DOI: 10.1002/cmdc.201500138

Biocatalysis at Work: Applications in the Development of Sagopilone

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For the antitumour agent sagopilone, an epothilone analogue, a large-scale synthesis was developed to synthesise the active pharmaceutical ingredient for clinical trials, exploring enzymatic and microbial methods to produce chiral building blocks on a multi-kilogram scale. The three building blocks were identified as key intermediates in the synthesis and needed to be produced with high optical purity in yields higher than those previously published. The improved syntheses of two of these building blocks are detailed herein. For building block A, the chemical research synthesis was abandoned, and a novel chemical route was developed leading to building block A via an enzymatic hydrolysis process. For building blocks C, replacement of a chemical catalytic procedure by a microbial process meant that the development of a new starting material could be avoided, thereby accelerating the development process. For the clinical development process, a human metabolite of sagopilone was required as a reference. To accelerate the synthesis of the metabolite, no chemical synthesis was investigated; rather, we relied solely on oxidoreductases. The human metabolite of sagopilone was synthesised on a multi-gram scale in a single-step process using genetically engineered *E. coli* expressing human cytochrome P450 enzyme 2C19. The integration of enzymatic and microbial processes provided tools that enable the synthesis of highly functionalised intermediates and metabolites.

Introduction

Searching the rich sources of nature to isolate and analyse novel compounds as candidates for pharmacological treatments is well established.[1] In the area of cancer therapy, a plethora of potent anticancer compounds have been isolated from natural sources[2] such as plants (e.g., paclitaxel from *Taxus brevifolia*)[3] and microorganisms (e.g., epothilones from *Sorangium cellulosum*, a myxobacterium strain).[4]

In addition to the exploitation of biological systems as a source of new bioactive lead compounds, microorganisms or isolated enzymes are widely used to perform chemical transformations as an alternative to traditional chemical catalysts.[5] There are many examples of the use of these biological tools, either as whole cells[6] or isolated enzymes.[7] In addition to originating from a renewable source, biological catalysts have the advantage of being highly selective[8] and exceptionally beneficial for introducing chirality into functionalised molecules.[9] Biotransformations have thus gained significant importance in industrial processes.[5g]

Epothilone B (**1**, Figure 1) has shown strong antiproliferative activity, but is accompanied by considerable toxicity in animal models at therapeutic doses.[10] Therefore, a research program aimed at identifying an optimised analogue as a development candidate was initiated in our company in 1997.[11] Although epothilone B is derived from a natural source, our research program was not limited to accessing new derivatives by semisynthesis, but instead it took a total synthesis approach. After the de novo synthesis of more than 350 epothilone analogues, sagopilone (ZK-EPO, **2**; Figure 1) was selected as the development candidate based on its most beneficial overall pharmacological profile.[11b] The synthesis of **2** was subjected to process optimisation and scale-up to provide the active pharmaceutical ingredient for clinical trials.[12] In phase II clinical studies, sagopilone was found to be highly efficacious in first- and second-line treatments of ovarian cancer,[13] prostate cancer,[14] melanoma,[15] and glioblastoma.[16]

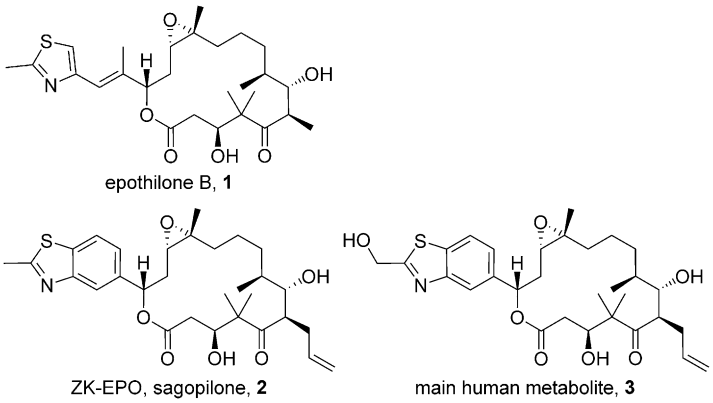

Figure 1. Epothilone B and analogues.

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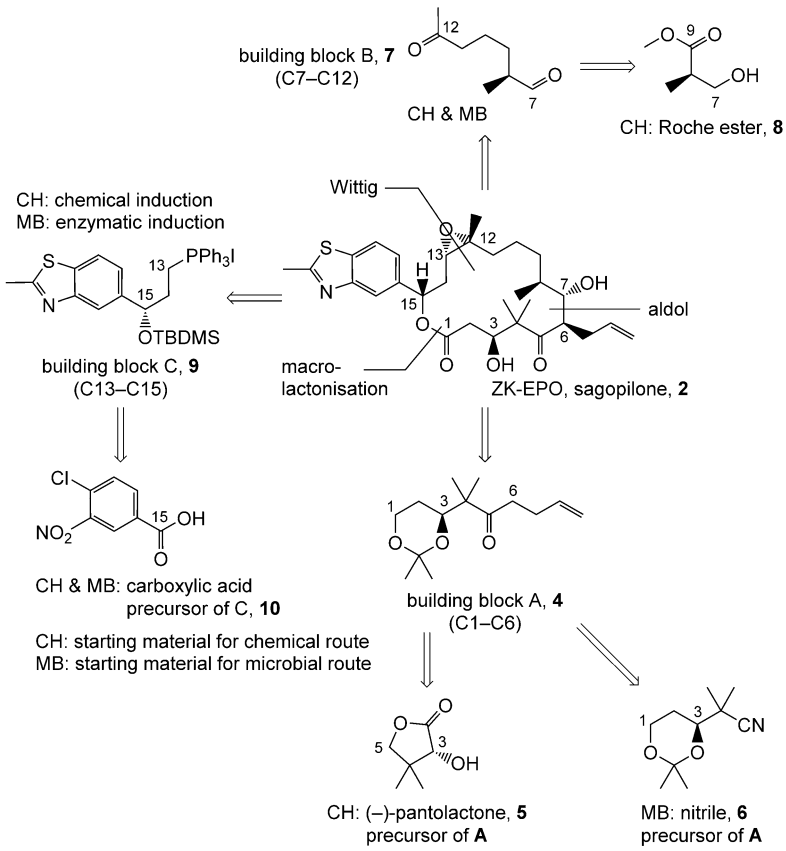


Scheme 1. Retrosynthetic analysis of sagopilone (**2**), with the resulting building blocks A, B, and C.

