Towards Biocatalytic Synthesis of β-Lactam Antibiotics

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Received May 23, 2001; Accepted June 25, 2001

Abstract: This review describes the remarkable transition in the manufacture of β-lactam antibiotics, which is driven by the desire to reduce or eliminate the production of waste and the dependence on organic solvents. To this effect, traditional chemical procedures are gradually being replaced by biotransformations. The β-lactam antibiotics industry has led the way in the introduction of biocatalysis in the fine chemicals industry by replacing the chemical multi-step process for the penicillin nucleus 6-aminopenicillanic acid (6-APA) by an enzymatic one in the early 1990's. Recently, bioprocesses have been developed for the synthesis of the cephalosporin nucleus, 7-aminodeacetoxycephalosporanic acid (7-ADCA) from a penicillin precursor and will shortly be commercialized. Thirty years of research have now resulted in viable enzymatic procedures for coupling the β-lactam nuclei with D-phenylglycine side-chains. The necessary adaptations in the synthesis of the side-chain donors have likewise resulted in more efficient procedures.

Keywords: ampicillin; amoxicillin; antibiotics; cephalexin; cefadroxil; enzyme catalysis

1 Introduction

There is a marked trend in the fine chemicals industry towards the replacement of classical organic methods by cleaner catalytic alternatives that minimize or eliminate the generation of (mainly inorganic) waste and avoid, where possible, the use of toxic and/or hazardous reagents and solvents. In the past, waste disposal was not a serious problem in the small-volume fine chemicals industry and only cursory attention was given to energy and environmental efficiency. However, if one considers the amount of waste formed per kg product[1,2] – the E factor[3] – there is a clear need for waste reduction in the fine chemicals and pharmaceutical industries (Table 1).

From the viewpoint of environmental acceptability, biocatalytic procedures offer obvious benefits: reactions are performed in water at neutral pH and temperature close to ambient, without the need for extensive functional group protection and/or activation, and high chemo-, regio-, and stereoselectivities are realized. In recent years many (hydrolytic) enzymes have become readily available at low cost owing to their large-scale application in, for example, laundry formulations. This has stimulated their application in the fine chemicals industry. In short, the unique characteristics of enzymes provide opportunities for developing environmentally acceptable procedures that are more efficient in the utilization of energy and raw

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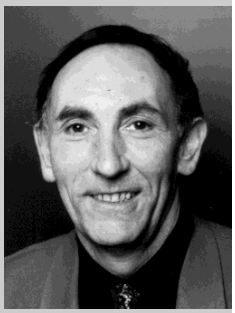
Margreth Wegman (1970) studied organic chemistry at the University of Nijmegen, where she did her diploma work under the supervision of Prof. B. Zwanenburg (1996). She moved to the Delft University of Technology, where she received her Ph.D. in 2001 for work on the synthesis of side-chain donors for semi-synthetic β-lactam antibiotics under the supervision of Prof. Roger Sheldon and Dr. Fred van Rantwijk, in collaboration with DSM Life Science Products. In 2001 she joined Avantium Technologies in Amsterdam.

Michiel Janssen (1974) studied bioprocess engineering at Wageningen Agricultural University. He did his diploma research under the joint supervision of Dr. Maurice Franssen, Laboratory of Organic Chemistry, Wageningen Agricultural University, and Dr. Harro Bouwmeester, Plant Research International, The Netherlands and received the M.Sc. degree in agricultural sciences in 1998. He moved to the Delft University of Technology in 1998 to do his Ph.D. research on the immobilization of penicillin acylase for application in enzymatic β-lactam antibiotic synthesis, under the supervision of Prof. Roger Sheldon and Dr. Fred van Rantwijk, in collaboration with DSM Life Science Products.

Fred van Rantwijk (1943) studied organic chemistry at the Delft University of Technology where he remained as a staff-member. He received his Ph.D. in 1980, for work under the guidance of Professor H. van Bekkum. Since the late 1980's he has been working on the application of enzymes in organic synthesis. His particular research interests are the use of enzymes in non-natural reactions, enzyme immobilization, and transformations using multi-enzyme systems.



Roger Sheldon was born in Nottingham (UK) in 1942. He received a PhD in organic chemistry from the University of Leicester (UK) in 1967. This was followed by post-doctoral studies with Prof. Jay Kochi in the U.S. From 1969-1980 he was with Shell Research in Amsterdam and from 1980-1990 he was R&D Director of DSM Andeno. In 1991 he moved to his present position as Professor of organic chemistry and catalysis at the Delft University of Technology (The Netherlands). His primary research interests are in the application of catalytic methodologies – homogeneous, heterogeneous, and enzymatic – in organic synthesis, particularly in relation to fine chemicals production. He developed the concepts of E factors and atom utilization for assessing the environmental impact of chemical processes. He is the author of three books on catalysis, one (with Jay Kochi) on catalytic oxidations, one on syn gas chemistry, and, more recently, one on chirotechnology. Amongst other awards he received the 1997 Paul Rylander Award of the Organic Reactions and Catalysis Society.

Table 1. Production of waste in various branches of the chemical industry[1,2]

Industry branch	Product (t/a)	E factor[a]
Oil refining	$10^6 - 10^8$	<< 0.1
Bulk chemicals	$10^4 - 10^6$	1 – 5
Fine chemicals	$10 - 10^4$	5 – 50
Pharmaceutics	$1 - 10^5$	25 – > 100

[a] By-product/product (kg/kg).

materials and rely much less on the use of stoichiometric amounts of auxiliaries and environmentally unacceptable solvents. Moreover, they often afford products of superior quality. This review focuses on the particularly relevant case of β-lactam antibiotics production, which is gradually evolving from virtually complete reliance on chemical methods to biocatalysis-based syntheses with substantial reductions in the number of reaction steps, usage of chemicals and solvents, and waste generated, combined with attractive cost price advantages.[4] The long market life-time of the semi-synthetic penicillins and cephalosporins – first introduced over 40 years ago – has greatly encouraged these developments.

2 Semi-Synthetic β-Lactam Antibiotics: Industrial Production Prior to 1985

Following Alexander Fleming's discovery in 1928[5] of the antibacterial activity of the mold *Penicillium notatum* more than a decade passed before Florey and Chain finally isolated penicillin G (Figure 1) in 1940.[6] This was quickly followed by its characterization and clinical application. Unfortunately, an increasing number of bacterial strains became resistant to penicillin G, which was phased out of clinical use in the late 1960's. Fortunately, acylation of the nucleus of penicillin G, 6-aminopenicillanic acid (6-APA), with various side-chains, afforded new active semi-synthetic penicillins. The most important of these are ampicillin and amoxicillin (Figure 1), in which the side-chain is D-phenylglycyl and a D-*p*-hydroxyphenylglycyl, respectively. Subsequently, the related semi-synthetic cephalosporins, derived from the 7-aminodeacetoxycephalosporanic acid (7-ADCA) nucleus, were introduced in clinical practice. The structures of cephalexin and cefadroxil, the cephalosporin analogues of ampicillin and amoxicillin, are shown in Figure 1.

Up to 1985, these semi-synthetic penicillins and cephalosporins, and related β-lactam antibiotics, were produced *via* chemical procedures, with the exception of the raw material, penicillin G, which was obtained *via* fermentation of *Penicillium chrysogenum*.[7] Subsequently, the phenylacetyl side-chain of penicillin G was cleaved off and the resulting 6-APA[8] was protected and condensed with the appropriate activated and protected side-chain derivatives. These were likewise synthesized using classical pro-

