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Industrial Enzymatic Production of Cephalosporin-Based β-Lactams

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Abstract Cephalosporins are chemically closely related to penicillins both work by inhibiting the cell wall synthesis of bacteria. The first generation cephalosporins entered the market in 1964. Second and third generation cephalosporins were subsequently developed that were more powerful than the original products. Fourth generation cephalosporins are now reaching the market. Each newer generation of cephalosporins has greater Gram-negative

antimicrobial properties than the preceding generation. Conversely, the 'older' generations of cephalosporins have greater Gram-positive (*Staphylococcus* and *Streptococcus*) coverage than the 'newer' generations. Frequency of dosing decreases and palatability generally improve with increasing generations. The advent of fourth generation cephalosporins with the launch of cefepime extended the spectrum against Gram-positive organisms without a significant loss of activity towards Gram-negative bacteria. Its greater stability to β-lactamases increases its efficacy against drug-resistant bacteria.

In this review we present the current situation of this mature market. In addition, we present the current state of the technologies employed for the production of cephalosporins, focusing on the new and environmentally safer 'green' routes to the products. Starting with the fermentation and purification of CPC, enzymatic conversion in conjunction with aqueous chemistry will lead to some key intermediates such as 7-ACA, TDA and TTA, which then can be converted into the active pharmaceutical ingredient (API), again applying biocatalytic technologies and aqueous chemistry. Examples for the costing of selected products are provided as well.

Keywords Biocatalysis · Enzymation · Cephalosporin C · Fermentation · *Acremonium chrysogenum* · β-Lactam

List of Abbreviations

7-ACA	7-Amino cephalosporanic acid
ACV	δ-(L-α-Aminoadipoyl)-L-cysteinyl-D-valine tripeptide
7-ADCA	Amino-desacetoxy cephalospranic acid
6-APA	6-Amino penicillinic acid
API	Active pharmaceutical ingredient
CA	Cephalosporin C acylase
CAH	Cephalosporin C acetyl hydrolase
CLEA	Cross-linked enzyme aggregates
CLEC	Cross-linked enzyme crystals
CPC	Cephalosporin C
DA-7-ACA	Deacetyl-7-ACA
DAC	Deacetylcephalosporin C
DAO	D-Amino acid oxidase
DO AC	Deacetoxycephalosporin C
GAC	Glutaryl acylase
GL-7-ACA	Glutaryl-7-ACA
HIC	Hydrophobic interaction chromatography
IEX	Ion exchange chromatography
KA-7-ACA	Ketoadipoyl-7-ACA
MMTD	2-Mercapto-5-methyl-1,3,4-thiadiazole
PGA	Penicillin G amidase
Pip-pHPG	D-2-(2,3-Dioxo-4-ethyl-1-piperazin-carbonylamino)-2-(4-hydroxy-phenyl)acetic acid
PMV	Packed mycelium volume
TDA	7-(Amino-3-(5-methyl-1,3,4-thiadiazole-2-yl)thiomethyl-3-cephem-4-carboxylic acid
TM	Metric ton
TTA	7-Amino-3-[(1,2,5,6-tetrahydro-2-methyl-5,6-dioxo-1,2,4-triazin-3-yl)-thiomethyl]-cephalosporanic acid
TZ-7-ACA	Tetrazolylacetyl-7-ACA

TZM Tetrazolylacetic acid methylester
$Y_{X/S}$ Yield coefficient consumption of amount substrate to yield amount product

1 Introduction

The discovery of cephalosporin C (CPC) goes back to Giuseppe Brotzu, working at an institute in Cagliari on the island of Sardinia. Upon isolation from seawater near a sewage outlet a microorganism that had antibiotic activity against Gram-positive and Gram-negative bacteria was obtained. Upon classification as *Cephalosporium*, this organism was sent to Oxford in 1948. Work started in 1953 with the isolation and purification resulting several years later in the published structure of CPC [1]. In 1964 cefalotin was launched as the first semi-synthetic CPC antibiotic. More than 50 semi-synthetic cephalosporins are being marketed today with sales of some US$9 billion annually.

The market for "antibiotics" (perhaps better defined as anti-infective agents) sold at dose form level worldwide is estimated to be close to $60 billion in 2002, taking into account materials supplied under various aid programmes, secular and religious charity aided programmes, national bilateral aid and UN assistance programmes. The market is broken down by product type in Table 1 and by region in Table 2.

In the context of this chapter, antibiotic means a chemical derived by fermentation or from a raw material obtained by fermentation, and having an-

Table 1 World anti-infectives market at ex-manufacturer/local primary distributor level

Product area	Estd. sales US$ Billion
Cephalosporins (all)	10
Penicillins (incl Amoxiclav)	8
All other betalactams	3
Quinolones incl fluoroquinolones	7
Macrolides (Erythromycin, Spiramycin and semi-synthetic derivatives thereof)	6
Aminoglycosides (natural and semisynthetic)	3
Tetracyclines	3
All other antibacterials (incl anti-TB, topicals etc.)	6
Antivirals (excl vaccines)	7
Anti-infective vaccines	4
Antifungals/antiparasitics	4
Total	61

Data is rounded to one significant figure and, for the smaller groups above, should be regarded as indicative, rather than definitive. Source: Michael Barber and Associates, Caterham, UK (2003).