Scheme 1 depicts the strategic bond formations and resulting building blocks that were used for the development process, as reported elsewhere.[12d] Key reactions for the coupling of building blocks involved an aldol reaction (formation of the C6–C7 bond), a Wittig olefination (formation of the C12–C13 olefin as a precursor for the epoxide moiety), and a macrolactonisation (ring closure).[12] All three building blocks A–C contain at least one stereogenic centre. Chemical and biocatalytic approaches were investigated in parallel in order to develop robust and economical syntheses for these building blocks. Details of the biocatalytic approaches are described herein.

In addition to implementing an efficient and scalable synthetic route to sagopilone, we were also faced with the need to provide gram quantities of its main human metabolite **3** (Figure 1). Traditionally, metabolites are synthesised via the same synthetic sequence as their parent, except that a different starting material or a modified intermediate with an additional functionality would be selected.[17] If this strategy was applied here, more than 37 steps for the synthesis of metabolite **3** would be required![12d] Clearly, such an approach would be time-consuming and uneconomical. Therefore, we relied on a biotransformation of sagopilone (**2**) to directly access its main human metabolite **3** in a single chemical transformation.

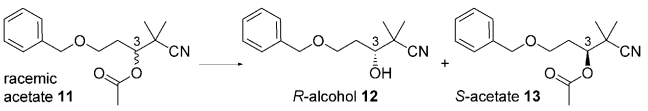
Results and Discussion

Preparation of building block A

An alternative access was required for the synthesis of building block A, as the research approach using optically active (−)-pantolactone (**5**) as the exchiral-pool starting material was not applicable for a development approach due to the use of hazardous reagents in several steps.[12d] A novel approach was therefore developed based on nitrile **6** (Scheme 1).[12d] This precursor was then transformed into building block A by a sequence of chemical transformations.[18]

First, however, the optically active nitrile precursor **6** had to be synthesised. To introduce the chirality, we relied on the enzymatic kinetic resolution of racemic acetate **11** (Table 1).[19] More than 20 enzymes were screened, and the lipase AY30 from *Candida rugosa* (EC 3.1.1.3, Amano) was found to be highly selective, providing an easy route to the desired *S*-enantiomer as acetate **13**.[20]

Table 1. Enzymatic kinetic resolution by hydrolysis of acetate **11** using *Candida rugosa* lipase AY30 (Amano).[a]

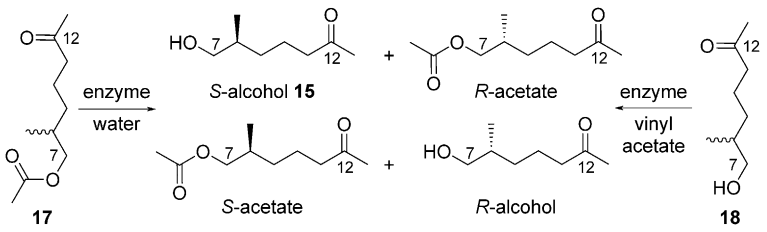


Incubation time [h]	*ee* [%][b] *R*-alcohol **12**	*ee* [%][b] *S*-acetate **13**	Yield [%][b] *R*-alcohol **12**
5	76	11	3
24	89	16	10
48	91	91	11
168	88	98	23

[a] Conditions: acetate **11** (1 mg), ethanol (50 μL), phosphate buffer (66 mM, pH 7, 1 mL), 37 °C, 1000 rpm. [b] Determined by chiral HPLC.

Even after days, only trace amounts of the *S*-acetate **13** were hydrolysed; thus, a rapid reaction workup was not required, minimising the possibility of a decreased yield of **13**. Without enzyme, acetate **11** was not hydrolysed. In the next step, the reaction needed to be performed on a gram scale. In the development process, higher concentrations ($>1\ g\ L^{-1}$) of acetate **11** were required, and additives to dissolve the acetate needed to be minimised. It was established that, at 40 °C, acetate **11** was soluble in the buffer without any additional solvent re-

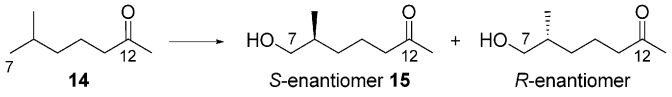
quired. The enzymatic hydrolysis could be performed at this temperature with the same excellent stereoselectivity and yield as before. Thus, to increase throughput, the temperature was raised to 40 °C which allowed an increase in the concentration of racemic acetate **11** to 40 g L^{-1}.[20] In bulk quantities, the lipase was supplied as a granulated product (lipase AYS, Amano) instead of a powder; nevertheless, similar results were obtained. To supply sufficient material for the initial development process, a 45-kg batch of the racemic acetate **11** in a buffer at pH 6 (actively controlled by the addition of 1 M sodium hydroxide solution) at 40 °C was hydrolysed to yield, after chromatography, 22 kg (yield: 98%, theoretically achievable maximum yield of 50%) of the *S*-acetate **13** with >99% *ee*.