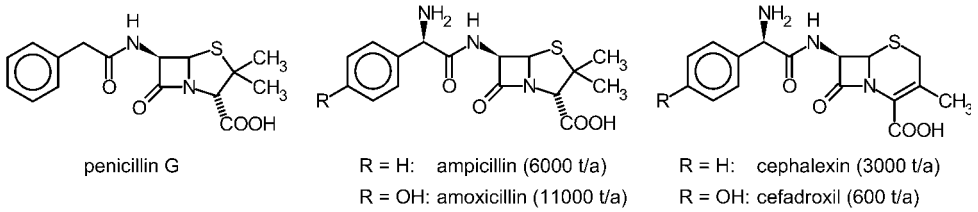
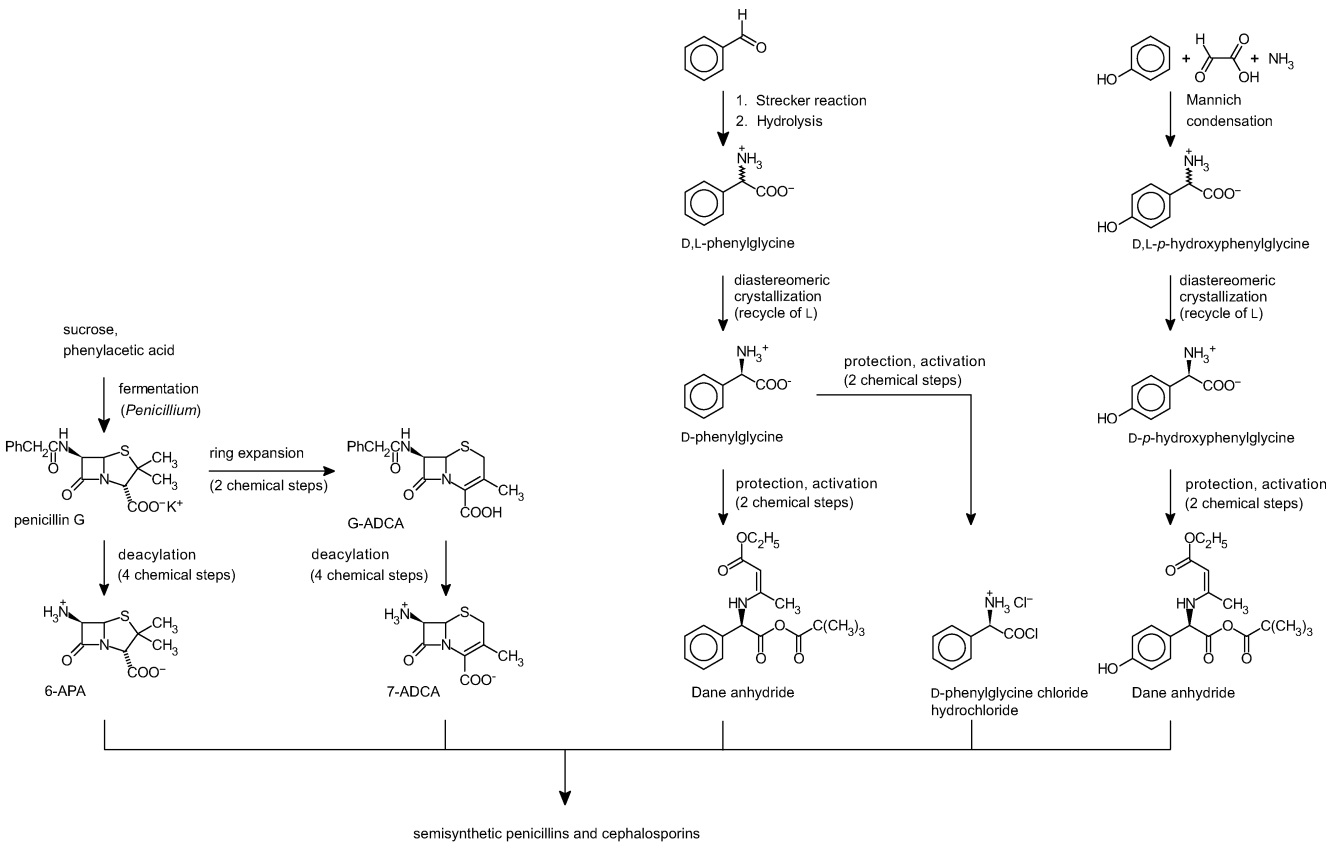


Figure 1. The four most important semi-synthetic penicillins and cephalosporins and their parent compound.



Scheme 1. An overview of the traditional, chemical synthesis of β-lactam antibiotics.

cedures (Scheme 1). The intermediate for the semi-synthetic cephalosporins, 7-ADCA, was obtained from penicillin G *via* a chemical ring expansion. In short, the triumph of Chemistry over Nature must have seemed complete. The processes involved will be discussed in more detail later in this review.

Nearly all of these procedures concerned "stoichiometric" chemical transformations and, hence, suffered from all of the shortcomings set out above. Even nowadays, 15 years hence, these conventional procedures still dominate the industrial-scale production of β-lactam antibiotics. As an illustration, the production of cephalexin which is, with an annual consumption of almost 3000 tons, the largest cephalosporin on the world market, generates 30 – 40 kg waste per kg of end-product.

It has become increasingly clear that new, more efficient synthetic methodologies for the production of β-lactam antibiotics are urgently required. This review will describe the accomplishments of the past 15 years and discuss the future prospects.

3 Biocatalytic Synthesis of β-Lactam Nuclei

3.1 6-Aminopenicillanic Acid

In the early 1960's 6-APA was produced[9] *via* enzymatic deacylation of penicillin G using penicillin G acylase (penicillin amidohydrolase, E.C. 3.5.1.11),[10,11,12,13] a heterodimeric Ntn serine hydrolase.[14,15] The procedure was inconvenient as well as inefficient by present-day standards: the productivity was low due to the large reaction volumes and the biocatalyst was discarded after use. Hence, improvement was sought by performing the cleavage by chemical means.

Efficient deacylation of penicillin G is not trivial, because it contains a tertiary as well as a secondary amide functionality. The secondary one should be cleaved whereas the tertiary one is more susceptible to basic and nucleophilic conditions. An efficient chemical deacylation process of penicillin G was ultimately developed[16] and commercialized by the Nederlandsche Gist en Spiritusfabriek (now a subsidiary of DSM). The selective cleavage of the secondary amide bond was accomplished *via* its transformation into an imine chloride, using phosphorus pentachloride in dichloromethane, followed by hydrolysis. The key step in this process was the use of the silyl group for the protection of the penicillin carboxyl group. This inexpensive one-pot synthesis of 6-APA is often referred to the "Delft Cleavage" (Figure 2).

This "Delft Cleavage" was fairly typical of its time: it was energy-intensive due to the low operating temperature, depended on hazardous, highly active chemicals, which were difficult to recover and reuse, and generated an excessive amount of waste. Nevertheless, it was universally favored over the microbial procedure mentioned earlier and remained in use for 15 – 20 years.

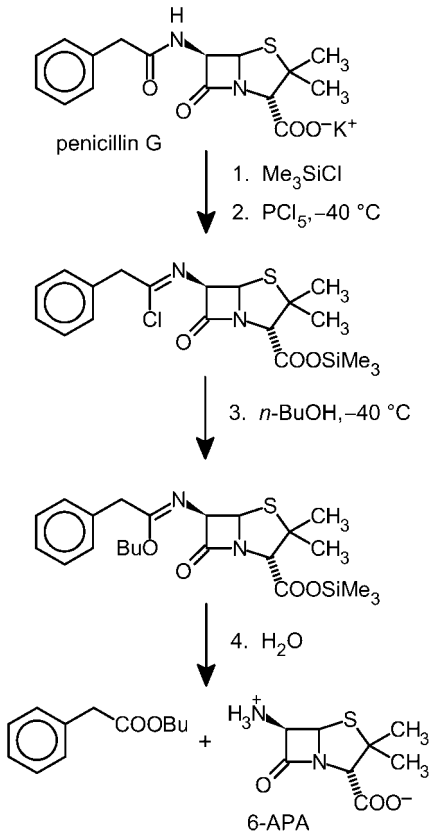


Figure 2. Chemical deacylation of penicillin G into 6-APA.

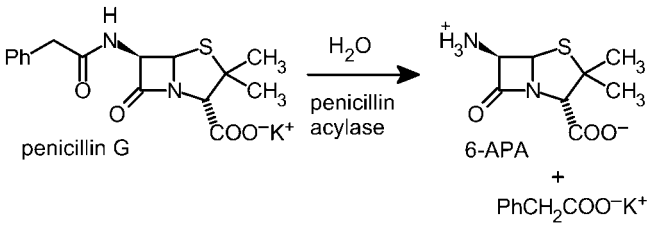


Figure 3. Enzymatic deacylation of penicillin G into 6-APA.

Meanwhile, developments in the enzymes field continued.[17] Penicillin G acylases with improved stability were obtained by screening and, by employing recombinant DNA technology, efficient production was achieved. Combined with efficient immobilization, which made recycling of the biocatalyst possible,[18,19] dramatic reductions in enzyme costs were forthcoming.[20]

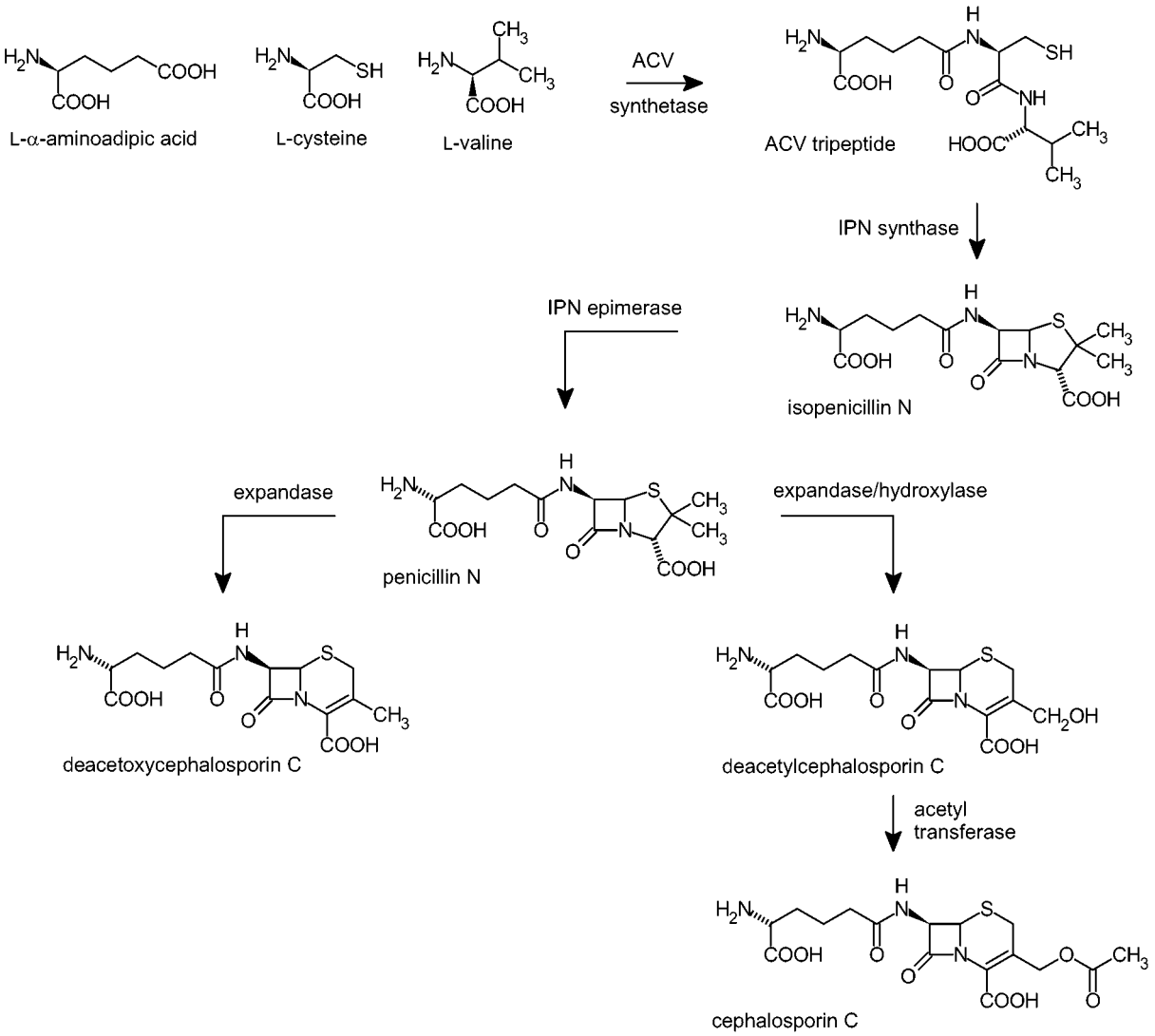
The enzymatic cleavage of penicillin G (Figure 3) is carried out in water at a slightly elevated temperature, rather than in halogenated solvent at –40 °C and affords 6-APA in excellent yield.[21] As an addi-