Table 2 World anti-infectives market at ex-manufacturer/local primary distributor level by region

Country/Region	Estd. sales US$ billion	% of total	Estd. popln. of region mill	Expenditure on anti-infectives per capita
USA	11	18	300	37
Europe (all other, incl Russia)	8	13	550	15
Europe (Germany, France, Italy)	7	12	200	35
Japan	7	12	150	47
S & E Asia/Australasia	7	12	750	9
China	5	8	1500	3.3
Africa	4	6	800	5
Americas incl Canada	4	6	550	7
India	4	6	1000	4
Middle East	3	5	350	9
Other Indian sub-continent	1–2	2	350	4
Total	61	100	6.5 bill	Ave 9.4

Data is rounded, particularly sales value data in the Developing World, where it is difficult to obtain reliably consistent sales data. Source: Michael Barber and Associates, Caterham, UK (2003).

tibacterial, antiviral or antifungal properties. By common association, totally synthetic molecules, such as the fluoroquinolones and sulfonamides, which have substantially the same effect, are included in the general term "antibiotic". There are, however, a number of important fermentation-derived antibiotics that have no antibacterial or similar activity. These include the anthracycline anticancer agents (e.g. doxorubicin, daunomycin, asparaginase, etc.) immunosuppressants (ciclosporin, mycophenolic acid, tacrolimus, sirolimus, etc.) vitamins, (e.g. ascorbic acid, pantothenic acid, cyano- and hydroxocobalamin, etc.). Those are not included in Table 1 and Table 2.

It is apparent from Table 1 that the two series of β-lactam antibiotics, the cephalosporins and the penicillins, are the largest selling antibiotics. Both of these contain the "β-lactam" structural unit. The important structural feature is the nature of any group attached to the N atom at the top left-hand corner of the diagram in Fig. 1. This atom carries the principal side chain R1 of the cephalosporin series. Semisynthetic analogues are made by replacing the natural side chain with synthetic variants. The term "cephalosporins" includes more than 50 semisynthetic antibiotics derived from cephalosporin C (CPC), a natural antibiotic with no clinically useful antibacterial activity in its own right.

β-Lactams exert their activity by inhibiting the cell wall synthesis of bacteria. The cephalosporins, the first of which, cefalotin, was introduced in 1964,

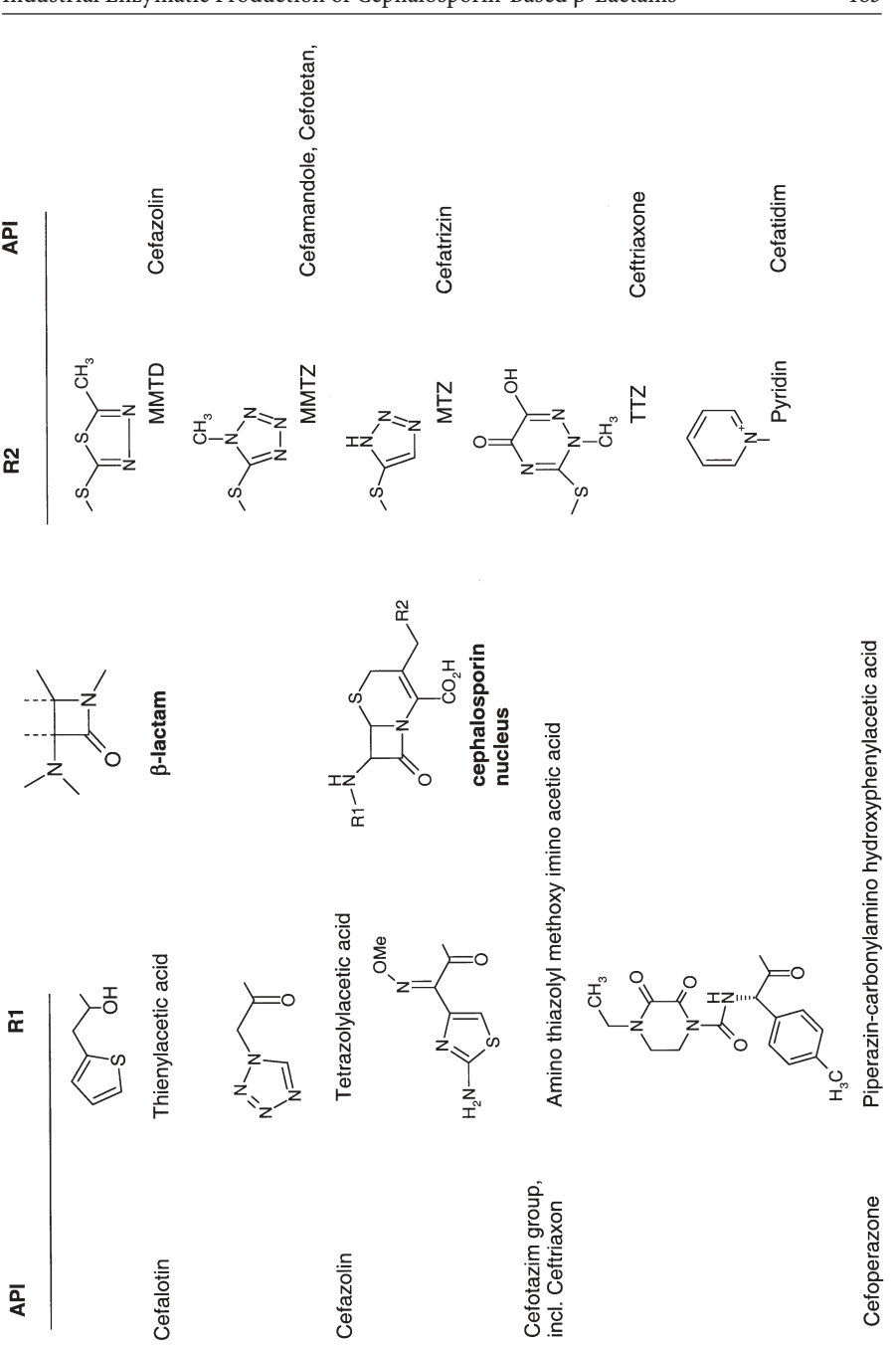

Fig. 1 Semi-synthetic β-lactam synthesis. The cephalosporin nucleus (*middle*) is derivatized on R1 and R2 to yield the active cephalosporin. Examples for 3′ and 7′ side chains are shown and the respective final products (API) indicated

collectively have the largest sales by value of any group of antibacterial agents. However, by volume, in terms of tonnes sold or the number of prescriptions written, penicillins outweigh cephalosporins by a factor of some four to one.

