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Evolving enzyme technology for pharmaceutical applications: case studies

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The case studies focus on two types of enzyme applications for pharmaceutical development. Demethylmacrocin *O*-methyltransferase, macrocin *O*-methyltransferase (both putatively rate-limiting) and tylosin reductase were purified from *Streptomyces fradiae*, characterized and the genes manipulated for increasing tylosin biosynthesis in *S. fradiae*. The rate-limiting enzyme, deacetoxycephalosporin C (DAOC) synthase/hydroxylase (expandase/hydroxylase), was purified from *Cephalosporium acremonium*, its gene over-expressed, and cephalosporin C biosynthesis improved in *C. acremonium*. Also, heterologous expression of penicillin N epimerase and DAOC synthase (expandase) genes of *Streptomyces clavuligerus* in *Penicillium chrysogenum* permitted DAOC production in the fungal strain. Second, serine hydroxymethyltransferase of *Escherichia coli* and phthalyl amidase of *Xanthobacter agilis* were employed in chemo-enzymatic synthesis of carbacephem. Similarly, echinocandin B deacylase of *Actinoplanes utahensis* was used in the second-type synthesis of the ECB antifungal agent.

Keywords: biosynthesis; tylosin; cephalosporin C; biocatalysis; carbacephem; ECB antifungal agent

Introduction

Case studies in enzyme technology can trace their roots to toluene dioxygenase from *Pseudomonas putida*. The monooxygenase with cytochrome p450 was the only recognized bacterial three-component oxygenation system in the early 1970s. The multi-component nature of toluene dioxygenase was not realized at the time, and this made its purification quite challenging. The subsequent resolution of the dioxygenase as a three-component system [30] was a significant milestone for this unique bacterial enzyme. The three-component system [24–26] was not only academically interesting but also industrially useful. The evolution of toluene dioxygenase was intimately connected with the evolution of multiple degradative enzymes of the β-ketoadipate pathway [28]. The metabolic evolution gave rise to practical enzyme technology in the areas of antibiotic biosynthesis, antibiotic biocatalysis and drug discovery.

The advances in enzyme technology have affected different aspects of enzyme biochemistry including: enzyme assay and purification, reaction optimization, enzyme characterization, substrate specificity, enzyme kinetics and mechanism, enzyme refolding and metabolic engineering. Examples of two types of enzyme technology in a number of these aspects of enzyme biochemistry are described below.

Results and discussion

Antibiotic biosynthesis

Antibiotic biosynthetic pathways in microorganisms are frequently complex and involve many enzymatic interconversions. Our primary focus has been the biosynthesis of the macrolide antibiotic, tylosin, and that of the beta-lactam compound, cephalosporin C.

Tylosin biosynthesis: Tylosin is an animal health product used agriculturally as a growth promotant. It is a secondary metabolite produced by the soil bacterium, *Streptomyces fradiae*. The tylosin biosynthetic pathway is shown in Figure 1 [3,12]. Our interest was focused on the two *O*-methyltransferases catalyzing the final two steps in the biosynthesis of tylosin, and on the aldehyde reductase mediating the conversion of tylosin to the less active compound, relomycin. Demethylmacrocin *O*-methyltransferase (DMOMT) catalyzes the 2′-*O*-methylation of the deoxyallose moiety forming macrocin, which in turn is the substrate for the 3′-*O*-methyltransferase (MOMT) that methylates the 3′-hydroxyl position of the same moiety to yield tylosin (Figure 1) [22]. Because of substrate (demethylmacrocin/macrocin) accumulation by high tylosin-producing strains, both enzymatic reactions were rate-limiting for tylosin biosynthesis [22]. Tylosin reductase (TR) catalyzes reduction of tylosin to relomycin, resulting in poor recovery of the tylosin product [12].

Through the efforts of several persons over a number of years, the three enzymes (DMOMT, MOMT and TR) were purified to homogeneity and characterized [4,12,16]. The physical, catalytic and kinetic properties of the three enzymes are listed in Table 1 [4,12,16]. DMOMT and MOMT catalyze two consecutive *O*-methylations in tylosin biosynthesis, and they are distinctive *O*-methyltransferases [4,16]. Both DMOMT and MOMT exhibit a low K_m (ie, high affinity) and a high V_{max} for their substrates and a very narrow substrate specificity (Table 1) [4,16], thus both enzymes may be specific to tylosin biosynthesis [12]. Interestingly, based on the K_i values of the macrolide inhibitors [4,16], the two *O*-methyltransferases are inhibited independently by different metabolites of the pathway, DMOMT