Scheme 4. Enzymatic kinetic resolution by hydrolysis of acetate **17** and acetylation of alcohol **18**.

Preparation of building block B

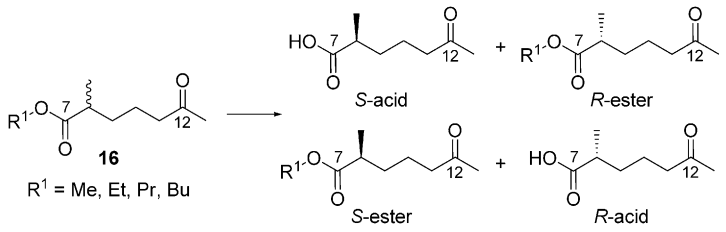
The chemical research synthesis of building block B also began with an ex-chiral-pool starting material, the well-known (−)-Roche ester **8**.[12d] During the development process, novel chemical and biocatalytic approaches to the *S*-enantiomer of building block B were evaluated. Initially, a biooxidation screening was performed to explore the option of transforming the commercially available ketone **14** directly into the desired *S*-enantiomer of keto alcohol **15** (Scheme 2). More than



Scheme 2. Stereoselective hydroxylation of ketone **14**.

135 strains from our corporate collection of fungi and bacteria were screened without success. This screening was hampered by the volatility of ketone **14**.

Next, the enzymatic kinetic separation of four racemic esters **16** was explored (Scheme 3).[21] These esters were accessed by



Scheme 3. Enzymatic kinetic resolution by hydrolysis of esters **16**.

oxidative ring opening of the inexpensive commercially available 2,6-dimethylcyclohexanone with, for instance, potassium permanganate and subsequent esterification under standard conditions.[22] A screening with 30 commercially available esterases and lipases was performed, but none of the enzymes showed a sufficient turnover rate with useful enantiomeric ratios. Therefore, this approach was terminated.

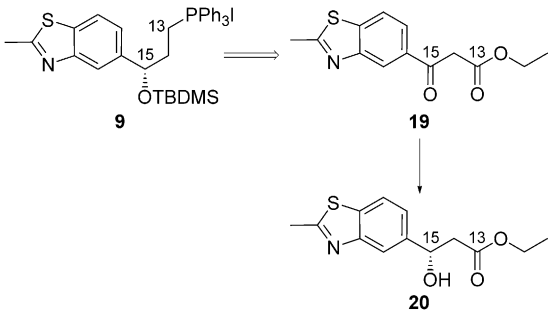
As the enzymatic kinetic resolution of esters **16** was unsuccessful, we turned to the enzymatic kinetic resolution of racemic acetate **17** and to acetylation of the corresponding alcohol **18** (Scheme 4).[23] The hydrolysis option was investigated with more than 60 enzymes, with the best result being obtained at pH 6 with lipase G50 from *Penicillium camembertii* (EC 3.1.1.3, Amano); however, only a moderate 46% enantiomeric excess of *S*-alcohol **15** was achieved. Acetylation of **18** with, for example, vinyl acetate was not selective, and this approach was abandoned. In the meantime, an improved chemical synthetic route starting from Roche ester **8** had been developed,[12d] so the search for an alternative enzymatic process was discontinued.

Preparation of building block C

In building block C a stereogenic centre substituted with a hydroxy group, protected as a silyl ether, was needed which was initially derived from carboxylic acid precursor **10** (Scheme 1). This precursor was first converted into the corresponding benzothiazole, and then the acid functionality was transformed into an aldehyde group. The aldehyde was subjected to an Evans aldol reaction to access diastereomeric oxazolidinone-based β-hydroxy amides.[12d] Isolation and purification of the desired stereoisomer were hampered by limited stereoinduction and facile epimerisation of the oxazolidinone stereocentre under deprotonation conditions. Therefore, a new approach was devised which avoided the Evans aldol step and instead used β-keto ester **19** as the key intermediate (Scheme 5).

Chemical as well as biocatalytic options were investigated for achieving the enantioselective reduction of **19** to the *S*-alcohol **20**. Asymmetric hydrogenation and transfer hydrogenation were hampered on a large scale by the fact that more than 1% of an expensive catalytic system [Ru/(*R*)-Xyl-SOLPHOS] was needed to obtain a highly enantio-enriched product.[12d] Additionally, and even more troubling, poisoning of the catalyst by traces of sulfur impurities, originating from the synthesis of the benzothiazole moiety, led to unreproducible results and thereby placed scale-up campaigns at risk.

Hence, the alternative biocatalytic approach for accessing β-hydroxy ester **20** became the last resort. The most economical process to introduce the required functionality is the stereoselective reduction of the keto group of **19**, as up to 100% of



Scheme 5. Building block C: retrosynthetic analysis and stereoselective reduction of the β-keto ester **19**.

the desired enantiomer is possible.[24] In contrast, in the case of enzymatic resolution of racemic hydroxy esters, for example, only up to 50% yield can be obtained, necessitating inversion of the undesired stereocentre to increase the overall yield.[25] Another possibility for obtaining up to 100% yield is through a dynamic kinetic resolution process, but special molecular features are required.[26] Therefore, we focused on establishing a direct biocatalytic reduction of β-keto ester **19**.

For a large-scale microbial reduction process, it is not sufficient to only achieve a high turnover rate and high enantiomeric purity of the desired enantiomer. In addition, ease of product isolation is equally important which, in whole-cell biotransformations, is sometimes hampered by the formation of lipophilic byproducts. Thus, a microorganism was needed which allowed high turnover and product isolation by simple extraction and crystallisation steps.