There are two main groups of cephalosporin antibiotics; one is derived from penicillin (G or V), the second from cephalosporin C (CPC). Most products that have intrinsic oral activity are better made from penicillin, while most products made from CPC are insufficiently absorbed from the human gut to be therapeutically adequate by the oral route, unless converted to pro-drugs by esterification [2, 3].

The penicillin-derived products are mainly based on 7-ADCA (hitherto made entirely from penicillin G) or on other more complex transformation products (made originally from penicillin V but now increasingly from penicillin G). The economics of the different processes involved are difficult to compare but essentially all (except for cefaclor as made by Lilly) depend on the price of commodity potassium penicillin G, principally supplied from China. Most products are multi-source at both the dose form and bulk active ingredient levels.

The first cephalosporins were only available as injections. Orally active products, cefalexin, cefradine, etc., followed some five years later. Though these were initially manufactured from cephalosporin C, Lilly realised early on that a cheaper synthesis was essential if API costs were to be reduced to a level that allowed cefalexin to be competitive, initially with ampicillin and later with amoxicillin. Accordingly, it developed a synthesis of a suitable precursor, starting from penicillin V, which enabled it both to compete on price and have sufficient material to meet the market demand it was creating. Gist Brocades made a further advance in the synthesis of the primary intermediates when it found that its "Delft" process for making 6-APA was equally applicable to the manufacture of the key cephalosporin intermediates, 7-ACA and 7-ADCA [4, 5].

The early cephalosporins had good activity against a wide range of Gram-positive bacteria, including a number of strains that produce penicillinase (but which remain methicillin sensitive). In contrast, they tended to have little activity against enterococci and weak and erratic activity against Gram-negative organisms. Cefazolin and cefradine (typical first generation products, Table 3) are still widely used in China but generally less and less elsewhere. The therapeutic limitations of the first generation of products led to the development of the so-called "second generation" products (cefamandol, cefaclor and cefuroxime, Table 3). These are characterised by a slightly poorer effect on Gram-positive bacteria but a significantly improved activity against enterobacteria and better resistance towards β-lactamases, especially those from Gram negative species.

The third generation products (e.g. cefotaxime, ceftriaxone and cefixime, Table 3), have even better activity against Gram-negative bacteria, especially enterobacteria species. Most of the third generation products have good activity against streptococci, which helps to compensate for the variably weaker activity against staphylococci. The newest, fourth generation, products (cefepime,

Table 3 Overview of cephalosporins 2002

	Names	Market volume MT bulk free acid equiv.[a]	Sales revenues $mill at dose form level[b]	Sum $mill
1st Generation	Cefalotin	70–80	80	2700–2800
	Cefatrizine (o)	105–115	110–120	
	Cefazolin	850–900	730–770	
	Cefapirin	25	<25	
	Cefalexin (op)	2800–2900	800–840	
	Cefadroxil (op)	450–480	430–450	
	Cefradine (op)	1500–1570	500–540	
		5800–6070	2700–2800	
2nd Generation	Cefuroxime	130–135	320–340	3200–3300
	Cefamandole	15	< 25	
	Cefonicid	10–15	60	
	Cefaclor (op)	340–350	450–480	
	Cefuroxime axetil (o)	190–200	700–740	
	Cefprozil (o)	75–85	390–410	
	Ceftibuten (o)	15	70–90	
	Cefteram pivoxil (o)	15	70	
	Cefditoren pivoxil (o)	25	170–200	
	Cefcapene pivoxil (o)	45–50	280–310	
	Cefetamet pivoxil (o)	20	40	
	Cefmenoxime	10	20	
	Cefotiam	50–55	200–220	
	Cefotiam hexetil (o)	25	50	
	Cefotetan	15	70	
	Cefoxitin	25	70	
	Ceftiofur	20	140–160	
	Loracarbef (o)	5	25	
		1030–1080	3140–3380	
3rd Generation	Cefotaxime	310–325	460–500	4000–4250
	Ceftizoxime	10	25	
	Ceftriaxone	280–290	1400–1440	
	Cefodizime	5	40	
	Cefdinir (o)	65	420–460	
	Cefixime (o)	50	270–290	
	Cefpodoxime Proxetil (o)	30	225–255	
	Cefoperazone and comb.	75–80	280–320	
	Ceftazidime	70–75	450–480	
	Cefsulodin	5	<25	
	Cefmetazole	10	30	
	Cefozopran	10	90	
	Flomoxef	25–30	270–300	
	Latamoxef	≤5	≤10	
		965–990	4000–4250	

(continued)

Table 3 (continued)

	Names	Market volume MT bulk free acid equiv.[a]	Sales revenues $mill at dose form level[b]	Sum $mill
4th Generation	Cefepime	40	285–315	390–420
	Cefpirome	15	100–120	
		55	385–425	
Totals			10300–10600	10400–10500

(o) Means the product is normally intended for oral administration.
(p) Means this product is normally made from penicillin.
[a] Data are rounded to 0 or 5 MT of free acid equivalent of requirement for dose form sales indicated. Sales data recorded in US$ at ex-manufacturer level; where a range is quoted, it covers variations such as exchange rate factors, errors in product sales recording, etc.
[b] Source: Micheal Barber & Associates 2003.

cefpirome, Table 3) couple the anti-Gram negative activity of the third (and some of the second) generation products with the anti-Gram positive activity of the first. Generally, too, the later generation products have better pharmacokinetics and pharmacodynamics than the earlier generation products.

Similar to penicillins, treatment with cephalosporins can cause possible side effects including hypersensitivity reactions, encephalopathy and tubulo-intestinal nephritis. Approximately 10% of penicillin-sensitive patients will also be allergic to cephalosporins.

The conversion of 7-ACA into the respective APIs, and in particular the addition of a new side chain at position 7′ can in many instances be performed in a mild enzymatic process catalysed by the penicillin G amidase (PGA). Addition of side chains at the 3′ position, often thiols, can be achieved in aqueous chemistry. Alternatively, in addition to the enzymatic routes, both removal of the 7- amino adipoyl side chain and the addition of new side chains at the two aforementioned positions can also be performed via the classical organic chemistry-based route. Both routes are in strong competition. While certain markets, traditionally in the Far East, favour the chemical routes and in part are willing to pay a premium price for this, European producers are more prone to use the enzymatic routes. This may in part be driven by environmental concerns and regulations that significantly add to the costs of production.

