the carbon skeleton of epothilones A and B.



[^{35}S]-Cysteine

Culture supernatant (10 ml) of a 4 day old preculture of So ce90 B2 grown in the presence of resin was pipetted into a sterile 100 ml Erlenmeyer flask. Protein synthesis was stopped by the addition of 20 μg/ml of tetracycline one hour before 10 μCi of [^{35}S]-cysteine (Amersham) was added. After 6 hours of incubation at 30°C on a rotary shaker, 1% of XAD-16 was added. The resin was harvested one hour later, eluted, and epothilone A isolated by HPLC. The radioactivity was measured with a scintillation counter.

Results and Discussion

Epothilones A and B are polyketides synthesized from acetate and propionate units, cysteine and one methyl group from methionine. The direction and sequence of biosynthesis can be derived from the labeling experiments, the arrowheads indicate carbon 1 of the respective precursors (Fig. 1). Together with the chemical structure of epothilone these experiments allow to predict the number of hypothetical modules of the responsible polyketide synthase (PKS) and the required enzymatic steps. These

Fig. 2. Incorporation of ^{35}S from labeled cysteine into metabolites adsorbed on XAD resin and into purified epothilone A.

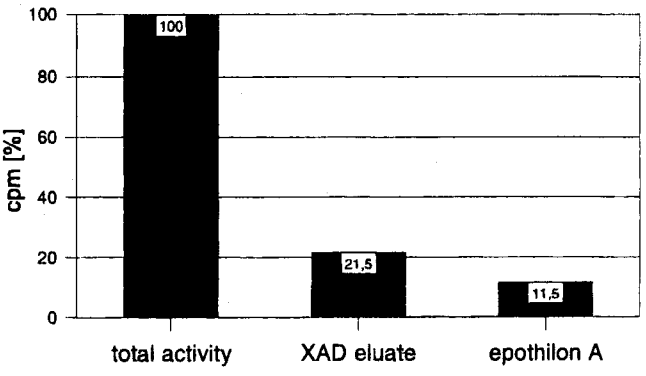


Table 2. ^{13}C NMR chemical shifts assignment of epothilone A (1) in CD_3OD, ^{13}C-labeling and ^{13}C-^{13}C coupling patterns in (1) biosynthesized from ^{13}C-labeled precursors.

carbon	δ (ppm)	[1,2-$^{13}C_2$]acetate rel. intensity	$J_{C,C}$ (Hz)[a]	[1-^{13}C]propionate relative intensity	[$^{13}CH_3$]methionine relative intensity
1	172.2	8.0	58.8	1	1
2	40.0	12.0	58.5	1	1
3	73.1	7.0	s	33	1
4	54.6	6.0	s	1	1
5	219.9	6.5	s	31	1
6	46.8	7.0	s	1	1
7	78.0	8.5	s	37	1
8	37.3	7.5	s	1	1
9	30.8	9.0	35.3	1	1
10	25.1	9.0	35.2	1	1
11	33.4	10.0	45.9	1	1
12	56.4	10.0	46.1	1	1
13	58.9	7.5	45.1	1	1
14	28.4	10.0	45.0	1	1
15	78.1	6.0	s	34	1
16	139.7	8.0	s	1	1
17	120.6	1	-	1	1
18	153.2	1	-	1	1
19	117.7	1	-	1	1
20	167.0	11.0	54.7	1	1
21	18.7	14.0	54.5	1	1
22	23.1	1.0	s	1	49
23	20.9	7.0	s	1	3.3
24	16.7	8.0	s	1	1
25	18.4	9.0	s	1	1
26	-	-	-	-	-
27	15.3	6.5	s	1	1

[a] Singlets (s) accompanied by doublets due to scrambling and incorporation of acetate into propionate units.

are, *e.g.*, ketosynthase, ketoreductase, dehydratase, enoylreductase, and methyltransferase[14].

Formation of the thiazole heterocycle from acetate (C20-C21) and cysteine are the first steps of epothilone biosynthesis by *Sorangium cellulosum* strain So ce90, which also worked when protein synthesis was inhibited by tetracycline. The incorporation of radioactivity from [^{35}S]-cysteine into epothilone A (Fig. 2) suggested that cysteine is actually the biosynthetic origin also of carbons C-17, C-18, C-19 which were not labeled by acetate or propionate (Tables 2, 3).

A combination of a non-ribosomal peptide synthetase (NRPS) and PKS has just been described as responsible for the formation of the thiazole ring of myxothiazol[15] and for glycine incorporation into antibiotic TA[16].