tional advantage, the liberated phenylacetic acid is recycled into the fermentation. The economic, environmental, and operational advantages of the enzymatic process[22,23] over the chemical "Delft Cleavage" are such that the latter process has been universally replaced by the former.[24,25]

3.2 7-Aminodeacetoxycephalosporanic Acid

In 1953 a new β-lactam antibiotic – cephalosporin C – was isolated from the fermentation broth of *Acremonium chrysogenum*.[26,27] Although its antibacterial activity was too low for therapeutic use, its spectrum appeared to be very interesting, displaying activity mainly against Gram-negative bacteria. However, production of cephalosporin C by fermentation of *A. chrysogenum* is not selective. Up to 20% of the structurally closely related deacetylcephalosporin C (see Scheme 2), as well as penicillin N, are formed besides cephalosporin C, which complicates the isolation of the latter.

The laborious isolation of cephalosporin C initiated studies into the relationship between cephalosporins and penicillins. It was found by Morin et al. that a cephalosporin nucleus could be obtained *via* sequential sulfoxidation, esterification, and dehydratation/expansion of the penicillin nucleus.[28] In consequence, the 3-substituent was a methyl group rather than an acetoxy group and 7-ADCA was obtained. The yields were low, initially, and a tremendous number of papers dealing with all aspects of the Morin procedure has appeared.[29] The carboxylic acid protecting group can be chosen such that deprotection takes place during isolation of the ring-expanded product. The best example is the use of trimethylsilyl protection.[30] For example, reaction of penicillin G sulfoxide with pyridinium hydrobromide and *N,N'*-bis(trimethylsilyl)urea in toluene afforded the cepha-



Scheme 2. Biosynthesis of cephalosporin C and related β-lactam compounds in *A. chrysogenum*.

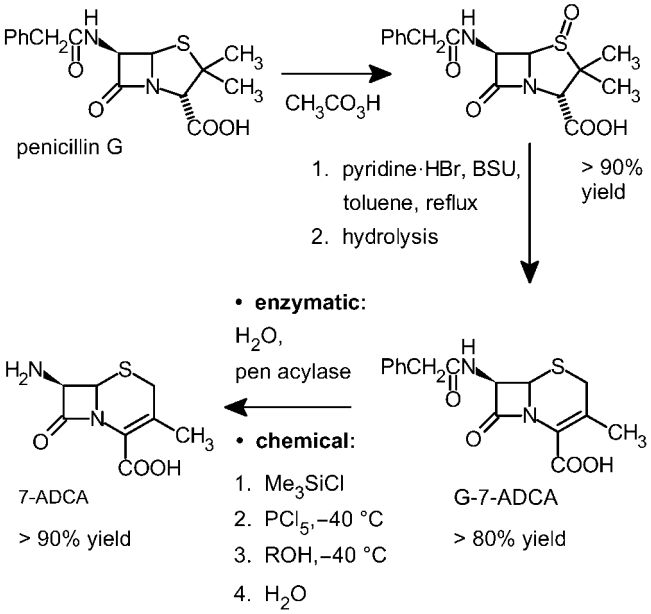


Figure 4. Chemical synthesis of 7-ADCA from penicillin G [BSU: *N,N'*-bis(trimethylsilyl)urea].

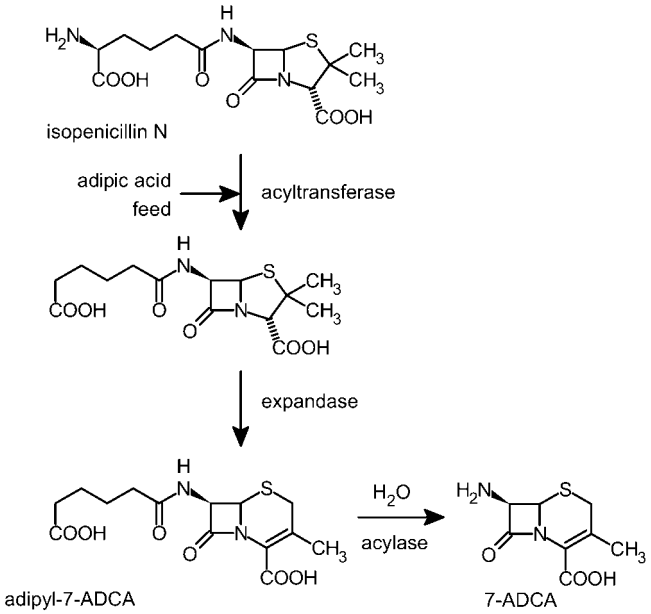


Figure 5. An all-bioprocess for 7-ADCA: biosynthesis of adipyl-7-ADCA in a recombinant *P. chrysogenum* and enzymatic hydrolysis of the adipyl group.

losporin derivative G-7-ADCA in > 80% yield (Figure 4).[31] "Delft Cleavage"[16] of the latter afforded 7-ADCA, the main starting material for cephalosporin antibiotics.

Analogous to 6-APA, the production of 7-ADCA by chemical deacylation was subsequently replaced by a simple enzymatic conversion, using an immobilized penicillin G acylase.[23,32] The worldwide production of 7-ADCA from penicillin G in 2000 amounted to almost 3000 tons, which was mainly used in the production of cephalexin.[24]

The replacement of the two-step chemical ring expansion by a biotransformation, preferably in the course of the fermentation, would have obvious economic and environmental benefits.[24] Developing a biocatalytic route to 7-ADCA proved to be far from trivial, however, due to a complete lack of strains that produce sufficient quantities of deacetoxycephalosporin C. The crucial step in the biosynthesis of the latter is the expansion, catalyzed by deacetoxycephalosporinase (expandase), of the 5-membered ring in penicillin N into the 6-membered ring in deacetoxycephalosporin C (see Scheme 2).[33] Penicillin N, however, is not commercially available and, unfortunately, expandase does not accept 6-APA, penicillin G, or penicillin V as a substrate. Even genetically modified expandase could not accomplish the ring expansion.[34] Clearly, the expandase activity is largely dependent on the structure of the substrate.

A solution was found by introducing the genes for isopenicillin N epimerase and penicillin N expandase from *Streptomyces clavuligerus* into *Penicillium chrysogenum*, which naturally does not produce either penicillin N or any cephalosporin. Stable transformants of *P. chrysogenum* that produced deacetoxycephalosporin C were obtained.[35] In addition, it had already been found in the early days of cephalosporin chemistry that, when adipic acid (instead of phenylacetic acid or phenoxyacetic acid) was fed to *P. chrysogenum* fermentations, the fungus synthesized adipyl-6-APA instead of penicillin G or V, respectively.[36] These two concepts were the lead for Crawford et. al.[37] to feed adipic acid to a transgenic *P. chrysogenum* strain that also expressed the expandase gene of *S. clavuligerus*, resulting in the synthesis of adipyl-7-ADCA (Figure 5). The production level of adipyl-7-ADCA was improved *via* site-directed mutagenesis of the expandase.[38] Related β-lactam compounds are also produced, however, because the ring expansion is neither complete nor selective. Hence, a considerable effort has been devoted to developing efficient procedures for the isolation of adipyl-7-ADCA from the culture broth.[39] A new enzyme, dicarboxylic acid acylase, was developed to remove the side-chain from adipyl-7-ADCA,[24,40] because the latter is not a substrate for penicillin G acylase.

It was subsequently shown that the precursor side-chain was not restricted to adipic acid,[41] although expansion of penicillin G proved elusive. Surprisingly, Gist-brocades recently reported that the expandase from *S. clavuligerus*, expressed in the penicillin G producing *P. chrysogenum*, could be dedicated to the ring expansion of penicillin G.[42] The method was subsequently improved by site-directed mutagenesis of the expandase[43] and the conditions that allow the ring expansion have been elucidated.[44] A

major advantage of this procedure is that the side-chain can readily be removed by penicillin G acylase.