The following chapter will present an insight into the current cephalosporin market and provide information on the current production processes for some selected compounds from the industrial perspective, while at the same time protecting proprietary processes.

2
The Cephalosporin Market

2.1
Market Dynamics

In any well-investigated therapeutic area, later compounds inevitably have to have some significant advantage over those already on the market. Typically, this will mean that the product is more effective than its predecessors. In reality many elements of this effectiveness are a factor of improved pharmacokinetics rather than of absolute biological activity. In such cases, the introduction of the new compound generally leads to a reduction in the daily dose of the therapeutic agent.

This is particularly true in the cephalosporin field. From their introduction in 1964 until around 1990 the increase in demand for cephalosporins, and thus the increase in bulk requirements, continued almost unhindered. Market demand seemed insatiable. However, in the 1985–1990 period, several of the older, high-volume, products became obsolescent, and were gradually replaced by newer products, which were both more potent and longer acting. Separately, therapeutic substitution, the replacement of a cephalosporin antibiotic with one having a different molecular structure and mode of action, occurred with increasing frequency. Examples of this include, in the US market, the steady replacement of the oral cephalosporins by Augmentin, and by azithro- and clarithromycin. In the penicillin-based cephalosporins, branded cefalexin was replaced by cefaclor in many markets, while later cefaclor came under pressure from 7-ACA derived products such as cefuroxime axetil, cefixime and cefdinir. In several markets these have, in turn, come under pressure from the latest in the series, cefprozil. Cefprozil is, in fact, very unusual, if not actually unique, in having been originally manufactured from penicillin and only later from CPC.

With the 7-ACA-based products, a similar effect was observable. Many of the branded first and second-generation products gave way during the late 1980s to, for example, cefotaxime, ceftazidime and ceftriaxone. (The last became the leading cephalosporin product of all time, by dose form sales, in 1998.) These newer products were generally longer acting and required lower dosages than the earlier products. This meant that the growth in demand for 7-ACA, (historically 8–10%/year) suddenly slowed, leading to an oversupply situation and a dramatic fall in the price of 7-ACA (from $260/kg in 1990/1 to $170/kg in 1995). In addition, several important products have lost patent protection over the last five years, notably cefotaxime and, increasingly, ceftriaxone. Patents on cefuroxime axetil will also largely expire by 2003. Those on cefprozil, the last major product still the subject of basic product protection, begin to expire in 2005. As a consequence of this, several producers left the business during this period, for example, Asahi Chemical, Cephagen, Eli Lilly, Merck and perhaps Takeda. Other companies, notably Antibioticos, consolidated their activities onto fewer sites.

It is almost inevitable that, after such market restructuring, the price obtained by the remaining producers tends to rise, aided by the continuing increase in demand for 7-ACA-derived cephalosporins. If these points are then coupled with the difficulties experienced by Antibioticos in commissioning its fully enzymatic process for 7-ACA, it is not surprising that the prices quoted for it rose sharply early in 1996, to around $210/kg 7-ACA, an increase of about $35/kg. An increase of this magnitude attracted the attention of companies to the now generous margins that were available; several new plants were built and a number of existing producers significantly expanded their capacity. Not surprisingly, the price fell dramatically during the third quarter of 1999 to barely half the level at the middle of that year. Yet again, this price collapse has taken its toll, leading to the withdrawal of at least two producers and probably more before the end of 2005. The current main producers of bulk cephalosporins are listed in Table 4. Quantities are given as equivalents of 7-ACA, the common key intermediate. Also indicated is whether the companies use the classical chem-

Table 4 Worldwide production of 7-ACA equivalent 2002

Manufacturer	Total production of 7-ACA equivalent:	Process used E=enzymatic C=chemical	Comments
Europe			
ACS Dobfar	230–270	E (& C??)	>90
Antibioticos	500–550	E & C	~50:50
Biochemie	570–630	E & C	~60:40
GSK	170–210	E & C	~30:70
Subtotal	1530–1610		
Korea			
Cheil Jedang	250–300	C	
Chong Kun Dang	120–160	E	
Subtotal	390–440		
Japan			
Fujisawa	160–200	C	
China			
Hebei (Shiyao Group)	600–800	E & C	70:30 (year end)
Shandong Lukang	150–200	C	All producers have the
Fuzhou	0–50	C	enzymatic route
Harbin (Hayao Grp)	0–50	C	under review as
North China PC	0–50	C	replacement for zinc
Hisun	0	E	salt method currently
Nanyang	0	C	used and now con-
Subtotal	850–950		sidered obsolete
All others	<50	Various	Small production
Total production	3000–3100		

Source: Michael Barber & Associates 2003.

ical route to 7-ACA for modern enzymatic conversion, which has been developed and first introduced by the European companies.

Notwithstanding the continued expansion of the overall 7-ACA-based products market, it seems unlikely that any increase in demand will make a significant impact on the current oversupply situation. Accordingly, no significant increase in the price of 7-ACA, in real terms is expected for at least three years, and certainly not to pre-1999 levels, since expectations of low cost active ingredients are now well established and will provide the benchmark for all future price discussions. Hence, by 2005–2007, there will be only about five major producers of 7-ACA and downstream intermediates, all of whom will use efficient new technology on a large scale.

These observations strongly suggest that the cephalosporins business is in a period of transition, which should be substantially complete by 2007. This transition manifests itself in two essentially opposite ways. In the dose form business, it is moving from a situation in which it used to be driven by a few large, research based companies discovering new molecules, to one in which the number of producers of dose forms is increasing (on a worldwide basis). This trend is assisted by a tendency on the part of the bulk producers to increase supply beyond what the market can absorb. At the other end of the manufacturing chain, the number of producers of the basic raw materials is falling, under the influence of commercial and cost pressures on the one hand and technological improvement on the other.