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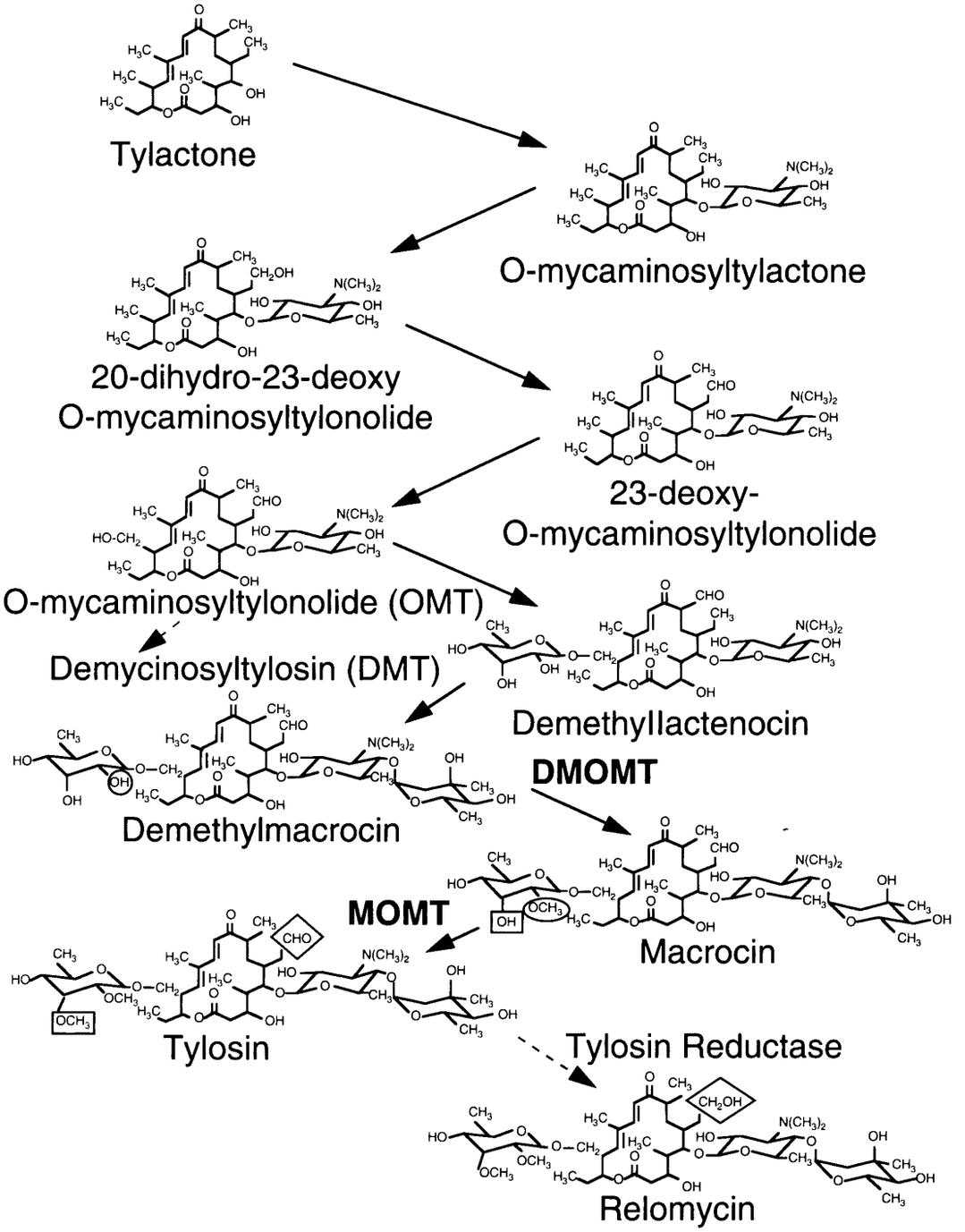


Figure 1 Tylosin biosynthesis in *S. fradiae*. A solid arrow indicates a known metabolic step and a dashed arrow indicates a shunt route.

Table 1 Biochemical properties of DMOMT, MOMT and TR

Property	DMOMT	MOMT	TR
Substrate	Demethylmacrocin	Macrocin	Tylosin
Co-Substrate	AdoMet	AdoMet	None
Oligomer	122–126 kDa	65 kDa	270 kDa
Monomer	42 kDa	32 kDa	26- and 10-kDa
Structure	Trimer	Dimer	Octamer
Optimal pH	7.8–8.5	7.5–8.0	7.0–7.2
Optimal temp	42°C	31°C	40°C
Metal ion	Mg^{2+}	Mg^{2+}, Mn^{2+}, Co^{2+}	None
Cofactor	None	None	Flavin
Electron donor	None	None	NADPH
K_m (mM)	0.006	0.005	1.4
V_{max} (mU mg^{-1})	230	150	920
Substrate specificity	Narrow	Narrow	Broad

strongly by macrocin and MOMT strongly by demethylmacrocin (Figure 2). In contrast to DMOMT or MOMT, TR shows a very high K_m (ie, low affinity), a higher V_{max} for its substrate and a broad substrate specificity (Table 1) [12]. This indicates that TR may not be specific for tylosin conversion [12]. Also, several chromatographic forms of TR activity were observed from *S. fradiae* [12]. In addition, TR activity was observed from mutants of *S. fradiae* which lack most or all of the tylosin-biosynthetic activities as well as from other actinomycetes which do not produce tylosin [12]. Since relomycin is less active as an antibiotic than tylosin, bacteria may have recruited several aldehyde reductases from primary metabolism to carry out tylosin reduction as a detoxification mechanism [12]. The project on the enzymology of tylosin biosynthesis initiated enzyme technology at Lilly from two aspects. Specifically, the amino-terminal sequence of MOMT was used in the first successful bacterial gene cloning via reverse genetics at Lilly [7,11]. In general, by studying and defining the three rate-limiting steps biochemically and the metabolite inhibition patterns of DMOMT and MOMT, enzyme technology has guided the program on the gene manipulation of tylosin biosynthesis [4,12,16].

Beta-lactam biosynthesis: Cephalosporin C is used as starting material for semi-synthetic cephalosporin antibiotics. The known biosynthetic pathway of cephalosporin C by *Cephalosporium acremonium* with its corresponding enzymes is shown in Figure 3 [31]. The accumulation of penicillin N and DAOC (ie, two substrates), suggested that the expandase/hydroxylase activities were rate-limiting for cephalosporin C biosynthesis in the production strain [31]. The purified expandase/hydroxylase is a bifunctional enzyme of about 41 kDa [2,9]. An extra copy of the expandase/hydroxylase gene, cloned by reverse genetics [21], was added to the production strain of *C. acremonium* [23]. Addition of the extra gene copy led to increased expandase/hydroxylase activity *in vivo* and a significant