A new method was published recently, which provides a solution for the cross-contamination of the recombinantly produced 7-ADCA derivative alluded to above. Inactivation of the expandase/hydroxylase gene (see Scheme 2) in the industrial *Acremonium chrysogenum* strain caused the accumulation of large amounts of penicillin N.[45] Further expression of the expandase gene from *S. clavuligerus* in these knock-out strains caused the *in vivo* expansion of penicillin N into deacetoxycephalosporin C without cross-contamination with other cephalosporins.

The transformation of deacetoxycephalosporin C into 7-ADCA is somewhat complicated due to the lack of an enzyme for removing the D-α-aminoadipyl side-chain. This can be accomplished, however, by using a two-step enzymatic bioconversion that had originally been developed for the manufacture of 7-ACA from cephalosporin C.[46] In the first step a D-amino acid oxidase (EC 1.4.3.3) mediates the oxidative deamination of the D-α-aminoadipyl side-chain into the corresponding α-keto residue, which undergoes oxidative decarboxylation into the glutaryl derivative.[47] The latter is subsequently hydrolyzed by a glutaryl acylase.[48] The glutaryl acylase bears a close resemblance to the dicarboxylic acid acylase used in the enzymatic synthesis of 7-ADCA from adipyl-7-ADCA.

In conclusion, the key β-lactam nuclei 6-APA and 7-ADCA can now be entirely synthesized *via* biotransformations at great savings in chemicals, solvents, and energy.

4 Biocatalytic Routes to Side-Chains

4.1 Synthesis of the Side-Chain Building Blocks

Océ-Andeno (now DSM Fine Chemicals) developed, in the early 1970's, a process for the manufacture of D-phenylglycine *via* crystallization of a diastereomeric salt of its racemate. The latter is readily available from a Strecker reaction on benzaldehyde, followed by hydrolysis of the nitrile. Optically pure (+)-camphor-8-sulfonic acid in aqueous medium is used as the resolving agent (Figure 6). L-Phenylglycine is racemized in a separate step and recycled.[49]

Kanegafuchi (nowadays Kaneka Corporation) developed a more elegant procedure, following the example of D-*p*-hydroxyphenylglycine (see later), which involved a D-hydantoinase-mediated, enantioselective hydrolysis of 5-phenylhydantoin. The latter is readily prepared from benzaldehyde, HCN, and urea. The hydrolysis can be turned into a dynamic kinetic resolution by racemization of 5-L-phenylhydantoin under mildly alkaline conditions to give *N*-carbamoyl-D-phenylglycine in 100% yield. The *N*-carbamoyl group was subsequently removed *via* treatment with nitrous acid (but see later).

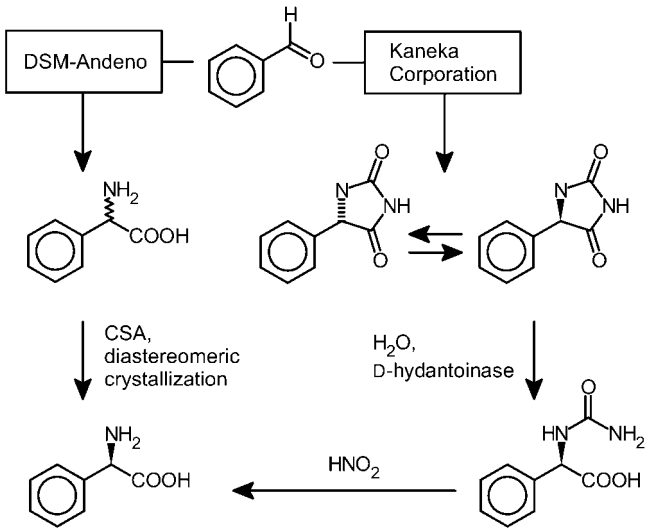


Figure 6. Production routes to D-phenylglycine [CSA: (+)-camphor-8-sulfonic acid].

All procedures for D-*p*-hydroxyphenylglycine start from phenol, since *p*-hydroxybenzaldehyde is too expensive. A Mannich-type condensation with ammonia and glyoxylic acid afforded racemic *p*-hydroxyphenylglycine, which was resolved via crystallization with 3-bromocamphor-8-sulfonic acid, combined with racemization and recycling of the L-enantiomer. Kaneka established, in 1979 (after initial research results by Snam Progretti in Italy), the industrial production of D-*p*-hydroxyphenylglycine *via* a dynamic kinetic resolution of D,L-5-*p*-hydroxyphenylhydantoin, again using a D-specific hydantoinase. Decarbamoylation was initially performed by treating *N*-car-

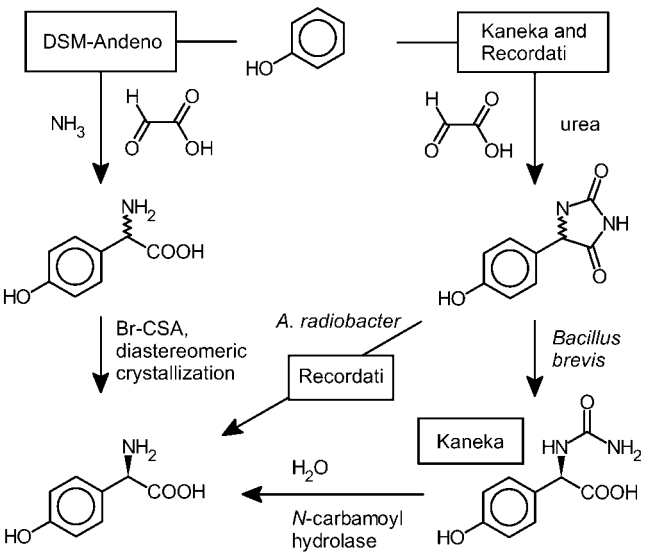


Figure 7. Processes for D-*p*-hydroxyphenylglycine [Br-CSA: (+)-3-bromocamphor-8-sulfonic acid].

bamoyl-D-*p*-hydroxyphenylglycine with nitrous acid[50] (Figure 7).

Recordati (Italy) developed a closely related, more elegant route that employed the microorganism *Agrobacterium radiobacter*, which contains a D-specific hydantoinase as well as a D-carbamoylase.[51] Hence, the complete conversion of the racemic hydantoin into D-*p*-hydroxyphenylglycine was accomplished in a one-step, two-enzyme process (Figure 7). Kaneka Corporation responded by cloning a D-carbamoylase gene derived from *Agrobacterium* sp. KNK712 and expressing this into *E. coli*. Subsequent mutagenesis resulted in a more stable enzyme with respect to temperature and pH.[52,53] The immobilized mutant D-carbamoylase has been used since 1995 in the commercial production of D-*p*-hydroxyphenylglycine.[54] Nowadays, all D-*p*-hydroxyphenylglycine is produced using a hydantoinase process.

4.2 Synthesis of Activated Side-Chain Donors

All methodologies for coupling the phenylgycine side-chains to the β-lactam nucleus, chemical as well as biocatalytic ones, require an activated side-chain donor. This is due to the zwitterionic nature of α-amino acids, which at near-neutral pH represents the minimum on the chemical energy surface, rendering the carboxyl function unreactive towards any nucleophile.

Activation of D-phenylglycine has been accomplished *via* transformation into the Dane anhydride (see later) or its acid chloride hydrochloride. The latter reaction was carried out in two steps, with HCl and PCl_5, respectively, in a halogenated hydrocarbon solvent (Figure 8).

By analogy, the side-chain donor for amoxicillin and cefadroxil would be *p*-hydroxyphenylglycyl chloride hydrochloride, but the latter is not available in the desired purity at an economically attractive price. Hence, the so-called Dane salt method[55] was universally adopted for D-*p*-hydroxyphenylglycine. The Dane salt is prepared from D-*p*-hydroxyphenylglycine and methyl acetoacetate and is subsequently converted to a mixed anhydride with, e.g., pivaloyl chloride[55] or methyl chloroformate[56] in the presence of a base (Figure 9). The Dane anhydride cannot be isolated, however, and must be converted *in situ* at low temperature. Subsequently, the Dane anhydride also became the preferred donor for D-phenylglycine.[57]

The shift from traditional multi-step chemical procedures for β-lactam antibiotics to biocatalytic ones, which will be discussed later, also influenced the nature and the production method of the side-chain donors. The key intermediates in the chemical coupling are D-phenylglycine and its *p*-hydroxy derivative, which are subsequently subjected to an activation step. Enzymatic coupling also requires an activated side-chain donor[58,59] such as an ester, amide, or mixed anhydride. However, preparing these from the amino acid, which represents a minimum on the chemical energy surface, would be evidently inefficient. The synthetic route should rather be designed in such a manner that the chemical energy in the primary intermediate is conserved. Hence, D-phenylglycine amide rather than the ester is the preferred donor in an industrial context, as it is prepared from a precursor of D-phenylglycine, whereas the ester is synthesized from D-phenylglycine itself and, hence, would require an extra step.