Toward this end, an explorative screening of 30 yeast strains was conducted. Pure samples of both enantiomers of the β-hydroxy ester product were synthesised by alternative methods to allow direct HPLC-based analyses of the screening samples. In all cases, β-keto ester **19** was nearly completely consumed after 24 h, and, in most cases, the *S*-enantiomer **20** was detected as the main enantiomer formed; however, degradation of product **20** was observed with certain strains if incubation was continued for 72 h.

After these encouraging first results, the screening was expanded, and more than 300 strains of yeasts and anaerobic bacteria from our corporate collection of microorganisms were screened. We began with a substrate concentration of 100 and 200 mg L^{-1} of β-keto ester **19** for yeasts and anaerobic bacteria, respectively. For promising hits from this initial screening, fermentation conditions were further optimised with a focus on substrate concentration and the testing of various fermentation media. Several subsets of strains were incubated in different media and with β-keto ester **19** at 200–10000 mg L^{-1}. The best results were achieved with a medium consisting of 50 g L^{-1} glucose and 20 g L^{-1} corn steep liquor.[27] Our goal was to establish high turnover and enantioselectivity with substrate concentrations >1 g L^{-1}.

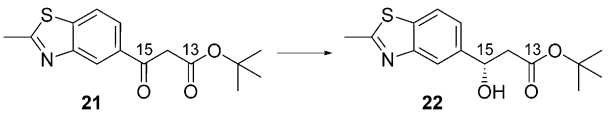
Four strains showed good to excellent preference for formation of the *S*-enantiomer **20**. *Issatchenkia orientalis* strains needed more than 24 h to consume more than 1 g L^{-1} of starting material **19**. Even after 72 h at a concentration of 2 g L^{-1}, 1–4% of the starting material could still be detected in the fermentation mixture.

With *Pichia* strains, the β-keto ester **19** was reduced nearly completely, even at substrate concentrations as high as 2 g L^{-1}. *Pichia wickerhamii* (IFO 1278) was identified as a highly productive biocatalyst for the desired transformation, as 2 g L^{-1} of substrate **19** were reduced within 24 h, and the product **20** was obtained with $>99\%$ *ee*.

In a next step, we studied further substrate concentration increases with this yeast strain. In all samples, the β-keto ester **19** was reduced exclusively to the *S*-enantiomer with $>99\%$ *ee*. At a concentration of 4 g L^{-1} all of the β-keto ester **19** was reduced, but at a concentration of 6 g L^{-1} and higher, more than 20% of the β-keto ester was still detected after 72 h. Therefore, we opted to use 4 g L^{-1} in a shake-flask fermentation process. In this way, β-keto ester **19** (4 g) was incubated for 114 h. The culture broth was then extracted with ethyl acetate, and the extracts were purified by column chromatography, which gave the *S*-enantiomer **20** in 78% yield (3.14 g) with $>99.5\%$ *ee*.

After this favourable result, the fermentation process was scaled up further. In one of the first batches, the β-keto ester **19** (109 kg) was incubated with *P. wickerhamii* (IFO 1278) in a 50 m^3 fermenter. β-Hydroxy ester **20** was extracted with methyl isobutyl ketone and crystallised from diisopropyl ether and cyclohexane, affording the *S*-enantiomer in 79% yield (86.3 kg) with 99% purity and 99.8% *ee* ($<0.1\%$ of keto ester **19**).[28]

In an alternative approach, the corresponding β-keto *tert*-butyl ester **21** could be reduced with *Issatchenkia orientalis* (NCYC 45) in a shake-flask fermentation process to yield the *S*-enantiomer **22** in 59% yield (Scheme 6). Highly selective reductions of this class of β-keto esters with yeasts proved to be a quite general process for accessing the corresponding chiral hydroxy esters, but the process does require substrate-specific optimisation of the exact parameters.[12c]



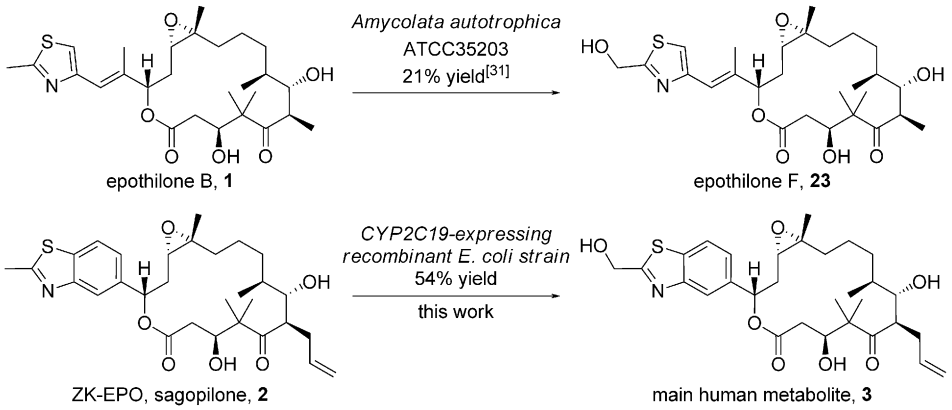
Scheme 6. Stereoselective reduction of the β-keto *tert*-butyl ester **21**.