2.2
Bulk Active Ingredients and Sterile Products

The supply of oral dose forms presents few problems and can be readily augmented at short notice. Regulatory issues tend to be less significant outside, principally, the USA, North-West Europe and Japan than within those markets. Thus the wider availability of non-sterile oral grade bulk materials has led to a growth in the number of formulators in many countries. This is an example of how demand-pull is hidden and the prime market determinant appears to be supply push (see below). The reality is that unfulfilled demand creates a market vacuum into which the excess supply is sucked. India and Korea have emerged as two important suppliers of oral dose form cephalosporins to the open market in recent years, while China has a huge industry geared towards the needs of its enormous home market, historically but not exclusively importing the bulk active ingredients.

The position with the sterile dose forms is different. Here the limiting steps are the supply of sterile bulk and/or the supply of filled vials. The number of companies able to produce such products to the exacting standards necessary is limited. Nevertheless, more than ten sterile bulk plants were commissioned in Korea during 1994–1998, as well as twice that number in India. China has upwards of 100 plants claiming to be able to make injectable cephalosporins, although the GMP status of many of these is uncertain. For high levels of GMP

compliance, Italian producers are still predominant. However, the position could change as competition in the "free" market intensifies. Finally, the production of bulk non-sterile active ingredients has historically been an Italian speciality. This role is increasingly being assumed by India and Korea, with varying degrees of success.

Just as the market for dose form antibiotics is generated by the incidence of infectious disease and the demand for treatment for it (demand pull), the market for the bulk active ingredients is driven, pulled by the demand for the dose form products. In many of the developing markets, the demand for treatment is still unfulfilled. In the case of the 7-ACA cephalosporins, reasons for this have included:

1. Perceived high price for the products compared with older, established treatments
2. Lack of foreign exchange to pay for high priced medicines
3. Lack of local manufacturing capability and facilities for sterile product manufacture
4. Lack of technology for such manufacture
5. Lack of sources of intermediates and synthons (especially side chains)
6. Lack of capital resources to fund any of the foregoing operations

Improved prosperity in many markets, combined with trade liberalisation moves, has relieved the problems of item 6, which has made item 5 of particular importance. The expiry of so many patents has allowed companies to engage in the production of items hitherto closed to them, with a reasonable prospect of being able to sell their output into a demand-led market. As a result, India and, insofar as the 7-ACA-based products are concerned, Korea, emerged as major sources of bulk non-sterile and sterile active ingredients, while China became a leading source of side chains and synthons. Korea and Italy remain two of the leading sources of 7-ACA and advanced intermediates to the free market. Italy, of course, remains the largest single supplier of 7-ACA-based cephalosporins to the world market, in the form of bulk sterile and non-sterile products, bulk active ingredients and of 7-ACA and intermediates derived from it.

It should be noted that the fall in bulk prices referred to above is a factor of increasing competition and over supply. The ultimate result will be the emergence of a few large producers, each with sustainably large shares of the world market, or of a few niche players with major positions in small, specialist sectors of the overall market.

3 Production of 7-ACA

A generalized flowchart outlining the enzymatic processes leading to 7-ACA is presented in Fig. 2. The overall process comprises the following main stages:

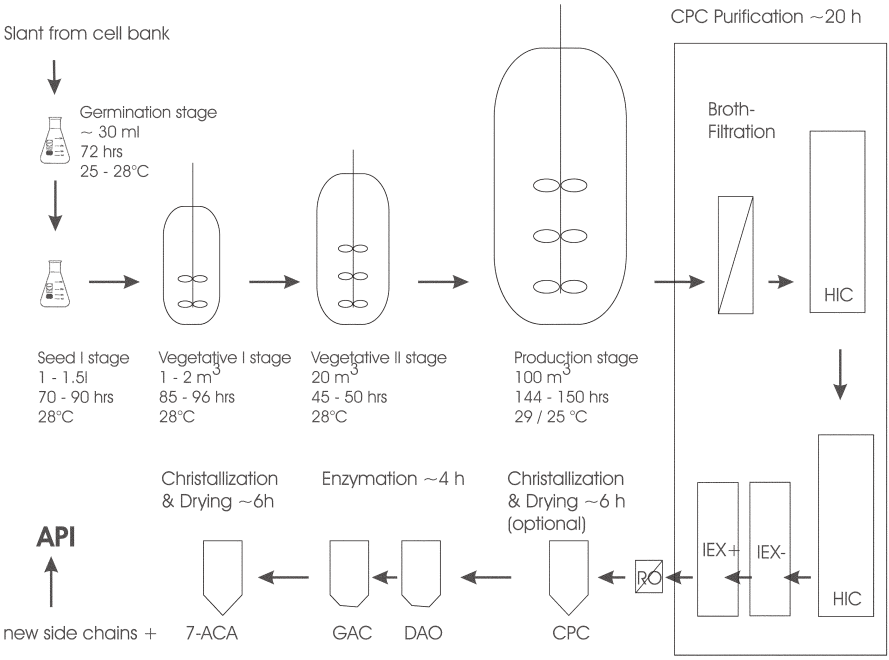

Fig. 2 Production scheme for the fermentative production of CPC (*upper*), purification of CPC from the broth (*right box*), and the enzymatic two step conversion into 7-ACA (*lower row*). Derivatization of 7-ACA will yield the API (Fig. 1). HIC, IEX+, IEX-, and RO represent hydrophobic interaction chromatography, cation-, anion exchange chromatography and reverse osmosis, respectively

- *Fermentation* of a high-yielding strain of *Acremonium chrysogenum* to produce a broth containing CPC
- *Purification* of the fermentation broth to produce an aqueous solution of CPC
- *Enzymation* of the purified aqueous extract to produce an aqueous solution of 7-ACA.
- *Precipitation and isolation* of 7-ACA of appropriate purity (ca. 98%) for use in the preparation of advanced cephalosporin intermediates and/or active pharmaceutical ingredients.