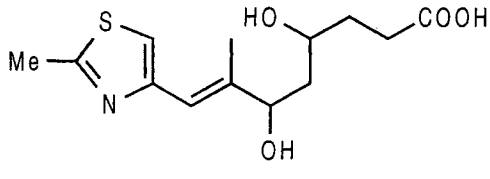
During the next two steps of biosynthesis a propionate

Table 3. ^{13}C NMR chemical shifts assignments of epothilone B (2), ^{13}C, ^{18}O-labeling and ^{13}C-^{13}C coupling patterns in (2) biosynthesized from ^{13}C-labeled precusors.

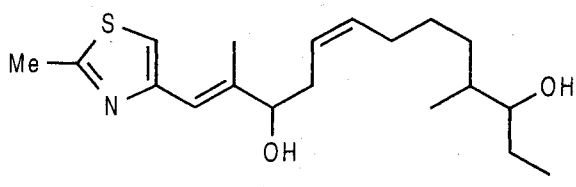
carbon	δ (ppm)	[1,2-$^{13}C_2$]acetate rel. intensity	$J_{C,C}$ (Hz)[a]	[1-^{13}C]propionate rel. intensity	[2-^{13}C]propionate and $^{18}O_2$ rel. intensity	[$^{13}CH_3$]methionin rel. intensity
1	172.1	8.5	57.9	1	1	1
2	40.2	12.5	58.0	1	1	1
3	72.6	8.0	s	28	4.0	1
4	54.8	6.0	s	1	9.5	1
5	220.1	6.0	s	31	3.0	1
6	46.3	6.0	s	1	8.0	1
7	77.7	8.5	s	25	3.0	1
8	37.4	8.0	s	1	16.0	1
9	31.0	12.5	34.8	1	1	1
10	24.4	12.5	34.8	1	1	1
11	33.4	5.5	s	25	3.0	1
12	63.2	6.0	s	1	14.0[b]	1
13	63.8	10.0	45.0	1	1	1
14	34.2	10.0	45.2	1	1	1
15	78.3	6.0	s	27	3.0	1
16	139.6	1	-	1	19.0	1
17	120.8	1	-	1	1	1
18	153.2	1	-	1	1	1
19	117.8	1	-	1	1	1
20	167.0	18.0	54.6	1	1	1
21	18.7	16.0	54.5	1	1	1
22	19.9	4.5	s	1	1	48
23	23.0	8.0	s	1	6.0	3.6
24	16.5	7.0	s	1	5.5	1
25	18.5	9.0	s	1	6.0	1
26	22.6	6.0	s	1	7.0	1
27	15.3	5.0	s	1	8.0	1

a) Singlets (s) accompanied by doublets due to scrambling and incorporation of intact acetate units into propionate units.
b) Accompanied by a ^{13}C-12 ^{18}O signal of 30% rel. intensity at $\Delta\delta - 0.035$ ppm.

Fig. 3. Structures of trace compounds **38** and **40**, from epothilone fermentation.



38



40

and an acetate unit (C-16 to C-13) are condensed. The substrate specificity of the subsequent loading module is decisive for epothilone A or B formation. Incorporation of acetate (C-11, C-12) results in epothilone A, and of propionate (C-11, C-12, C-26) in epothilone B formation. The results of our labeling experiments disprove the speculation of a post-PKS methylation of C-12 to B[13]. With the exception of methyl group C-22 on C-4, which has its biosynthetic origin in the methyl group of methionine (Tables 2, 3), all other methyl branches are derived from C-3 of propionate and were not labeled by methionine. A methyltransferase (MT) domain, which is believed to be responsible for the methylation of C-4, was found integrated into module 7 (numbering of modules according to MOLNAR *et al.*[13]). Simultaneous feeding of [2-^{13}C]-propionate and $^{18}O_2$ proved that the epoxide of the epothilones is introduced by an oxidative step from molecular oxygen (Table 3). This means, that the precursor should have a double bond between carbons C-12 and C-13. Results from sequencing the PKS gene cluster[13,14] show however clearly that there is no dehydratase (DH) sequence present in the module 3, responsible for elaboration of this ketide unit. Rare functional inaccuracies of the PKS result in products which could be isolated from fermentation broths and gave some insights into this reaction (Fig. 3). Compound **38**, set free by a mistake occuring in module 4, has a hydroxy group at position C-13 which is consistent with the finding that module 3 had no DH domain. Compound **40** is liberated from the PKS as a result of a defect which occured in module 6. In contrast to **38** we find here a double bond at the corresponding position. Perhaps one of the dehydratases of modules 1, 4 or 5 are reponsible for this dehydration. Thus the results from sequencing the epothilone gene cluster and from our labeling experiments are in good agreement. The structures of two byproducts **38** and **40** support the hypothesis of TANG *et al.*[12] that dehydration could also be performed by one of the neighbouring modules of the PKS resulting in an atypical reaction sequence.

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