Biooxidation of sagopilone to access its main human metabolite

Safety profiling of major human metabolites is a key component in the development of each new drug candidate and is strictly regulated.[29] In general, metabolites resulting from deconstructive pathways (e.g., dealkylative pathways) are easily accessible from intermediates in the synthetic routes to the parent compounds. In contrast, metabolites resulting from complexity-increasing pathways (e.g., hydroxylative pathways) require the development of a new synthetic route. Depending on the overall complexity of the drug, this may result in signifi-

cant capacity requirements. Therefore, major efforts have been undertaken in recent years to develop methodologies for the direct oxidation of drugs to their metabolites. Currently, all major pharmaceutical companies use biocatalytic platforms to generate human metabolites by the direct biooxidation of drug candidates. In more recent years, newly developed chemical CH functionalisation methodologies have started to complement these biocatalytic processes. An excellent overview of current technologies for accessing metabolites is available.[30]

As the most efficient option, we elected to investigate the direct oxidative biotransformation of sagopilone (**2**). Encouragingly, there were recent reports of the successful biooxidation of epothilone B (**1**; 610 mg) to epothilone F (**23**; 126 mg, 21% isolated yield) using *Amycolata autotrophica* ATCC 35203 (Scheme 7).[31] In this case, isolation of the desired product was complicated by the formation of a second, regioisomeric hydroxylation product. With a second, not freely accessible microorganism the isolated yield of epothilone F could be increased to 33%.[31]



Scheme 7. Biooxidation of epothilones to hydroxylated derivatives.

Unfortunately, incubation of **2** with eight different *Amycolata* strains gave disappointing results. Even after extended incubation times, the desired hydroxylation product was only formed with less than 5% turnover.[32] With *A. autotrophica* ATCC 35203, sagopilone remained mostly unchanged, with trace amounts of two hydrolysis products being formed.[33] Screening of a further set of wild-type microorganisms, which had already been used successfully for other drug biooxidations, also failed to reveal any hits.

Sagopilone is metabolised in the human body by the cytochrome P450 enzyme CYP2C19 which requires a complementary P450 reductase as well as NADPH for closing the catalytic cycle. Several efforts toward designing *E. coli* strains recombinantly expressing human CYP enzymes together with P450 reductases and their use as whole-cell biooxidation catalysts have been reported.[34] Based on these pioneering studies, a biooxidation toolbox was developed by the LINK consortium which is still used by several pharmaceutical companies.[35]

Along the same lines, we have constructed a toolbox of *E. coli* strains which recombinantly express the major human CYP enzymes along with complementary P450 reductases. In addition to CYP isoform diversity, our toolbox comprises further diversity dimensions resulting, for example, from using different *E. coli* host strains and employing different expression strategies (different N-terminal modifications, use of dual as well as bicistronic CYP-P450 reductase constructs). For example, we had 12 different engineered *E. coli* strains recombinantly expressing CYP2C19 at hand, which were screened in a microtitre plate setup under standardised conditions. Under the screening conditions at pH 7.4, sagopilone (**2**) was stable, and two strains showed promising results with nearly 10% of the main metabolite **3** being formed. For further optimisation we focused on one strain, which is based on *E. coli* DH5a-LPSd as host organism and contains a bicistronic plasmid with N-terminally modified hCYP3A4 and the human P450 oxidoreductase POR.

We applied a process for metabolising sagopilone which is known as 'resting cell' fermentation.[5g,36] In this process, the microorganism that functions as the biocatalyst is incubated in a shaking flask or in a fermenter for a defined period of time and is harvested by centrifugation. Then, in a second fermentation step, this cell mass is used as the biocatalyst by incubation with the substrate of choice. The cell mass can be washed with aqueous solutions containing salts and nutrients or can be used without washing. For the transformation process, the cell mass is suspended in the transformation medium. The biocatalysts can be produced ready for immediate use or may be stored at −80 °C in cryo buffer to prevent decomposition. For the optimisation of a resting cell fermentation, both steps need to be investigated: the fermentation that produces the biocatalyst, and also the transformation step.

Initially, we incubated our *E. coli* strain in a 10-L steel fermenter to isolate cell material as resting cells which could be used for further evaluation of the transformation step. Using fresh cell material and ^{14}C-labelled sagopilone to allow quantification of the turnover, we were able to achieve 90% turnover into the desired metabolite within 22 h.

Then, we investigated various fermentation conditions for the production of the active biocatalyst (on a 10-L scale). The time point for harvesting the cell mass was varied (i.e., from 144 h to 120 h) and different media were tested (see the Experimental Section below for details of the optimal medium). As an example, in the case of cells from batch 3 (Table 2), a medium with an altered nitrogen and carbon source was tested: peptone from meat (2 g L^{-1}) was omitted, and the glycerol concentration was increased (from 4 to 5 g L^{-1}), but the amount of tryptone (12 g L^{-1}) and yeast extract (24 g L^{-1}) were unchanged. Under the screening conditions with a sagopilone

Table 2. Influence of biocatalyst quality on sagopilone turnover.[a]

Cell material batch no.[b]	Fermentation time for cell mass [h]	[Sagopilone] [mg L^{-1}]	Sagopilone (**2**) [%][c]	Metabolite **3** [%][c]
1	144	25	<1	81
1	144	50	<1	56
1	144	75	15	62
1	144	100	40	46
2	120	25	<1	80
2	120	50	<1	74
2	120	75	<1	77
2	120	100	<1	72
3	120	25	<1	79
3	120	50	<1	75
3	120	75	12	67
3	120	100	26	56

[a] Conditions: 27 °C, 20 h, at OD_{550} 10, 165 rpm. [b] For batches 1 and 2, an optimal medium was used (see Experimental Section). For batch 3, an altered medium was used: tryptone (12 g L^{-1}), yeast extract (24 g L^{-1}), glycerol (5 g L^{-1}). [c] Determined by HPLC.