Adding up the times of all steps, an industrial scale production takes roughly three weeks, of which two weeks are devoted to the fermentation and about one week is required of the down stream processing. Derivatization at positions 3′ and 7′ to yield an API is not included. Starting from 7-ACA, these processes may take one day each for the derivatization plus the time for purification, crystallization and drying. The resulting bulk active cephalosporin can then be sterilized and formulated for marketing.

Some of the individual steps of the outlined production scheme can be changed. In particular the second ion exchange column and the crystallization step of CPC are not absolutely required even though some producers will keep these steps in an existing process. Crystallization of CPC is needed, however, for the chemical route to 7-ACA, a process mainly performed by suppliers in Korea and China (Table 4). CPC can be precipitated as zinc salt, which is environmentally problematic, or as sodium or potassium salt. The latter two are recommended for enzymatic splitting into 7-ACA.

The following sections will describe the production in more depth and the subsequent enzymatic conversion of CPC into 7-ACA, which has become standard for the European producers. The onward conversion into advanced intermediates that already contain a new side chain or an API that carries altered side chains on both positions (3′ and 7′) will be described on selected examples.

3.1 Fermentation

3.1.1 Strains

Starting point for the synthesis of cephalosporins is CPC obtained as secondary metabolite from large scale fermentations of the filamentous fungus *Acremonium chrysogenum* (Fig. 3). High yielding industrial production strains are used for production. These strains require continued improvement in both the titre achieved at the end of fermentation and in their stability. In practice, industrial strains are constantly mutated and re-isolation of best performing strains is conducted routinely, as even prolonged storage of high producing strain can occasionally result in the loss of its productivity. Genetic engineer-

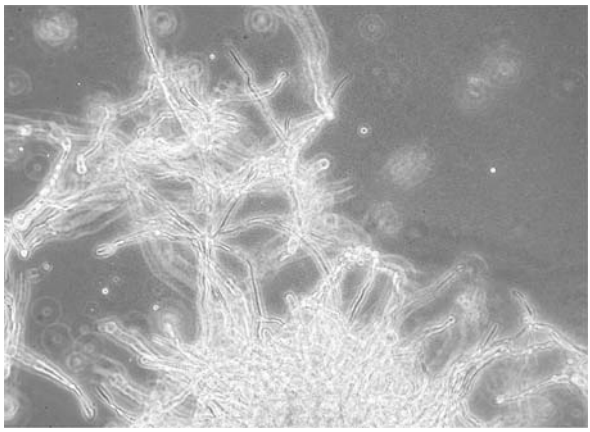

Fig. 3 Microphotograph of *Acremonium chrysogenum* grown in liquid culture

ing is increasingly being used to improve productivity or to direct the fermentation to new products [6, 7].

In contrast to industrial strains of *Penicillium*, which carry multiple copies of the penicillin biosynthesis gene cluster, *Acremonium* only has one biosynthesis cluster [8]. Protoplast fusions have yielded recombinant strains with improved CPC production. Unfortunately, these strains were genetically unstable and hence not suitable for industrial production. As an alternative to a duplication of the entire gene cluster, cloning and expression of genes that represent rate limiting steps in the CPC biosynthesis [9] has been employed with some success. Of particular interest are those genes encoding export functions (*cef*T [10]) and the late steps (ring expansion form penicillin N→deacetoxy CPC→deacetyl CPC→CPC, *cef*EF and *cef*G) in biosynthesis of CPC [11].

Another problem in industrial production is the accumulation of deacetyl CPC (DAC) in the fermentation broth. The accumulation of DAC in cultures of *A. chrysogenum* ought to be minimized since DAC is useless as an antibiotic and interferes with CPC purification. Some of the accumulated DAC may originate from the lack of acetylation of this biosynthetic intermediate since the level of expression of the *cef*G gene, which encodes the DAC-acetyltransferase, is very low [12] and overexpression of *cef*G gene results in a more efficient acetylation [11]. The remaining accumulated DAC may result from extracellular enzymes with CPC acetyl hydrolase (CAH) activity or by chemical hydrolysis particularly at alkaline pH. Recently Martin's group has purified and characterized an extracellular CAH (E.C. 3.1.1.41) of *A. chrysogenum*, and cloned the respective gene, *cah*B, [13]. It remains to be seen whether a deletion of this esterase will positively effect the CPC production.

Many medically useful semisynthetic oral cephalosporins (cefalexin, cefradin, cefadroxil) are made from 7-aminodeacetoxycephalosporanic acid (7-ADCA). These are usually produced through a chemical conversion of penicillin G or penicillin V. Recently a *Penicillium chrysogenum* strain has been genetically engineered by cloning and expression of the *cef*E gene from *Streptomyces clavuligerus* encoding for the enzyme catalysing the ring expansion [14]. This recombinant strain produced high titres of deacetoxycephalosporin C (DAOC). Production level of DAOC is nearly equivalent (75–80%) to the total β-lactams biosynthesised by the parental overproducing strain. DAOC deacylation is carried out by two final enzymatic bioconversions catalysed by D-amino acid oxidase (DAO) and glutaryl acylase (GAC) yielding 7-ADCA. In contrast to the data reported for recombinant strains of *Penicillium chrysogenum* expressing ring expansion activity, no detectable contamination with other cephalosporin intermediates occurred. The industrially amenable bioprocess for 7-ADCA production has been described could replace the expensive and environmentally unfriendly chemical method classically used. Nevertheless, genetic instability of the recombinant has been observed.

While it would be possible to direct *Acremonium* to produce 7-ADCA by disrupting and one-step replacement of the *cef*EF gene with *cef*E from *S. clavuligerus*, it would not be an economically viable process.

3.1.2 Culture Conditions

Similar to *Penicillium chrysogenum* fermentations (penicillin) or fermentations of filamentous bacteria such as *Saccharopolyspora erythraea* (erythromycin), *A. chrysogenum* cultures also undergo a series of barely understood changes in their morphology and physiology before the secondary metabolite is made. While the role or the function of these metabolites in nature is mostly unresolved – for antibiotics, self defence looks reasonable while the for clinical use desired antitumour or immunosuppressive activity may only be a side effect in the natural habitat – their production is initiated after exponential growth has ceased and the culture enters into stationary phase. Slowed growth, caused by depletion of nutrients, or accumulation of side products may induce this metabolic shift to the production of the secondary metabolites [15]. In modern processes, however, CPC production is already observed during the late growth phase during the productive fermentation stage [16–18].