A synthetic procedure for D-phenylglycine amide, *via* the enantioselective hydrolysis of the Strecker-derived racemic amide[60] in the presence of an L-specific amidase, had already been reported by DSM in the mid-1970's.[61] A process (see Figure 10), using the L-specific aminopeptidase in *Pseudomonas putida* ATCC 12633,[62,63] was subsequently developed.[64] The inherent disadvantage of this resolution process is that the desired product is obtained, or rather re-

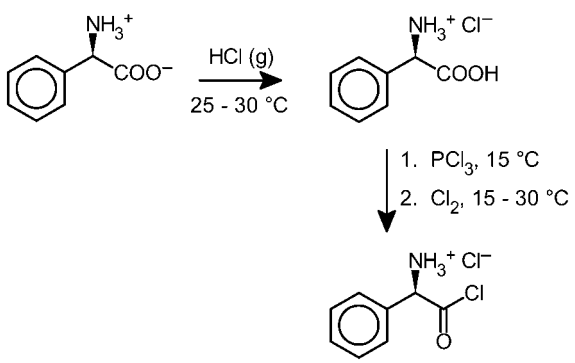


Figure 8. Synthesis of D-phenylglycyl chloride hydrochloride.

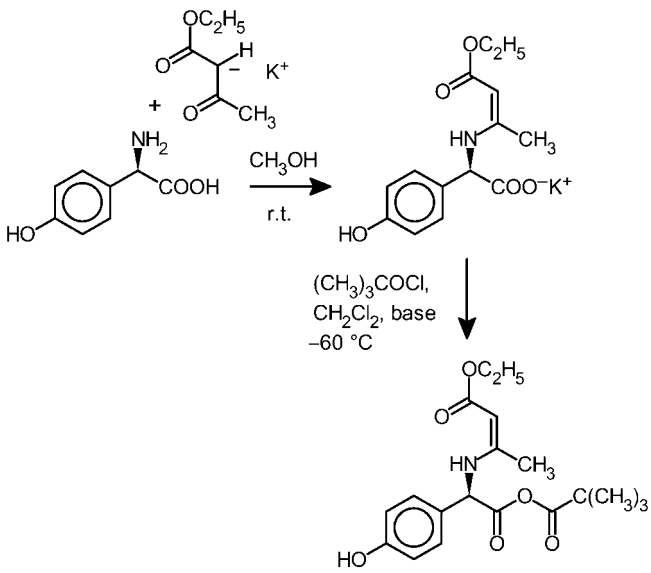


Figure 9. Activation of D-*p*-hydroxyphenylglycine as its Dane anhydride.

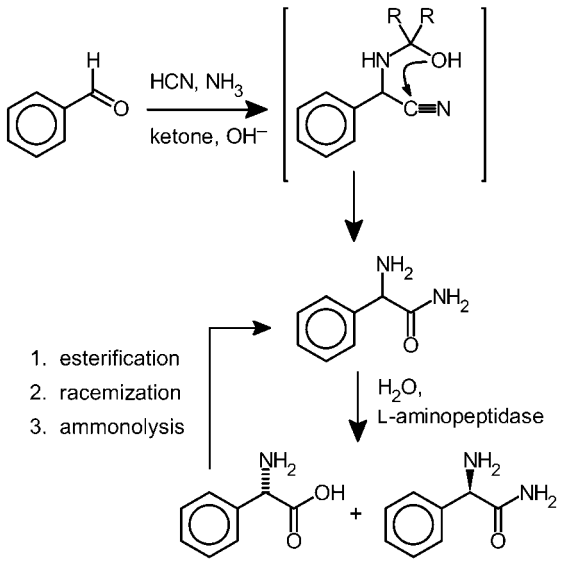


Figure 10. The DSM aminopeptidase process for D-phenylglycine amide.[64]

tained, in a maximum yield of 50%. Recycling the unwanted L-phenylglycine into the D,L-amide *via* a conventional procedure would require 3 steps – esterification, racemization, and ammonolysis.[64a,65] It would also generate copious amounts of salts because the esterification of α-amino acids requires a stoichiometric, rather than a catalytic, amount of acid since the reactant as well as the product are basic. Hence, such a conventional recycle is economically (as well as energetically) unattractive. The short-term solution is to racemize the L-phenylglycine and add the racemate to the existing process for resolution of phenylglycine by diastereomeric salt crystallization. However, this option may not be available in a longer-term scenario where it is expected that that the classical resolution process will be phased out.

A catalytic, salt-free, approach to the reactivation of L-phenylglycine would require a methodology for performing an acid-catalyzed reaction in a bulk basic medium. The latter problem was solved by using a solid acid catalyst, the naphtha cracking catalyst zeolite H-USY[66], in which access to the acidic sites is restricted. Thus, phenylglycine, as well as other α-amino acids, were esterified in the presence of a catalytic amount of H-USY.[67] The reaction was accompanied by racemization due to the basic medium.

We had previously shown that *Candida antarctica* lipase B catalyzes the enantioselective ammonolysis of phenylglycine ester into D-phenylglycine amide.[68] Combining this procedure with *in-situ* racemization of the slow-reacting L-phenylglycine ester, mediated by an aromatic aldehyde such as pyridoxal, resulted in a dynamic kinetic resolution. The enantioselectivity of the lipase-catalyzed ammonolysis, which initially was rather low,[69] was improved by performing the reaction at –20 °C and D-phenylglycine amide was obtained in 85% yield and 88% ee after 66 h[70] (Figure 11). To become economically feasible, however, further improvement of both the enantioselectivity and the reaction rate is required.

DSM developed a dynamic resolution of phenylglycine amide by elegantly combining a diastereomeric crystallization of the L-mandelic acid salt of D,L-phenylglycine amide with a benzaldehyde-catalyzed *in situ* racemization of the L-amide[71] (Figure 12). D-Phenylglycine amide is obtained in nearly quantitative yield with high ee. The L-mandelic acid can be readily recycled after cleavage of the salt, but the need for recycling stoichiometric quantities of resolving agent remains a limitation of this process.

The direct conversion of racemic phenylglycine nitrile (2-amino-2-phenylacetonitrile) into D-phenylglycine amide, using a D-selective nitrile hydratase in a dynamic kinetic resolution (the nitrile is known to racemize easily), would clearly be superior to all existing methodologies (see Figure 13). This route has been more or less disregarded because of a lack of enantioselective nitrile hydratases[72]. We screened

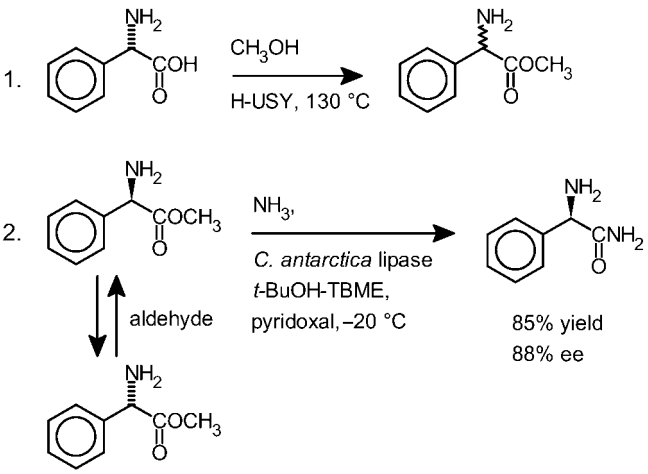


Figure 11. D-Phenylglycine amide *via* salt-free esterification[68] and dynamic kinetic resolution[70].

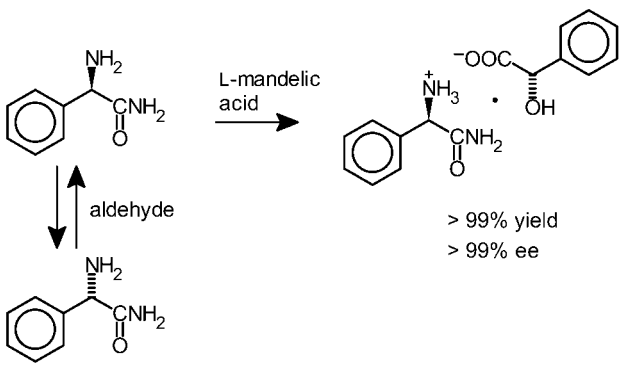


Figure 12. Dynamic resolution of D-phenylglycine amide *via* diastereomeric crystallization[71].

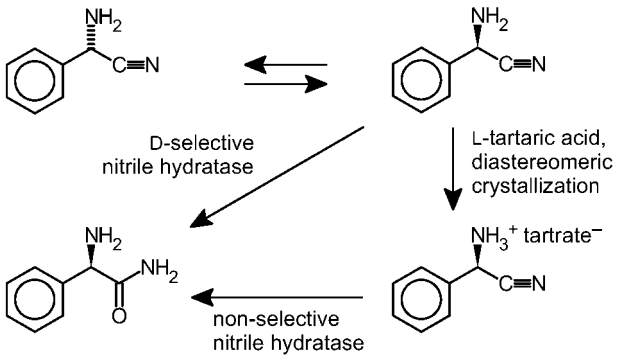


Figure 13. Nitrile hydratase routes towards D-phenylglycine amide.

60 nitrile hydratase-harboring strains and found five that converted D,L-phenylglycine nitrile.[73] None of these was enantioselective, however; the observed enantioselectivity was entirely due to the amidase, as is generally the case.[72]

Alternatively, a less attractive but still interesting proposition would be the stereoretentive nitrile hydratase-mediated hydration of D-phenylglycine nitrile. The latter is accessible *via* asymmetric transformation of the L-tartaric acid salt of the racemic nitrile.[74] Careful optimization was required to minimize the facile racemization and degradation *via* a retro-Strecker reaction. The latter takes place readily at pH ~7, conditions that are optimum for the enzymatic reaction.