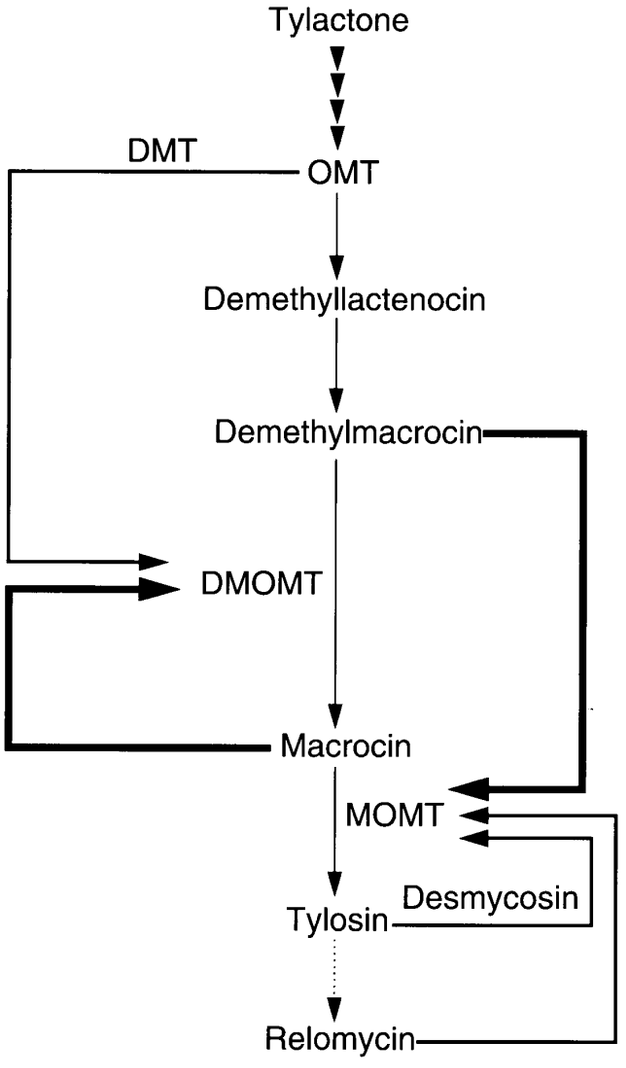


Figure 2 Independent metabolite inhibition of MOMT and DMOMT *in vitro*. A heavy side-arrow indicates a strong inhibition and a regular side-arrow indicates a weak or moderate inhibition. See OMT and DMT in Figure 1 and the detailed metabolite inhibition data in Reference 16.

improvement in the rate of cephalosporin C production [23]. This confirmed that the expandase/hydroxylase was rate-limiting for cephalosporin C biosynthesis in the production strain.

In contrast to the bifunctional fungal expandase/hydroxylase (41 kDa), the bacterial expandase (35 kDa) and hydroxylase (37 kDa) for cephamycin C biosynthesis are separate enzymes (Figure 4) [1,10,13]. With similar molecular mass and extensive sequence homologies, the fungal and two bacterial enzymes appear to represent products from a divergent evolution [1,10].

DAOC can be enzymatically deacylated to 7-ADCA, which is also starting material for semi-synthetic cephalosporin antibiotics [29]. DAOC could be produced by direct inactivation of the bacterial hydroxylase gene [6,14]. Alternatively, DAOC biosynthesis by a new approach was undertaken. The bacterial expandase and epimerase were purified [10,27] and their genes cloned to a production