Table 3. Influence of biocatalyst concentration on sagopilone turnover.[a]

OD_{550}	[Sagopilone] [mg L^{-1}]	Sagopilone [%][b]	Metabolite **3** [%][b]
3	25	20	77
3	50	30	29
3	75	60	33
3	100	70	24
5	25	<1	97
5	50	<1	91
5	75	9	80
5	100	35	65
10	25	<1	100
10	50	<1	86
10	75	<1	88
10	100	<1	90

[a] Conditions: biocatalyst production as for batch 2 in Table 2; biotransformation conditions: 27 °C, 20 h, 165 rpm. [b] Determined by HPLC.

concentration of 25 mg L^{-1}, these two changes (fermentation time and medium) did not influence the turnover rate into the human metabolite (79–81%); however, differences in the biocatalyst quality became apparent upon investigation of higher sagopilone concentrations (Table 2).

Cells from batch 2 (harvested at an earlier time point) allowed for an increase in the sagopilone concentration to 100 mg L^{-1} without the turnover being compromised (Table 2). Older cells (batch 1) or cells from fermentations with the changed medium (batch 3) showed significantly decreased turnover rates at sagopilone concentrations beyond 50 mg L^{-1}. These results underscore the importance of optimising fermentation time and the medium for biocatalyst production. We now routinely use the sagopilone hydroxylation reaction as an in-house quality-control assay for each CYP2C19 biocatalyst batch before it is used with new substrates.

We also investigated variations in the transformation step; for example, we determined the necessary concentration of the biocatalyst for complete sagopilone turnover. The concentration of cells, as gauged by the optical density (OD), was set at OD 10 (determined at 550 nm) for the above studies. For the large-scale fermentation, we wanted to decrease the amount of biocatalyst required by decreasing the cell concentration; however, as shown from the results in Table 3, an OD_{550} of 10 was necessary for complete transformation of 100 mg L^{-1} of sagopilone. When the biocatalyst concentration was decreased to OD_{550} 5, up to 50 mg L^{-1} of sagopilone could be transformed. This demonstrated that the process was already running at the limit of oxidation capacity of the cells, and hence a decrease in cell concentration was not feasible.

To verify the robustness of our optimised biotransformation conditions, we performed a 1-L preparative-scale transformation with sagopilone (90 mg) which, after extractive workup and chromatography, gave the desired metabolite **3** in 66% yield (61 mg).

Finally, cryo-conserved cells (4 L; 50 L combined fermentation volume, four individual batches) were resuspended in 100 mM pH 7.4 potassium phosphate buffer (100 L) containing 1% glucose. Sagopilone (**2**; 9 g) dissolved in *N*,*N*-dimethylformamide (DMF; 367 mL) was added to the transformation medium, which was incubated for 23 h under a partial oxygen concentration of 50%. Then, the culture broth was harvested and extracted twice with methyl isobutyl ketone. HPLC analysis of the organic layers showed complete turnover of sagopilone into the desired metabolite **3**; notably, no other regioisomeric hydroxylation products were detected. The combined organic layer was concentrated and purified by silica gel chromatography to afford the desired metabolite **3** (5.03 g) in 54% isolated yield, in analytically pure form.

Conclusions

We have shown that parallel investigation of chemical and enzymatic processes can be integrated into the development process of a complex chiral drug candidate on a large scale. For two of the three required chiral building blocks for the synthesis of sagopilone, superior processes were developed based on biocatalytic key steps. In addition, we have described the multi-gram-scale direct biotransformation of a highly complex drug into its human metabolite by using a recombinant hCYP biocatalyst. To our knowledge, this hCYP biocatalytic process is the most complex application of such a biocatalyst, as well as the largest scale, reported to date.

Experimental Section

All microorganisms were handled under a clean bench or under sterile conditions using the corresponding equipment. All solutions were either sterilised at 121 °C or sterile-filtered. Screening procedures are described in the literature: enzymatic ester hydrolysis,[37] enzymatic esterification,[38] and microbial transformations.[39]

(3S)-1-(Benzyloxy)-4-cyano-4-methylpentan-3-yl acetate (13) and (3R)-5-(benzyloxy)-3-hydroxy-2,2-dimethylpentanenitrile (12) by enzymatic kinetic resolution

A 2000-L reactor was charged with water (1115 kg), Na_2HPO_4 (10.16 kg), and KH_2PO_4 (3.92 kg). An 81-kg solution of the racemic acetate **11** (44.5 kg, 161.6 mol) in MTBE was added, and the solution was adjusted to pH 6 with 1 M NaOH. The temperature was set at 40 °C, and the MTBE was removed by distillation under reduced pressure. Then, lipase AYS (Amano, 22.3 kg) was added, and the mixture was stirred at 40 °C for 68 h. The pH was controlled and kept at pH 6.0 by adding 1 M NaOH. The reaction mixture was extracted with EtOAc (2×1785 L). The organic phase was filtered over diatomaceous earth and concentrated under reduced pressure. The crude material was separated by chromatography [Kromasil NP 10 μm (11 kg), *n*-hexane/MTBE gradient] yielding the *S*-acetate **13** (21.87 kg, 98%; >99.5% *ee*) and the *R*-alcohol **12** (14.21 kg, 75%; >97.5% *ee*).

S-Acetate **13**: t_R (Chiralpak AD 20 μm, 250×4.6 mm, 30 °C, 1.5 mL min^{-1}, hexane/EtOH 98:2, UV 204 nm) = 5.91 min; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.26 (s, 3H), 1.31 (s, 3H), 1.69–1.79 (m, 1H), 1.99–2.08 (m, 4H), 3.34–3.41 (m, 1H), 3.44–3.50 (m, 1H), 4.40 (sym m, 2H), 4.98 (dd, J = 10.61, 2.02 Hz, 1H), 7.24–7.37 ppm (m, 5H); MS (ESI) *m/z* (%): 293 (100) $[M+NH_4]^+$, 276 (71) $[M+H]^+$.