We found that the retro-Strecker reaction could largely be circumvented by conducting the reaction in a fed-batch mode (to maintain a low concentration of nitrile) at pH 7 and 5 °C. D-Phenylglycine amide was obtained, at 0.1 M concentration, in >95% yield and >95% ee in the presence of our *Rhodococcus* species or Novo SP361.[75] The enantioselectivity could be further improved, with marginal loss in yield, by prolonging the reaction time. This resulted in selective hydrolysis of the small amounts of L-amide in the product, catalyzed by the L-selective amidase present in the microorganism.

Efficient synthesis of a D-*p*-hydroxyphenylglycine-derived donor is more problematic. Present designs of enzymatic processes for amoxicillin and cefadroxil depend on D-*p*-hydroxyphenylglycine methyl ester, because it can be prepared from the amino acid in one step, using two equivalents of acid and a large excess of methanol. A salt-free esterification, using H-USY, is not feasible because the reaction rate is too low and racemization is predominant.[68]

In short, the shift to biocatalytic coupling procedures for β-lactam antibiotics is accompanied by a change to more efficient processes for the synthesis of the side-chain donors, mainly because the former obviates the protection of the α-amino function that is required in the chemical coupling process.

5 Enzymatic Coupling of the Side-Chains to the β-Lactam Nuclei

5.1 Chemical Procedures

The industrial-scale coupling of 6-APA and 7-ADCA with the appropriate side-chains has a development history of 40 years, which has recently been reviewed in some detail.[4] Ampicillin, the first α-aminobenzylpenicillin to be commercialized (by Beecham), was initially synthesized using conventional peptide synthesis techniques.[76] The industrial-scale synthesis of ampicillin has been dominated by two processes: the one *via* the acid chloride hydrochloride of D-phenylglycine and the one *via* the Dane anhydride (see later).

The acid chloride route involved silyl protection of the carboxyl group in 6-APA *via* treatment with a slight excess of a silylating agent, such as trimethylsilyl chloride or hexamethyldisilazane. Subsequently, the acid chloride was added to the silylated 6-APA at low temperature (Figure 14). Ampicillin was obtained after aqueous acidic removal of the protecting group.[77]

Amoxicillin synthesis *via* the acid chloride was not feasible, as explained above; hence, the Dane anhydride procedure[55,56] was adopted. 6-APA was originally solubilized in the reaction medium as a silyl ester,[56] but this was later found to be unnecessary. The Dane mixed anhydride is prepared *in situ* from the Dane salt at low temperature (see Figure 9) and the triethylamine salt of 6-APA is added. After the condensation, the pH is adjusted to 1 in order to remove

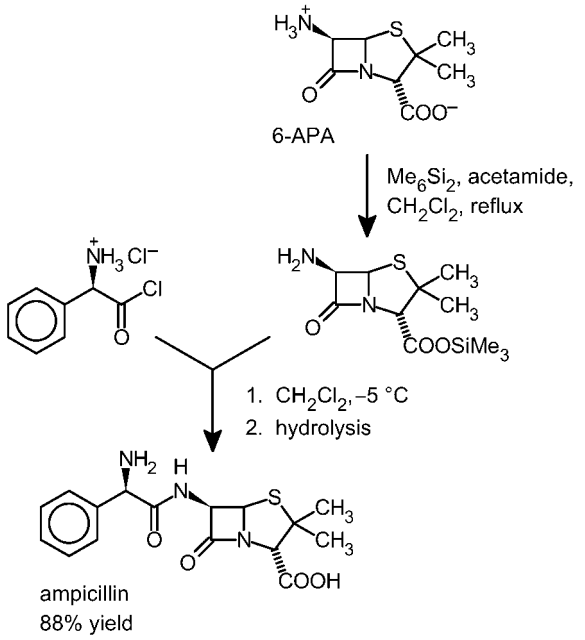


Figure 14. Synthesis of ampicillin *via* the acid chloride hydrochloride of D-phenylglycine.

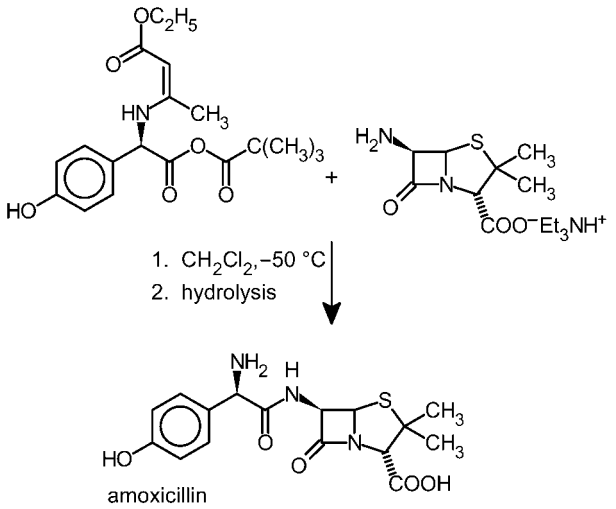


Figure 15. Synthesis of amoxicillin *via* the Dane anhydride; see Figure 9 for the *in-situ* synthesis of the latter.

the amino protecting group and the product is obtained in excellent yield (Figure 15).[56]

Subsequently, the Dane anhydride route was nearly universally adopted for all semisynthetic β-lactam antibiotics, including ampicillin.[4] Solvents and auxiliary reagents are recycled where possible, but the manufacture of 1 kg of cephalexin still generates 30 – 40 kg of non-recyclable waste, as noted above. A large proportion of this originates in the activation, coupling, and deprotection steps. The major part of this waste is not biodegradable and has to be disposed of in an environmentally and socially acceptable manner. Moreover, highly active reagents, such as pivaloyl chloride and silylating reagents, are consumed in stoichiometric amounts. Hence, the desirability of a more efficient procedure, preferably an enzymatic one in aqueous medium, became increasingly recognized in the early 1990's.

5.2 Enzymatic Coupling

5.2.1 Basic Considerations and Process Concepts

The enzymatic synthesis of β-lactam antibiotics can, in principle, be accomplished either *via* – thermodynamically controlled – reversal of the hydrolytic reaction or *via* – kinetically controlled – transacylation.[78] Water is the reaction medium of choice.

The thermodynamically controlled reaction proceeds towards equilibrium, whereas the kinetically controlled reaction may overshoot the equilibrium (see Figure 16), depending on the reaction conditions and the properties of the biocatalyst. The generally accepted catalytic mechanism[79,80] involves an acyl-enzyme intermediate; coupling results when the latter is intercepted by the β-lactam nucleus. Water acts as a competing nucleophile and causes parasitic hydrolysis of the reactant (primary hydrolysis) as well as of the product (secondary hydrolysis). The reaction should be stopped when the synthesis rate (r_S) becomes equal to r_{-S} (see Figure 16) and the product concentration passes through a maximum, otherwise kinetic control is lost and thermodynamic control will take over.

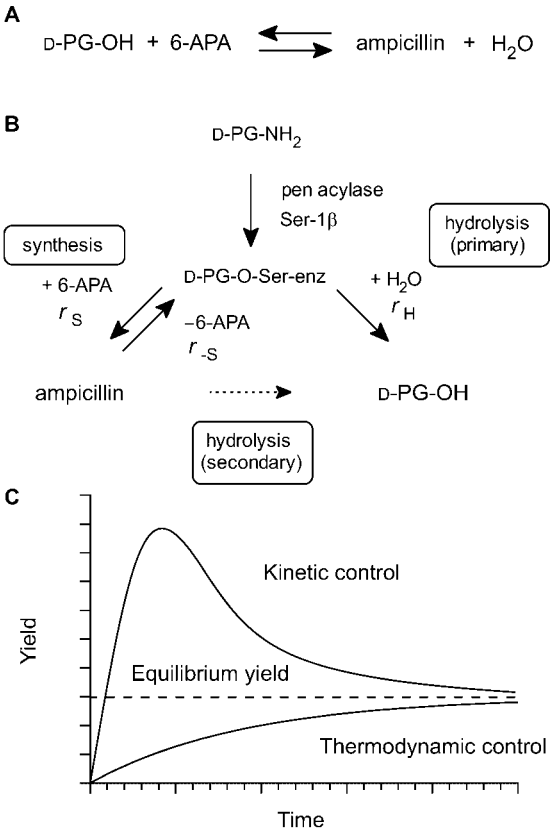


Figure 16. Thermodynamically (**A**) *vs.* kinetically controlled (**B**) synthesis of ampicillin and the time-course of both (**C**).

The thermodynamically controlled condensation of, e.g., phenylacetic acid and 6-APA in the presence of *E. coli* penicillin G acylase had already been described in the early 1960's.[10,11,81] Hence, the condensation of 6-APA and D-phenylglycine was for long thought to be feasible and has been claimed in a patent application.[82] Recent studies, however, proved that equilibrium is overwhelmingly on the side of hydrolysis,[58,59] due to the low chemical energy of zwitterionic phenylglycine. We note that thermodynamic coupling of an *N*-protected derivative of D-phenylglycine should be feasible. Thus, the condensation of the Dane salt of D-phenylglycine and 6-APA in the presence of *E. coli* ATCC 9637 has been claimed,[83] but could not be repeated in our hands[84] as would be expected on the basis of current insights[85] in the steric restrictions of the acyl subsite in penicillin G acylase.[86]

The main obstacle confronting the kinetically controlled synthesis is the accompanying hydrolysis al-

luded to above, which necessitates the use of an excess of the side-chain donor and renders the downstream processing more laborious. Hence, the synthesis/hydrolysis ratio (S/H, mol product per mol hydrolyzed side-chain donor formed) is often used as an indicator of the viability of the process.[87]

The S/H generally declines as the reaction proceeds, due to exhaustion of the starting compounds, accumulation of the product, and the change in ionic strength. Hence, it would seem advantageous to economize on the donor by stopping the reaction at low conversion and recycling the unconverted starting compounds. The latter approach is feasible with 7-ADCA, although the downstream processing becomes more complex, but 6-APA should rather be completely converted on account of its limited stability.