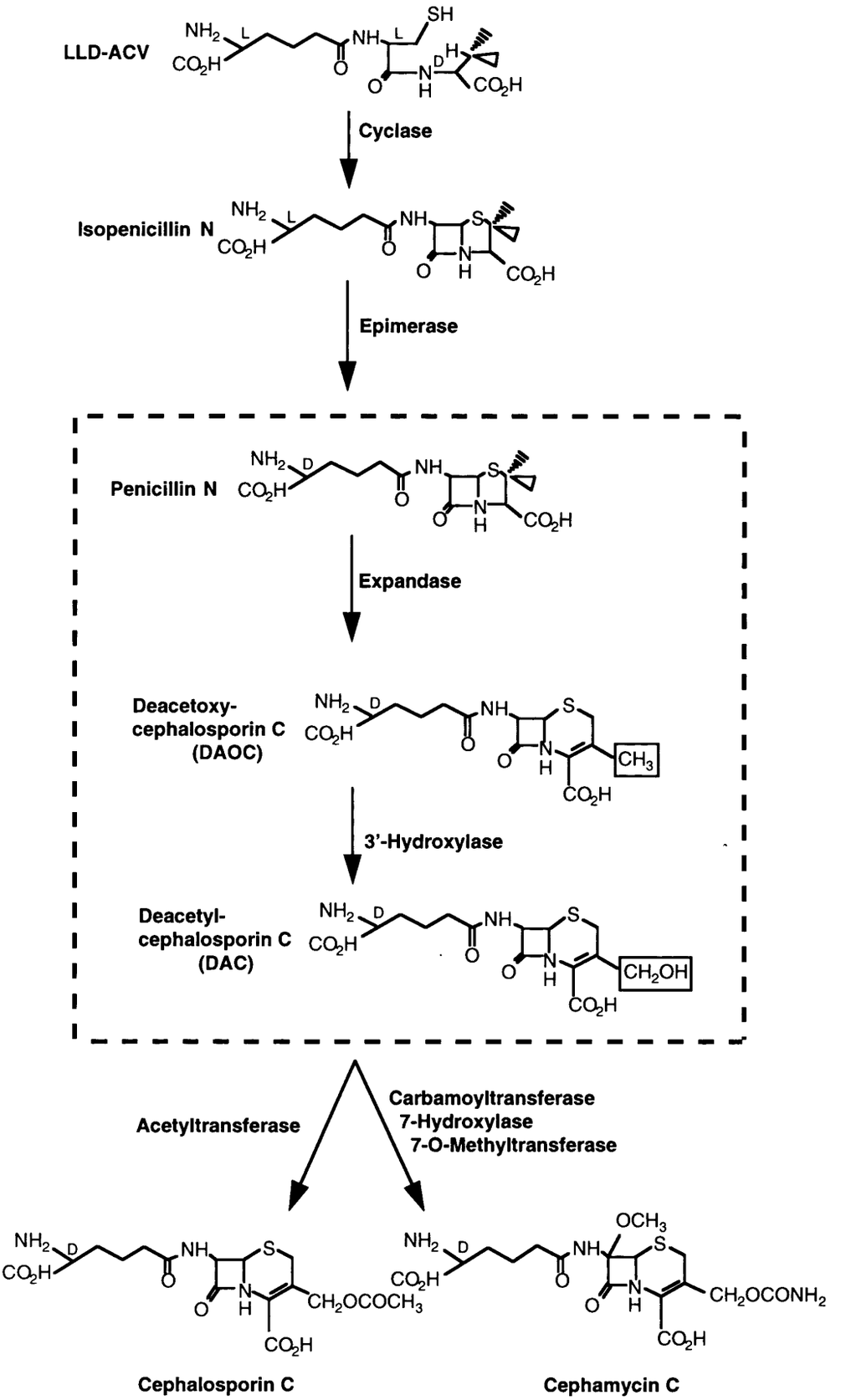


Figure 3 Cephalosporin C biosynthesis in *C. acremonium* and cephamycin C biosynthesis in *S. clavuligerus*. LLD-ACV, δ-(l-aminoadipyl)-l-cysteinyl-d-valine.

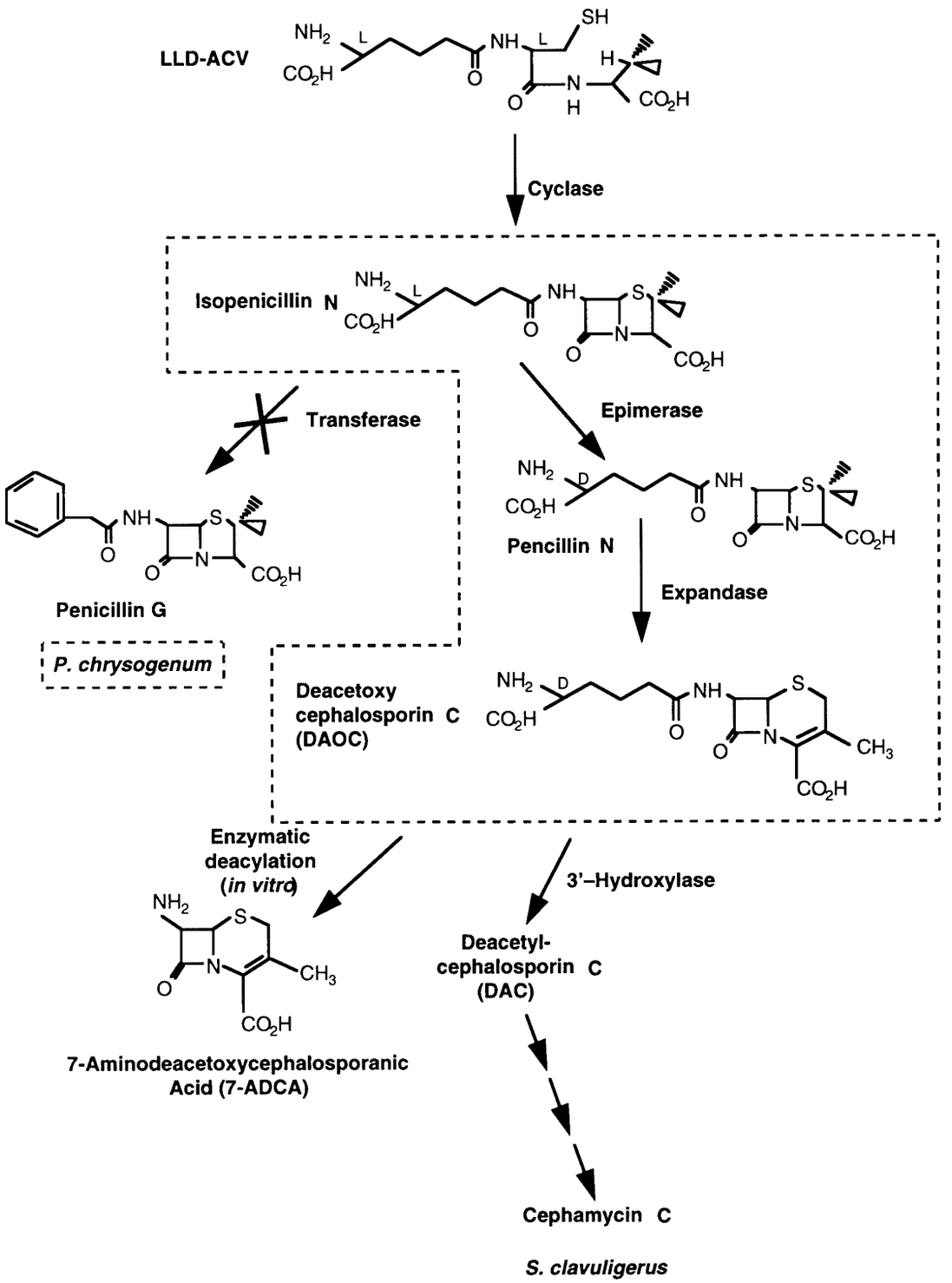


Figure 4 Metabolic engineering for DAOC biosynthesis in *P. chrysogenum*.

strain of *Penicillium chrysogenum* [6,15,20]. The cloned genes were targeted to integrate into and disrupt the endogenous transferase gene [6]. Since the level of isopenicillin N, substrate for the epimerase, is much higher in *P. chrysogenum* than in the *S. clavuligerus* available to us [6], addition of the two bacterial genes to *P. chrysogenum* resulted in moderate DAOC accumulation [6]. The metabolic engineering for the production of DAOC in *P. chrysogenum* is a unique example of the production of a complex chemical intermediate by manipulation of the cellular enzyme composition.

Antibiotic biocatalysis

Three enzymes involved in two pharmaceutical synthetic pathways will be described: serine hydroxymethyltransferase (SHMT) and phthalyl amidase (PA) for carbacephem synthesis; and echinocandin B (ECB) deacylase for the synthesis of a new anti-fungal agent.