R-Alcohol **12**: t_R (Chiralpak AD 20 μm, 250×4.6 mm, 30 °C, 1.5 mL min^{-1}, hexane/EtOH 98:2, UV 204 nm) = 15.54 min; ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.22 (s, 3H), 1.25 (s, 3H), 1.48–1.57 (m, 1H), 1.83–1.92 (m, 1H), 3.46 (ddd, J = 10.67, 6.25, 2.02 Hz, 1H), 3.52–3.62 (m, 2H), 4.46 (s, 2H), 5.39 (d, J = 6.32 Hz, 1H), 7.24–7.37 ppm (m, 5H); MS (ESI) *m/z* (%): 251 (100) $[M+NH_4]^+$, 234 (46) $[M+H]^+$; Anal. calcd for $C_{14}H_{19}NO_2$: C 72.07, H 8.21, N 6.00, found: C 71.85, H 8.41, N 5.87.

Ethyl (3S)-3-hydroxy-3-(2-methyl-1,3-benzothiazol-5-yl)propanoate (20) by reduction of β-keto ester 19

The yeast strain *Pichia wickerhamii* with access number IFO 1278 was purchased from the culture collection of the Institute for Fermentation, Osaka, Japan. A 2-L Erlenmeyer flask was charged with an aqueous solution (500 mL) containing glucose (50 g L^{-1}) and corn steep liquor (20 g L^{-1}). The solution was adjusted to pH 6.25 with 16% NaOH. The flask was sterilised in an autoclave at 121 °C for 20 min. The culture medium was inoculated with a cryo culture (10 mL; 50% glycerol) of *P. wickerhamii* IFO 1278. The culture broth was incubated on a shaker at 30 °C for 24 h at 180 rpm. The culture broth (500 mL) from the 2-L flask was added to a sterile 5000-L fermenter containing glucose (50 g L^{-1}), corn steep liquor (20 g L^{-1}), and an antifoam agent (0.1 g L^{-1}). The mixture was incubated at 28 °C for 38 h. The culture broth was transferred to a 50000-L fermenter containing an aqueous solution (50000-L) of glucose (50 g L^{-1}) and corn steep liquor (20 g L^{-1}) that had been sterilised at 121 °C for 30 min. The mixture was adjusted to pH 6 with 16% NaOH. After 0.5 h, the β-keto ester **19** (180 kg, 683.6 mol) dissolved in DMF was added as a solution (200 g L^{-1}) over 15 h. The mixture was incubated at 28 °C. After 22 h, the concentration of the *S*-alcohol **20** had reached 3.5 g L^{-1}, and the fermentation process was terminated.

The culture broth was extracted with methyl isobutyl ketone (1:1). The layers were separated, and the organic layer was partly concentrated. The fermentation product was filtered over charcoal, and the filtrate was concentrated under reduced pressure. A solution of diisopropyl ether and cyclohexane (1:1.5) was added to the residue at 40 °C. The mixture was cooled to 5 °C. The product was isolated using a plate filter and dried at 50 °C, yielding the desired *S*-alcohol **20** (160 kg, 88%; >99% *ee*): $[\alpha]_D^{20} = -36.4$ (c = 1.1 in $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.27 (t, J = 7.15 Hz, 3H), 2.73–2.86 (m, 5H), 3.40 (d, J = 3.51 Hz, 1H), 4.20 (q, J = 7.03 Hz, 2H), 5.27 (dt, J = 8.34, 3.98 Hz, 1H), 7.41 (dd, J = 8.28, 1.51 Hz, 1H), 7.80 (d, J = 8.28 Hz, 1H), 7.95 ppm (d, J = 1.25 Hz, 1H); MS (ESI) *m/z* (%): 265 (38) $[M]^+$, 178 (100), 176 (37), 150 (86), 109 (33).

(1S,3S,7S,10R,11S,12S,16R)-10-Allyl-7,11-dihydroxy-3-[2-(hydroxymethyl)-1,3-benzothiazol-5-yl]-8,8,12,16-tetramethyl-4,17-dioxabicyclo[14.1.0]heptadecane-5,9-dione (sagopilone metabolite, 3)

Expression of the P450 enzyme CYP2C19 was performed in *E. coli* DH5a-LPSd pCW_d3_2C19 (#6), a strain engineered in our laboratory in 2004. The strain was stored at −80 °C in a 50% glycerol solution.

Oxford trace elements solution: $FeCl_3{\cdot}6H_2O$ (27 g L^{-1}), $ZnCl_2$ (1.31 g L^{-1}), $CoCl_2{\cdot}6H_2O$ (2.87 g L^{-1}), $CuCl_2{\cdot}2H_2O$ (1.27 g L^{-1}), $B(OH)_3$ (0.5 g L^{-1}), $CaCl_2{\cdot}2H_2O$ (1.32 g L^{-1}), $Na_2MoO_4{\cdot}2H_2O$ (2.35 g L^{-1}), and 37% HCl (100 mL) in water (1 L).