5.2.2 Developments in Kinetically Controlled Synthesis

The enzymatic synthesis of ampicillin from 6-APA and D-phenylglycine amide or ester, in the presence of penicillin G acylase from *E. coli*, was first reported in 1963[88] and a more extensive investigation was published in 1969[89]. There was scant commercial interest in enzymatic coupling at that time due to the low concentration used and because chemical procedures were considered to be quite satisfactory.

Studies in kinetically controlled synthesis continued during the 1980's[90,91,92] and several methodologies for suppressing the competing hydrolysis were investigated. Performing the reaction in aqueous methanol resulted in a modest improvement;[93] a somewhat better result was obtained by adding poly(ethylene glycol).[94] Attempts to selectively inhibit the hydrolytic activity of penicillin acylase, with, for example, thiopheneacetic acid met with some success but the cost in terms of reaction rate was prohibitive.[95] We have found that D-phenylglycine nitrile effected a selective inhibition of hydrolysis in the synthesis of cephalexin, resulting an improvement in S/H by a factor of 2.5.[96,97]

Alternatively, product hydrolysis could be suppressed by selectively removing the product from the reaction mixture. In the case of the cephalosporins this has been accomplished *via* cocrystallization with, for example, 2-hydroxynaphthalene.[98] The effect on S/H was impressive,[99,100] but the downstream processing would become more complex and contamination of the β-lactam product with traces of complexant cannot be excluded.

Improvement has also been sought, by analogy with enzymatic peptide synthesis,[101] by conducting the reaction at subzero temperature. Increased yields of β-lactam antibiotics were indeed observed – at a heavy penalty in rate – when the reaction was conducted in frozen medium, but only at low (20 mM) concentrations and in the presence of dissolved catalyst. At industrially relevant concentrations or when an immobilized penicillin acylase was used the advantage vanished.[102]

A breakthrough in the kinetic synthesis of β-lactam antibiotics was finally achieved when it was discovered that performing the reaction at high, i.e., 0.3 – 0.5 M, concentrations dramatically improved the yield.[103,104] A general procedure emerged[105] in which the desired β-lactam nucleus is acylated with an excess of an activated side-chain donor, e.g., an ester or amide, at 0.2 – 0.8 M concentration in water at pH 7 – 8 at zero to ambient temperature (Figure 17).

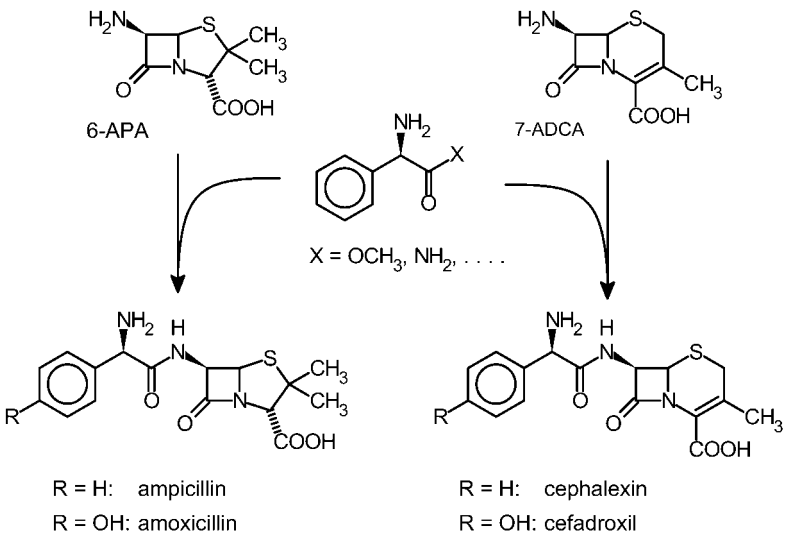


Figure 17. Kinetically controlled enzymatic synthesis of β-lactam antibiotics.

5.2.3 Developments in the Biocatalyst Field

A considerable number of penicillin acylases and α-amino acid esterases catalyze the acylation of 6-APA and 7-ADCA.[106] The α-amino acid ester hydrolases,[107] such as those from *Acetobacter pasteurianus*, *Xanthomonas citri*, and *Acetobacter turbidans* have had scant practical impact because they are restricted to ester donors, suffer from an undesirably narrow pH range (pH 5 – 6),[108,109] and have limited stability as well as a low S/H under industrially relevant conditions. Hence, the penicillin G hydrolysis catalyst, penicillin G acylase from *E. coli*, which is a relatively efficient acyl transfer catalyst and a very well-studied and readily available enzyme, has remained the catalyst of choice. Some improvement of its acyl transfer characteristics has been accomplished *via* mutagenesis.[110]

In industrial practice the use of an immobilized enzyme is a *conditio sine qua non* for reasons of downstream processing and recyclability, although immobilization has a detrimental effect on S/H. The β-lactam nuclei and their products are much larger molecules than water and the diffusional limitations in an immobilized enzyme are expected to lead to a lower S/H. It had already been shown in the early days of the enzymatic coupling that the magnitude of this effect depends on the immobilization method.[111] DSM has claimed that its proprietary gelatin-derived carriers perform much better in this respect than other ones.[112]

We found that cross-linked enzyme aggregates (CLEAs) of penicillin G acylase mediated the synthesis of ampicillin with an S/H that was close to that of the dissolved enzyme (see Figure 18), whereas cross-linked crystals (CLEC-EC®) afforded approximately half that value.[113]

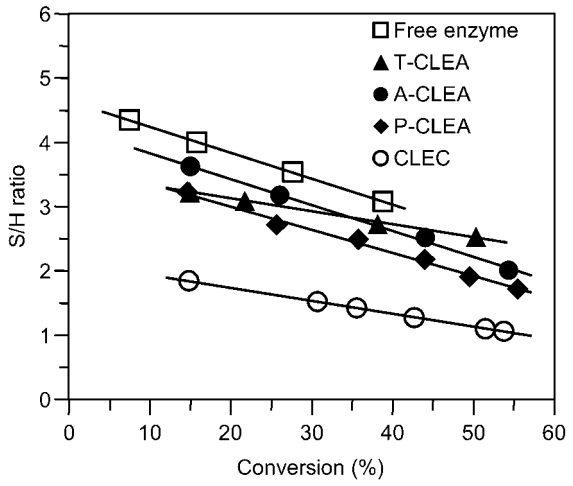


Figure 18. Relationship of S/H with the conversion of 6-APA in the synthesis of ampicillin, catalyzed by free and cross-linked penicillin G acylase from *E. coli*.[113]

5.3 Practical Procedures for Enzymatic Coupling

5.3.1 General Issues

In spite of the high yields on the β-lactam nucleus – as high or even better than in the traditional chemical condensations[87] – and the further obvious advantages of the enzymatic procedure, its widespread adoption by the pharmaceutical industry has been delayed, mainly for reasons of process economy. The hydrolysis problem, as well as the downstream processing, which is much more complex than that in the chemical process, proved to be formidable obstacles in this respect.

The downstream processing is further complicated by the presence of solid products due to the high concentrations that are required for efficiency.[103,104] Sieving is an easy method for separating the – microcrystalline – reaction products from the solid catalyst[114] and the sieve-bottom reactor has become a standard feature in process concepts for enzymatic semi-synthetic β-lactam antibiotic synthesis.[87] Alternatively, a low-density catalyst that will float when stirring is stopped[115] may be used.

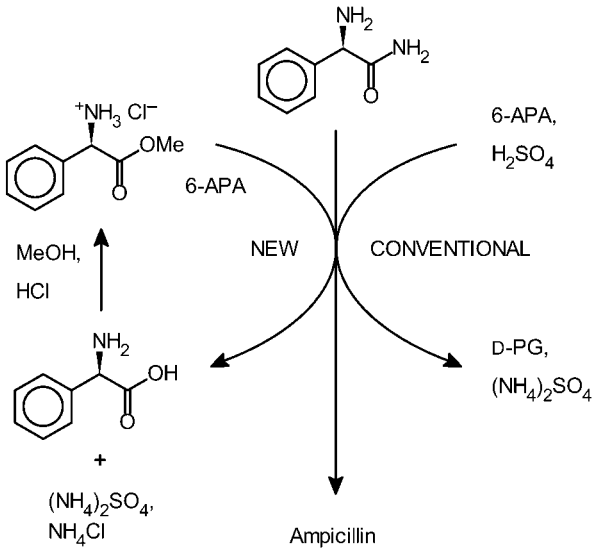
Product isolation will generally result in a mother liquor, containing a mixture of unconverted β-lactam nucleus, residual product, and excess side-chain donor. Recovery of the valuable β-lactam nucleus has been accomplished by subjecting the mother liquor to penicillin acylase-catalyzed hydrolysis.[116]

Much effort and ingenuity has been devoted to the efficient separation of the β-lactam product from the excess of side-chain donor and its hydrolysis product, as is attested by the large number of patent applications. The effort has resulted in carefully optimized procedures that accommodate the different characteristics of the various semi-synthetic β-lactam antibiotics as well as the economic criteria.