A SHMT-/PA-based route to a carbacephem is shown in Figure 5. SHMT catalyzes a C–C bond formation between an aldehyde and glycine leading to the diastereospecific intermediate (l-allo-AHHA). The phthalyl-blocking group is then added to protect the amino-group in subsequent steps. Because of its chemical stability, the phthalyl group is difficult to remove chemically. A phthalyl amidase, that was discovered and purified from *Xanthobacter agilis*, mediates the cleavage of the phthalyl carbacephem efficiently [5]. The *E. coli* SHMT exhibits a broad substrate specificity for aldehyde substrates and a more specific substrate specificity for amide co-substrates [see Table 5 in Ref. 18]. As an example, the enzyme has a high k_{cat} and low K_m for pentene aldehyde leading to a very high diastereospecificity of the product (l-allo-AHHA) [see Table 5 in Ref. 18]. The phthalyl amidase, though highly specific for phthalic acid, shows a broad substrate specificity for the amide portion [5]. As an example, its kinetic properties for five different substrates are shown in Table 2 [5]. Because of chemical utility of the phthalyl group and the broad substrate specificity of phthalyl amidase, the enzyme may become applicable widely in chemo-enzymatic synthesis of molecules such as carbacephems and peptides.

ECB is a six-member cyclic lipopeptide that is produced by *Aspergillus nidulans*. A putatively membrane-bound deacylase, that catalyzes the hydrolysis of the acyl side-chain of ECB to form the free cyclic peptide nucleus (Figure 6), was solubilized, purified and characterized from *Actinoplanes utahensis* [17]. The cyclic peptide nucleus is then reacylated chemically with a different side-chain for-

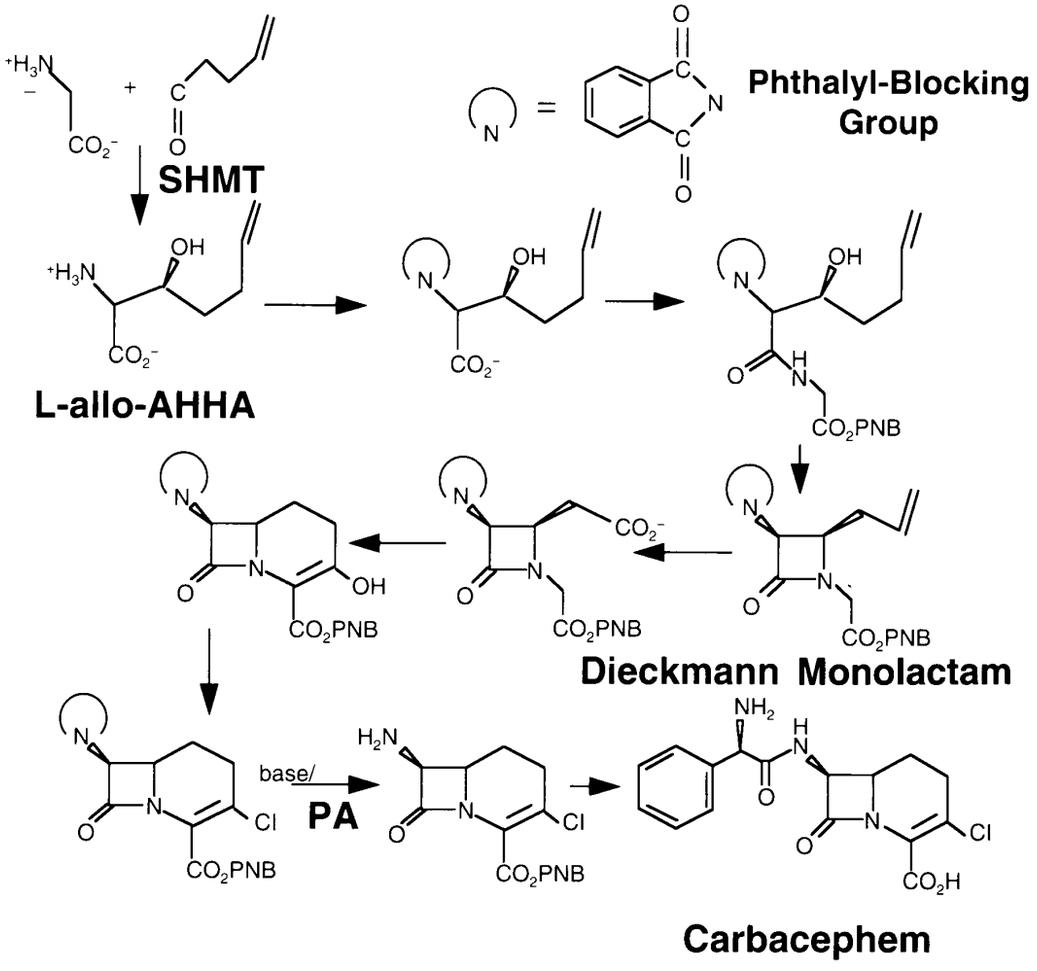
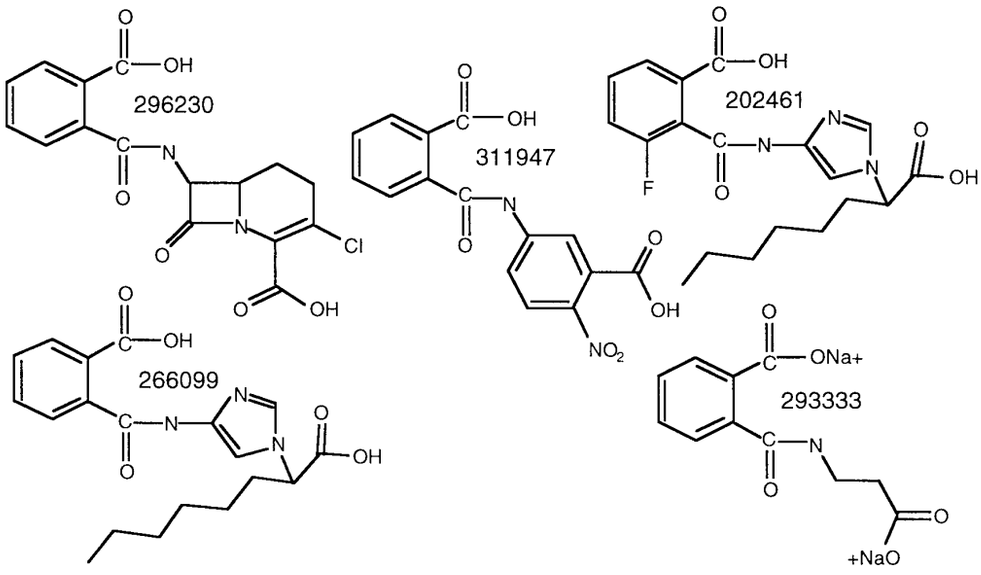