Common industrial fermentation media are complex media containing corn steep liquor as a main source for nitrogen and phosphate, soy bean, cotton seed or peanut flour as source for protein (Table 5). A detailed description of the medium composition and the feed profiles cannot be given, as both vary depending on the strain. Often medium composition is driven by the available raw material and will also depend on the supplier and the batches of raw material used. In particular the quality of corn steep liquor, a major and critical constituent, varies depending on the origin of the corn (northern hemisphere for summer and southern hemisphere for the winter harvest), which can strongly affect CPC production. Carbon sources include rapidly consumable glucose and slow-release carbon sources, such as starch or soy oil. Descriptions of optimised media and feed strategies have been detailed in numerous scientific publications [19–22], but each of the currently used industrial strains is sufficiently different from each other that there are no universal recipes.

Two genotypes of production strains have evolved. For one (older) lineage addition of methionine is needed for induction of CPC production, while the other requires sulfate which is reflected in the 'generic' medium compositions listed in Table 5. The effect of methionine is twofold: its main effect on CPC production results from a regulatory role, as methionine induces four of the enzymes of CPC biosynthesis at the level of transcription. Namely transcription of *pcb*AB, *pcb*C, *cef*EF, and to a lesser extent *cef*G, is induced [12]. L-Methionine is also converted to L-cysteine, one of three ACV precursors of cephalosporin C, by cystathionine-gamma-lyase. Eliminating cystathionine-gamma-lyase prevents the enhancing precursor effect of methionine, and moderate cystathionine-gamma-lyase overproduction increases CPC formation [23]. The sulfur is incorporated into CPC. Initially these media contain a high percentage of undissolved matter and the fungus will colonize these particles which results in an increase in viscosity during the cultivations. Considering the high vis-

Table 5 Key components of industrial media for CPC fermentations. Depending on the strain lineage, requiring methionine or not, Medium 1 or 2 may be used

Production medium	Compound	Medium 1 kg/m^3	Medium 2 kg/m^3
	Corn steep liquor		92.5
	Peanut seed flour	100	
	Cotton seed flour		17.5
	Soya oil		14
	Methyl-oleate	3	
	$MgSO_4 \cdot 7H_2O$	2.5	8
	$(NH_4)_2SO_4$		14
	Ammonium acetate	6	
	$FeSO_4 \cdot 7H_2O$		0.1
	$MgSO_4 \cdot 7H_2O$	2.5	0.04
	$CaSO_4 \cdot 7H_2O$	2.5	
	$CaCO_3$	5	10
	Soya flour		34
	D,L-Methionine	3	
	Urea		3
	Starch		20
	Gulcose	11	65
	Desmophen (antifoam)	3	0.5
pH 5.8 Feed	Soya oil		
	Glucose syrup	500	66%
	$(NH_4)_2SO_4$		20%
	Methionine	15–20	

cosity in combination with a fast growing culture, care must be taken to ensure sufficient supply of oxygen. Even though *Acremonium* is not particular sensitive to oxygen limitation, CPC production will be reduced and precursors, in particular PenN, will accumulate. Experiments with an early generation industrial strain demonstrated that by reducing the pO_2 in the production phase from 40 to 5% of its saturation value, the CPC concentration diminished from 7.2 to 1.1 g l^{-1} and the PenN concentration increased from 2.57 to 7.65 g l^{-1} [19, 24, 25]. Alternative reactor and agitator designs, but more importantly adaptations of the feed and temperature profiles, are used to control and maintain sufficient oxygenation of the culture. The end of the fermentation is defined by a drop in CPC production rate and possibly be a beginning fragmentation of the mycelium.

The beginning of the four stage fermentation process outlined in Fig. 2 is fresh biomass scraped off an agar slant to inoculate the seed cultivation which will provide the biomass for the inoculation of the subsequent, so-called vegetative fermentation stages. These stages are merely required to produce large quantities of biomass as inoculum for the productive fermentation which is

performed in batches of about 100 m^3. Mostly rapidly metabolised carbon and nitrogen sources will support rapid growth during the vegetative stages with little to no CPC being made. In contrast, the productive fermentation stage at which the culture changes to the secondary metabolism requires a carefully controlled balance between rapidly consumed and slowly metabolised carbon sources. Glucose concentration has to be controlled in a narrow range since high glucose levels interfere with numerous catalytic and anabolic pathways. Controlled to a sufficiently low concentration, usually below the 5 g/l, production starts, while overfeeding represses CPC production when higher concentration permit glucose uptake rates to increase beyond 0.5 g/l/h [26]. The relationship between carbon source, its concentration and CPC production has recently been summarized [27]. While avoiding glucose repression a sufficient supply of energy rich carbon source in the form of oil is provided to sustain culture viability and production. Oil represents the major source of energy during the later stages of the fermentation and CPC production. In order to minimize the cost of fermentation and the downstream purification of CPC, oil levels should not exceed 1 vol.%.