5.3.2 Ampicillin

Ampicillin was the first member of its family that was synthesized enzymatically[88] and ampicillin synthesis has served, for over 30 years, as a test-bed for enzymatic coupling procedures. D-Phenylglycine methyl ester has commonly been used as donor in academic studies, although the amide is the donor of choice in an industrial setting, as explained above. Hence, the penicillin acylase from *E. coli*, rather than an α-amino acid ester hydrolase, is the catalyst of choice for industrial application.

The major problems in the enzymatic synthesis of ampicillin are its rather low intrinsic S/H, which rapidly decreases in the course of the reaction (see Figure 18), because the high solubility of ampicillin accelerates its (secondary) hydrolysis. The pH, usually kept at 7, is a critical parameter in this respect.[117] At-



Process	Input (kg/kg)[a]	Waste (kg/kg)[a]		
	D-PGA	D-PG	Salts	Total
Conventional	0.62	0.26	0.25	0.51
Mixed-donor	0.37	–	0.16	0.16

[a] Per kg ampicillin

Scheme 3. Enzymatic mixed donor process for ampicillin.

tempts to improve the yield by performing the reaction in aqueous methanol met with scant success;[118] ethylene glycol gave some improvement.[119] The effect of nearly every process parameter has been investigated and the number of papers on the subject is very considerable.[120]

Recently, a suspension-to-suspension reaction system has been developed on the basis of a detailed study into the effect of the reactant concentrations.[121] Repetitive addition of the reactants, to avoid exhaustion of 6-APA, resulted in a further improvement.[122] It has also been pointed out that there is no single optimum pH for the coupling reaction but that the former rather depends on the concentration of the reactants. Hence, it proved to be advantageous to decrease the pH in the course of the reaction, instead of maintaining a fixed value.[123]

In spite of these improvements the coupling reaction still is accompanied by the formation of approx. 0.5 equivalent (relative to ampicillin) of D-phenylglycine. Its recycling *via* the amide would require two steps[64a] and the generation of more than one equivalent of salt.[124] We considered, moreover, that in the synthesis of ampicillin ammonia is liberated and neutralized by the addition of approx. 1.5 equivalents (relative to ampicillin) of acid. Using the HCl salt of D-phenylglycine methyl ester in a double role, as titrant and as secondary donor (see Scheme 3), would economize on acid and donor consumption, as well as on salt generation. In the resulting mixed-donor procedure the amounts of waste generated was reduced by a factor of 3 compared with the standard coupling procedure.[125]

The limitations of the α-amino acid ester hydrolases alluded to above have severely reduced their practical impact, although they have been much studied in academic settings. An improved, fed-batch procedure, using a specially stabilized immobilizate of the *A. turbidans* enzyme in 40% aqueous methanol has recently been described.[126] Ampicillin was obtained in high yield (95% on 6-APA and 79% on D-phenylglycine methyl ester) but the final product concentration was only 50 mM.

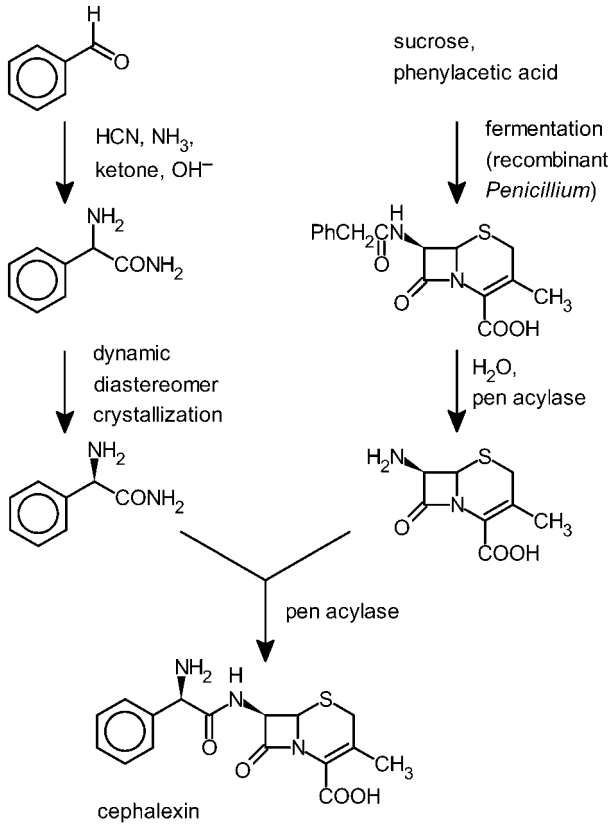
The high solubility of ampicillin and the presence of large amounts of D-phenylglycine render the isolation of the former more laborious. Procedures that have been reported by DSM involve acidification of the reaction mixture to dissolve all solids, followed by selective crystallization of ampicillin using a pH shift.[127] The inherently low S/H, as well as the lagging demand for ampicillin have, thus far, prevented the commercialization of an enzymatic process for this rather outdated therapeutic.

5.3.3 *Amoxicillin*

The solubility of D-*p*-hydroxyphenylglycine methyl ester, the preferred donor in an enzymatic process for amoxicillin, is undesirably low. The use of the more hydrophilic 2-hydroxyethyl ester as the donor has been presented as a solution to the latter problem.[128] The solubility of amoxicillin also is very low, in contrast with ampicillin. Consequently, the S/H is higher and, moreover, the degradation of amoxicillin is minimal. A process has been described in which the crystalline product is continuously removed by filtration and subsequent centrifugation; the filtrate is resaturated with side-chain donor and 6-APA and fed back into the reaction.[104] Amoxicillin was obtained in 90% yield (based on 6-APA) with an S/H of 2.4. A suspension-to-suspension reaction system for amoxicillin has recently been described and modeled.[129]

5.3.4 *Cephalexin*

The first enzymatic synthesis of cephalexin, from D-phenylglycine ester and 7-ADCA in the presence of *X. citri* as well as a number of related organisms, dates from 1972.[107] Hydrolysis of the side-chain donor predominated at the low – 20 mM – concentrations that were commonly used in early studies[130] but the intrinsic S/H is quite high at industrially relevant concentrations[131] and hydrolysis is much less of a problem than with the other β-lactam antibiotics.



Scheme 4. A biocatalytic production route to cephalexin.

The downstream processing is complex, on the other hand, due to the small differences in acid/base properties and solubilities between cephalexin, 7-ADCA, and D-phenylglycine, which may result in co-precipitation. Pure cephalexin has been recovered by a number of pH shifts and crystallizations of cephalexin and 7-ADCA.[132]

Cephalexin is the first, and so far only, β-lactam antibiotic to be produced enzymatically, by Chemferm (a DSM subsidiary). The introduction of various biocatalytic steps in the production of cephalexin (Scheme 4) reduced the number of reaction steps from 10 (see Scheme 1) to 6.

The number of reaction steps could be reduced further, in principle, by combining two or more transformations in a cascade reaction, obviating the need for isolation and purification of the intermediate. Thus, we have combined the stereoretentive enzymatic hydration of D-phenylglycine nitrile[75] and the penicillin acylase-mediated coupling of the resulting D-phenylglycine amide with 7-ADCA in a two-enzyme cascade.[96] The nitrile hydratase from *R. rhodochrous* MAWE[73] and an immobilized penicillin G acylase mediated the formation of cephalexin in 60% yield at S/H 2.7. By adding a precipitant – 1,5-dihydroxynaphthalene[98] – the yield was increased to 79% and S/H to 7.7.[96]

5.3.5 Cefadroxil

The main problem in the enzymatic synthesis of cefadroxil is that the side-chain and the β-lactam nucleus are sufficiently soluble only at a completely different pH. Moreover, the solubility of cefadroxil is high, resulting in chemical degradation and enzymatic hydrolysis of the product. Consequently, high conversions are necessary to obtain a mixture from which cefadroxil can be crystallized. The optimum pH for cefadroxil is approximately 7 but 7-ADCA is poorly soluble at this pH; hence, the S/H is low (approx. 1). The solution was found in the use of supersaturated solutions.[133] The reaction was started with a solution of 7-ADCA, containing the catalyst, at pH 8.5. A concentrated solution of D-*p*-hydroxyphenylglycine methyl ester at pH 4 was added at such a rate that crystallization did not take place. Coupling was accomplished with S/H ~5 and 85% conversion of 7-ADCA. Cefadroxil crystallizes in the course of the reaction.

6 Conclusion and Future Outlook

Driven by economic and environmental needs, traditional chemical procedures for the manufacture of β-lactam antibiotics are increasingly being replaced by shorter and cleaner biocatalytic alternatives. Much of this has been made possible by the application of modern developments in biotechnology. We envisage, therefore, that in the not too distant future production methods for β-lactam antibiotics will consist virtually entirely of biocatalytic steps, with the exception of the basic raw materials for the side-chains.

Acknowledgements

The authors wish to thank Dr. A. Bruggink (DSM Life Sciences R&D) for his encouragement and critical reading of the manuscript. The authors also are grateful to Dr. J. Verweij (DSM Anti-Infectives) for his critical comments. Financial support to M.A.W. by DSM Life Science Products and the Netherlands Ministry of Economic Affairs is gratefully acknowledged.

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