Figure 5 SHMT-/PA-based route to a carbacephem.

Table 2 Substrate specificity of phthalyl amidase[a]

Kinetic constant	296230[b]	311947[c]	202461	266099	293333
K_m (mM)	0.9	0.05	0.17	0.09	0.14
V_{max} (μmol min^{-1} mg^{-1})	7.6	5.95	1.94	1.41	0.27
k_{cat} (μmol min^{-1} μmol^{-1})	6.33	4.95	1.61	1.18	0.22
k_{cat}/K_m (μmol min^{-1} μmol^{-1} mM^{-1})	7	99	9.5	13.1	1.6

[a]Reaction conditions: 200 mM KPi, pH 8.2 and 0.017 or 0.102 nmol enzyme in 1-ml mixture at 32°C for 20 min.
[b]Quantitated by HPLC for the nucleus formation (one substrate).
[c]Quantitated by HPLC for the phthalic acid formation (four substrates).



ming a compound with potent, broad spectrum anti-fungal activity [8]. In addition to ECB, this deacylase mediates cleavage of aculeacin A (a natural lipopeptide with the same nucleus), of various semi-synthetic ECB derivatives, and of daptomycin and its three derivatives (Table 3) [17]. Daptomycin is a natural product consisting of a ten-member cyclic peptide with three additional amino acids in the side-chain linked to an aliphatic moiety. It should be noted that the substrate specificity of the (ECB) deacylase is readily distinguishable from that of industrially important penicillin G amidase. Whereas the amidase catalyzes side-chain removal from a typical three-member cyclic peptide such as a penicillin or a cephalosporin, the deacylase mediates side-chain cleavage from six- or ten-member cyclic peptides such as ECB/aculeacin and daptomycin. Thus, the ECB deacylase may become increasingly significant as a pharmaceutical biocatalyst. From an evolutionary viewpoint, the deacylase shares a detectable sequence similarity with the amidase [17].

In addition to biosynthesis and biocatalysis as described above, microbial enzymes have been used extensively as targets for screen development and inhibitor optimization. The latter two types of enzyme applications for pharmaceutical discovery and development are beyond the scope of the case studies and are expected to be described elsewhere.

Conclusion

Both microbial enzymes and their academic/industrial uses are evolving continuously. The case studies in enzyme technology have focused on enzyme biochemistry applied to pharmaceutical development. The industrial applications have ranged from enzymes of antibiotic biosynthesis to enzymes used in biocatalysis and, more recently, also to enzymes used in screen development and inhibitor optimization. The enzyme technology described in this review represents a small glimpse of enzymes as tools in pharmaceutical applications, and it is expanding in various directions such as directed evolution of enzymes [19]. Finally, as an enzyme is encoded by a gene(s), the evolving enzyme technology that has significantly contributed to pharmaceutical applications has been greatly facilitated by simultaneously evolving molecular biology.