A 500-mL Erlenmeyer flask was charged with an aqueous solution (100 mL) containing tryptone (16 g L^{-1}), NaCl (10 g L^{-1}), and yeast extract (10 g L^{-1}). The solution was adjusted to pH 7.2–7.4 with 16% NaOH and 16% H_3PO_4. The flask was sterilised in an autoclave at 121 °C for 20 min. An aqueous ampicillin solution was added to the flask so that the final concentration was 50 μg mL^{-1}. To the flask was added the glycerol stock solution (50 μL) containing the *E. coli* strain #6. The flask was kept in an incubator at 37 °C for 17 h at 165 rpm. A 10-L fermenter was charged with tryptone (12 g L^{-1}), yeast extract (24 g L^{-1}), peptone from meat (2 g L^{-1}) [enzymatic (tryptic) digested], KH_2PO_4 (2.2 g L^{-1}), K_2HPO_4 (9.4 g L^{-1}), and 87% glycerol (4.6 g L^{-1}). The mixture was sterilised at 121 °C for 30 min. At 37 °C, the following solutions were added: ampicillin (0.5 g) in water (20 mL), riboflavin (10 mg) in water (20 mL), thiamine hydrochloride (3.37 g) in water (10 mL), and the Oxford trace elements solution (2.5 mL). After 2 h, the inoculation culture (100 mL) from the 500-mL flask was added. The fermentation mixture was stirred at 315 rpm and aerated with 3.3 L min^{-1} of air at pH 6.7. After 3 h, the optical density (OD_{550}) reached 1.058. The temperature was decreased to 27 °C, and isopropyl β-D-1-thiogalactopyranoside (IPTG; 2.38 g) in water (40 mL) and 5-aminolevulinic acid (838 mg) in water (40 mL) were added. After 6 h, the pH decreased, and feeding with a sterile aqueous solution of glucose (50%) was started to maintain the pH at 6.7. After 123 h, the culture broth was centrifuged, yielding cell mass (527.7 g) which was resuspended in cryo buffer (50% glycerol in water; 500 mL). The cell mass was stored at −80 °C.

A 100-L fermenter was filled with water (~90 L), and K_2HPO_4 (1230 g), KH_2PO_4 (400 g), and Synperonic (2.5 mL) were added. The mixture was sterilised at 121 °C for 30 min. EDTA solution (0.5 M in water; 100 mL) and sterile-filtered glucose solution (50% in water; 2 L) were added. The solution was regulated between pH 7.2 and 7.4 with 16% NaOH. At 27 °C, the fermenter was inoculated with the cell solution in cryo buffer (4 L). The partial oxygen concentration was set at 20%. Sterile water was added to adjust to a 100 L volume. The mixture was aerated with 33.3 L min^{-1} of air and the partial oxygen concentration was set at 50% and maintained by automatic regulation of the stirrer velocity. Sagopilone (**2**; 9 g, 16.6 mmol) in DMF (367 mL) was added. After 23 h, the culture

broth was transferred to an extraction vessel. The fermenter was rinsed with water (50 L), which was also transferred to the extraction vessel. Methyl isobutyl ketone (50 L) was added to the reaction culture mixture which was gently stirred for 23 h. The layers were separated, and the culture was extracted again with methyl isobutyl ketone (10 L). The combined organic layers were concentrated to 50 mL. Methanol (150 mL) was added, and the solution was concentrated to dryness. The residue was subjected to silica gel chromatography with a gradient of EtOAc and hexane, yielding **3** (5.03 g, 54%) as a pale-yellow foam: ^{1}H NMR (600 MHz, CD_3CN): $\delta = 0.93$ (s, 3H), 1.01 (d, $J = 6.78$ Hz, 3H), 1.20 (s, 3H), 1.25 (s, 3H), 1.33–1.59 (m, 6H), 1.65–1.71 (m, 1H), 2.11–2.18 (m, 1H), 2.19–2.23 (m, 1H), 2.31–2.36 (m, 1H), 2.38–2.46 (m, 2H), 2.52–2.57 (m, 1H), 2.81–2.86 (m, 2H), 3.39 (td, $J = 6.78$, 4.52 Hz, 1H), 3.43 (d, $J = 6.78$ Hz, 1H), 3.70 (td, $J = 6.31$, 3.95 Hz, 1H), 4.11 (br s, 1H), 4.16 (ddd, $J = 9.22$, 6.96, 4.52 Hz, 1H), 4.92 (br s, 2H), 4.96–4.99 (m, 1H), 5.00–5.04 (m, 1H), 5.76 (ddt, $J = 17.22$, 9.98, 7.29 Hz, 1H), 6.03 (dd, $J = 9.41$, 2.64 Hz, 1H), 7.44–7.46 (m, 1H), 7.95–7.98 ppm (m, 2H); ^{13}C NMR (151 MHz, CD_3CN): $\delta = 18.1$, 20.2, 22.0, 22.6, 23.1, 30.9, 33.1, 33.2, 36.3, 36.7, 39.9, 50.7, 53.7, 62.4, 62.6, 62.7, 72.6, 74.6, 75.1, 117.0, 120.9, 122.9, 123.9, 134.9, 137.0, 140.5, 154.4, 171.3, 176.4, 217.4 ppm; MS (ESI) *m/z* (%): 560 (100) $[M+H]^+$, 367 (27).

Acknowledgements

The authors thank Petra Helfrich and Sabine Trenner for their excellent laboratory work. We thank Joerg Knaeblein and Thomas Faupel for the E. coli strains. We also thank Ingo Hartung for helpful discussions and suggestions.

Keywords: antitumor agents · biocatalysis · enzymes · gene expression · oxidoreductases

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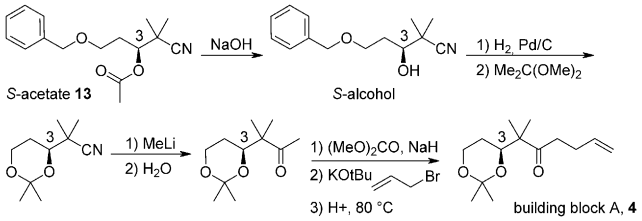

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Received: March 30, 2015
Published online on May 28, 2015