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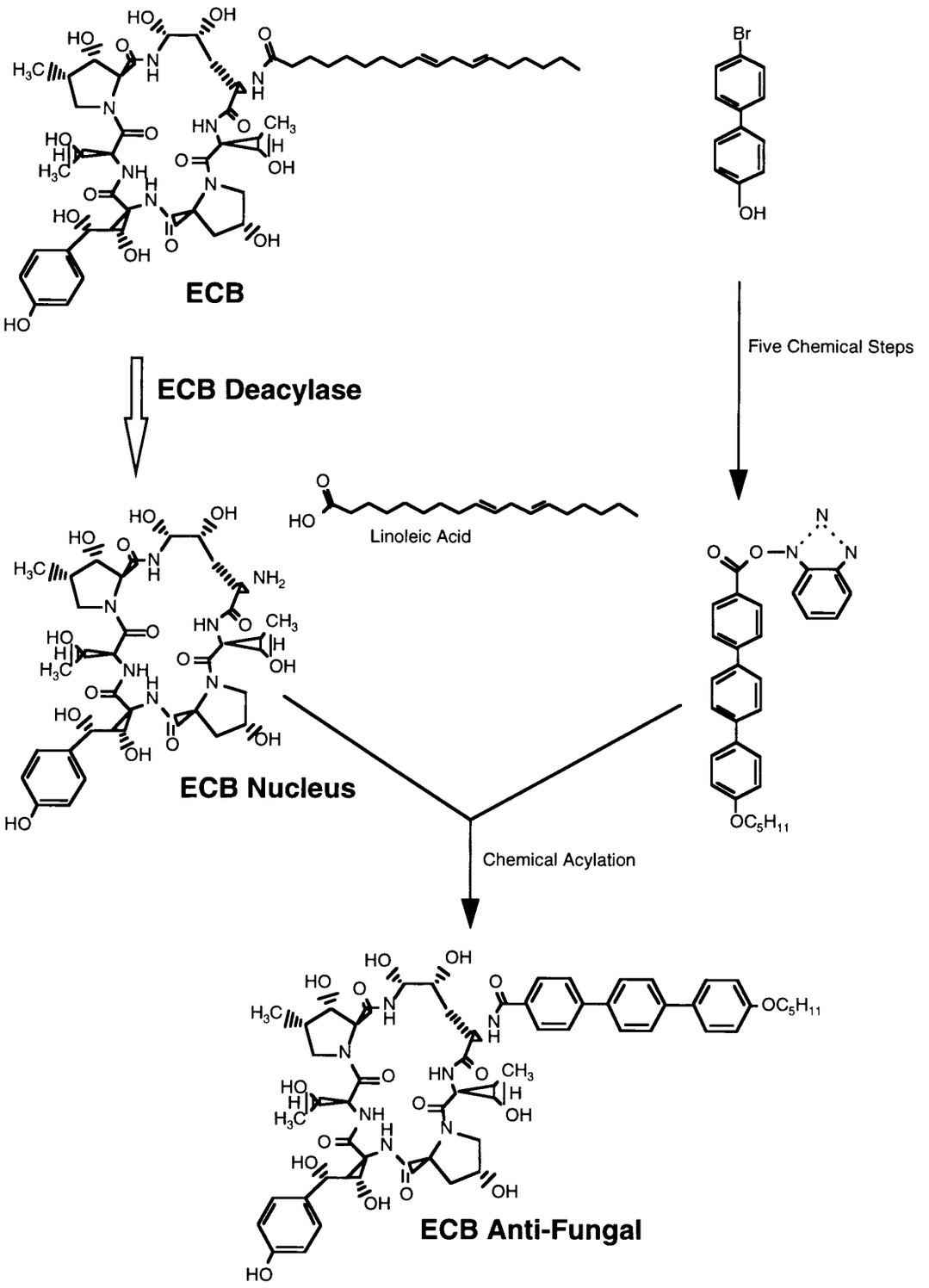
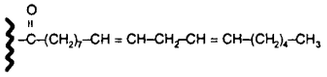
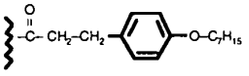
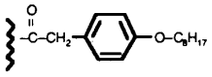
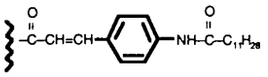
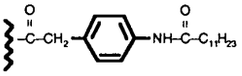
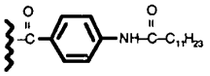
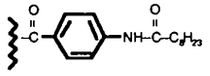
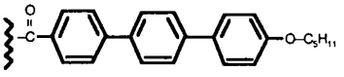
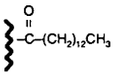
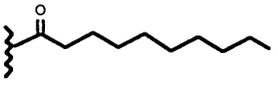
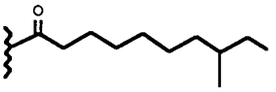
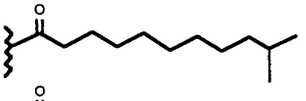
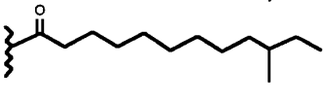


Figure 6 Chemo-enzymatic synthesis of an ECB anti-fungal agent.

Table 3 Substrate specificity of ECB deacylase

Substrate	R1 or R2	Activity (%)
ECB		100
ECB Analogue 1		30
ECB Analogue 2		30
ECB Analogue 3		15
ECB Analogue 4		5
ECB Analogue 5		5
Cilofungin		0
ECB anti-fungal		0
Aculeacin		208
Daptomycin		437
A21978C1		278
A21978C2		282
A21978C3		208

References

1 Baker BJ, JE Dotzlaf and WK Yeh. 1991. Deacetoxycephalosporin C hydroxylase of *Streptomyces clavuligerus*. Purification, characterization, bifunctionality, and evolutionary implication. J Biol Chem 266: 5087–5093.

2 Baldwin JE, RM Adlington, MJ Coats, C Crabbe, NP Crouch, JW Keeping, GC Knight, CJ Schofield, HH Ting, CA Vallejo, M Thorniley and EP Abraham. 1987. Purification and characterization of an enzyme with deacetoxycephalosporin C synthetase and hydroxylase activities. Biochem J 245: 831–841.

3 Baltz RH, ET Seno, J Stonesifer and GM Wild. 1982. Biosynthesis of the macrolide antibiotic tylosin: a preferred pathway from tylactone to tylosin. J Antibiotics 36: 131–141.

4 Bauer NJ, AJ Kreuzman, JE Dotzlaf and WK Yeh. 1988. Purification, characterization, and kinetic mechanism of S-adenosyl-l-methionine: macrocin *O*-methyltransferase from *Streptomyces fradiae*. J Biol Chem 263: 15619–15625.

5 Briggs BS, AJ Kreuzman, C Whitesitt, WK Yeh and M Zmijewski. 1996. Discovery, purification, and properties of phthalyl amidase from *Xanthobacter agilis*. J Mol Catalysis 2: 53–69.

6 Cantwell CA, RJ Beckman, P Whiteman, SW Queener and EP Abraham. 1992. Isolation of deacetoxycephalosporin C from fermentation broths of *Penicillium chrysogenum* transformants: construction of a new fungal biosynthetic pathway. Proc R Soc Lond B 248: 283–289.

7 Cox KL, SE Fishman, JL Larson, R Stanzak, PA Reynolds, WK Yeh, RM Van Frank, VA Birmingham, CL Hershberger and ET Seno. 1987. The use of recombinant DNA techniques to study biosynthesis and resistance in *Streptomyces fradiae*. J Nat Products 49: 971–980.

8 Debono M, WW Turner, L LaGrandeur, FJ Burkhardt, JS Nissen, KK Nichols, MJ Rodriguez, MJ Zweifel, DJ Zeckner, RS Gordee, J Tang and TR Parr Jr. 1995. Semisynthetic chemical modifications of the antifungal lipopeptide echinocandin B (ECB): structure-activity studies of the lipophilic and geometric parameters of polyarylated acyl analogs of ECB. J Med Chem 38: 3271–3281.

9 Dotzlaf JE and WK Yeh. 1987. Copurification and characterization of deacetoxycephalosporin C synthetase/hydroxylase from *Cephalosporium acremonium*. J Bacteriol 169: 1611–1618.

10 Dotzlaf JE and WK Yeh. 1989. Purification and properties of deacetoxycephalosporin C synthase from recombinant *Escherichia coli* and its comparison with the native enzyme from *Streptomyces clavuligerus*. J Biol Chem 264: 10219–10227.

11 Fishman SE, K Cox, L Larson, PA Reynolds, ET Seno, WK Yeh, RM Van Frank and CL Hershberger. 1987. Cloning genes for the biosynthesis of a macrolide antibiotic. Proc Natl Acad Sci USA 84: 8248–8252.

12 Huang SL, TC Hassell and WK Yeh. 1993. Purification and properties of NADPH-dependent tylosin reductase from *Streptomyces fradiae*. J Biol Chem 268: 18987–18993.

13 Jensen SE, DWS Westlake and S Wolfe. 1985. Deacetoxycephalosporin C synthetase and deacetoxycephalosporin C hydroxylase are two separate enzymes in *Streptomyces clavuligerus*. J Antibiot Tokyo 38: 263–265.

14 Kovacevic S and JR Miller. 1991. Cloning and sequencing of the β-lactam hydroxylase gene (*cef*F) from *Streptomyces clavuligerus*: gene duplication may have led to separate hydroxylase and expandase activities in Actinomyces. J Bacteriol 173: 398–400.

15 Kovacevic S, BJ Weigel, MB Tobin, TD Ingolia and JR Miller. 1989. Cloning, characterization, and expression in *Escherichia coli* of *Streptomyces clavuligerus* gene encoding deacetoxycephalosporin C synthetase. J Bacteriol 171: 754–760.

16 Kreuzman AJ, JR Turner and WK Yeh. 1988. Two distinctive *O*-methyltransferases catalyzing penultimate and terminal reactions of macrolide antibiotic (tylosin) biosynthesis: substrate specificity, enzyme inhibition and kinetic mechanism. J Biol Chem 263: 15626–15633.

17 Kreuzman AJ and WK Yeh. 1990. Purification and properties of cell-associated echinocandin B deacylase from *Actinoplanes utahensis*. In: Abstracts of The ASM Conference on Biotechnology, p 10, Chicago, IL.

18 Kreuzman AJ, JM Zock, JT Vicenzi, JE Dotzlaf, SW Queener and WK Yeh. 1997. Enzymatic synthesis of diastereospecific carbacephem intermediates using serine hydroxymethyltransferase. J Ind Microbiol 19: 369–377.

19 Moore J and FH Arnold. 1996. Directed evolution of a para-nitrobenzyl esterase for aqueous-organic solvents. Nat Biotech 14: 458–467.

20 Queener SW, RJ Beckman, CA Cantwell, RL Hodges, DL Fisher, JE Dotzlaf, WK Yeh and D McGilvray. 1992. High level expression of a bacterial *cef*F gene in *Penicillium chrysogenum*. In: ACS Conference Proceedings Series, Harnessing Biotechnology for the 21st Century, pp 49–51, New Orleans, Louisiana.

21 Samson SM, JE Dotzlaf, ML Slisz, GW Becker, RM Van Frank, LE Veal, WK Yeh, JR Miller, SW Queener and TD Ingolia. 1987. Cloning and expression of the fungal expandase/hydroxylase gene involved in cephalosporin biosynthesis. Bio/technology 5: 1207–1214.

22 Seno ET and RH Baltz. 1982. S-Adenosyl-l-methionine: macrocin *O*-methyltransferase activities in a series of *Streptomyces fradiae* mutants that produce different levels of macrolide antibiotic tylosin. Antimicrob Agents Chemother 21: 756–763.

23 Skatrud PL, AJ Tietz, TD Ingolia, CA Cantwell, DF Fisher, JL Chapman and SW Queener. 1989. Use of recombinant DNA to improve production of cephalosporin C by *Cephalosporium acremonium*. Biotechnology 7: 477–485.

24 Subramanian V, TN Liu, WK Yeh and DT Gibson. 1979. Toluene dioxygenase: purification of iron-surfur protein by affinity chromatography. Biochem Biophys Res Commun 91: 1131–1139.

25 Subramanian V, TN Liu, WK Yeh, M Narro and DT Gibson. 1981. Purification and properties of NADH-ferredoxinTOL reductase: a component of toluene dioxygenase from *Pseudomonas putida*. J Biol Chem 256: 2723–2730.

26 Subramanian V, TN Liu, WK Yeh, CM Serdar, LP Wacket and DT Gibson. 1985. Purification and properties of ferredoxinTOL: a component of toluene dioxygenase from *Pseudomonas putida* F1. J Biol Chem 260: 2355–2363.

27 Usui S and CA Yu. 1989. Purification and properties of isopenicillin N epimerase from *Streptomyces clavuligerus*. Biochim Biophys Acta 999: 78–85.

28 Yeh WK, P Flether and LN Ornston. 1980. Evolutionary divergence of coselected β-ketoadipate enol-lactone hydrolases in *Acinetobacter calcoaceticus*. J Biol Chem 255: 6342–6346.

29 Yeh WK, SK Ghag and SW Queener. 1992. Enzymes for epimerization of isopenicillin N, ring expansion of penicillin N, and 3′-hydroxylation of deacetoxycephalosporin C: function, evolution, refolding and enzyme engineering. Ann NY Acad Sci 672: 396–408.

30 Yeh WK, DT Gibson and TN Liu. 1977. Toluene dioxygenase: a multicomponent enzyme system. Biochem Biophys Res Commun 78: 401–409.

31 Yeh WK and SW Queener. 1990. Potential industrial use of cephalosporin biosynthetic enzymes and genes. Ann NY Acad Sci 613: 128–141.