The source and the concentration of nitrogen require special attention [28, 29]. Typically the ammonium feed will be controlled as well to prevent both gross excess and starvation. As expected for a protein rich medium, ammonium

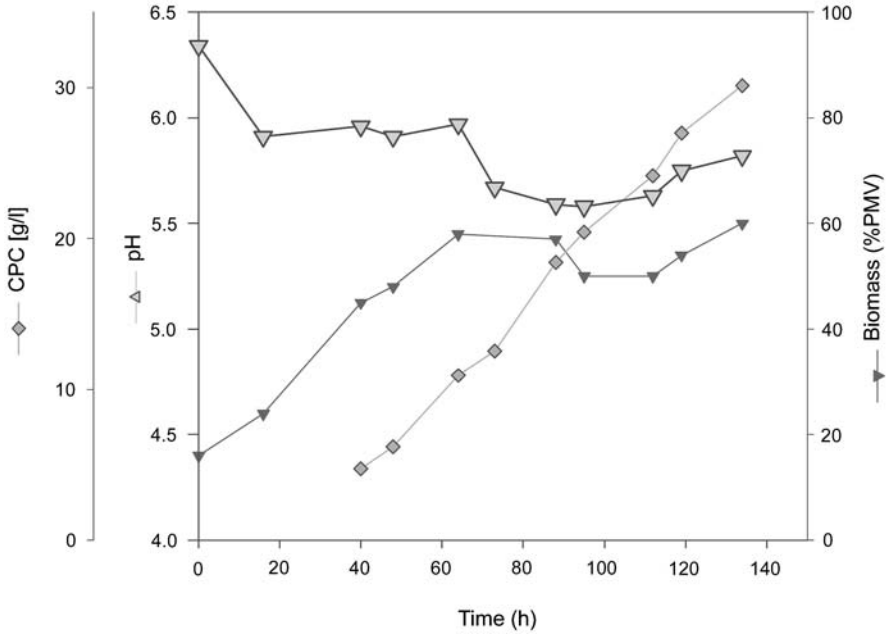

Fig. 4 Course of a production stage fermentation of *Acremonium chrysogenum*. Media optimisations led to the onset of CPC production already in mid-growth phase

is released during the initial growth phase, while acids are released during the productive phase, indicating a growth on sugars. During the latter stage, ammonia feed will also be used for pH control.

The productive fermentation stage takes roughly six days. On-line data on temperature, pH and pO_2, together with samples taken to determine reducing sugars, ammonia, oil, phosphate, sulfur, CPC, and biomass provide the basis for controlling the fermentation. Figure 4 depicts the course of some parameter during the production stage fermentation. In contrast to laboratory-scale experimental cultivations, the industrial setting asks for robust, quick, and simple methodology. A commonly used parameter to determine the biomass concentration is the packed mycelium volume (PMV) obtained by simply spinning down a sample or by having it sedimented under defined condition to determine the percentage of the volume occupied by the biomass. The volume occupied by solids initially present in the broth can be neglected as its contribution rapidly decreases. Similarly, the content of oil floating on top of the centrifuged broth is readily and precisely determined and expressed as a percentage of the total volume.

When the productive fermentation is stopped after approximately five to seven days, the CPC is isolated rapidly to avoid losses owing to its chemical instability in the broth and due to the action of esterases, which will also increase the level of side products, in particular DAC (7–15%), and the 3,4-lactone (~1%). While DAC would serve as precursor for 3-vinyl-derivates, such as cefdinir (Fig. 5) and DAOC, the second major side product (1–3%), could serve as precursor for cefuroxime, cefalexin, cefachlor or possibly cefradine, in practice it is difficult to isolate these products economically. Hence efforts are rather directed toward minimizing the side product formation. This can partly be achieved by the fermentation control; other attempts build on strain selection or genetic pathway engineering approaches as mentioned above.

In every company, numerous programs exist for improving the industrial production processes. Aimed to reduce production costs, strains are developed with increased CPC titres and fermentations are optimised with respect to their yield coefficient ($Y_{X/S}$). The details of the aforementioned processes are generally well guarded within the industrial environment. In particular the strain and their fermentation conditions and the titres obtained are not freely available. Nevertheless, several studies have been published from academic groups that were conducted with older industrial strains [19–22].

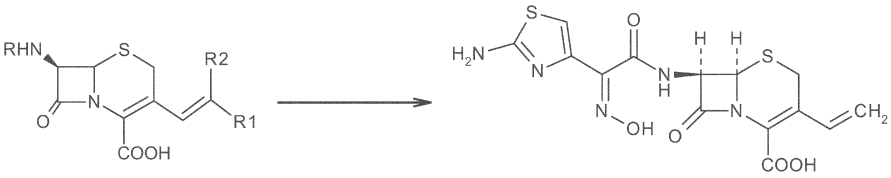

Fig. 5 3-Vinyl-7-ACA precursor for cefdinir

3.1.3 CPC Purification

In contrast to the penicillin where purification is done by a straightforward crystallization adding potassium acetate, CPC requires a more elaborate and expensive chromatographic purification (Fig. 2). Irrespective of process details, the separation of biomass and antibiotic-containing broth is generally achieved by filtration by which the biomass is removed from the CPC containing filtrate. The filtered broth is then passed through large scale hydrophobic interaction chromatography (HIC) columns to remove impurities, in particular proteins, peptides and salts. In addition, the undesired side products DAC and DAOC are removed. The first column, called scavenger, is filled with an adsorber resin, e.g. Diaion HP20 and operated at pH 5.5–6.0 at which hydrophobic coloured compounds bind while CPC adsorption is minimal (<3%). Typically 360 g CPC per litre resin are applied. The second so-called 'adsorber' column is filled with a hydrophobic resin such as Sepabeads SP700. After pH adjustment of 2.8–3, the percolate from the scavenger is loaded to the column. Elution of CPC separate from DAC and DAOC is achieved by a change of pH to 6. The expected capacity is 52 g CPC per litre resin. Ion exchange chromatography (IEX; anion exchanger) is employed to purify further the CPC solution and to remove the remaining colour. This 'decolourizer' column is filled with an anion exchanger, e.g. Diaion DAC11. Equilibrated in the acetate form the CPC percolate from the adsorber is passed through the column. The resin will progressively release CPC before coloured impurities break through. Often a merry-go-round system with three columns is advantageous. In the first column CPC is replaced by the coloured impurities; the released CPC displaces the acetate in the second column, while the third column is being regenerated to the acetic form. When the colour breaks through at the first column, the columns are turned to new positions: column 1 to position 3 for regeneration, column 2 to position 1 for decolourisation and column 3 to position 2 for equilibrating to. Typically 90 g CPC per litre resin distributed in three columns are applied. A weak cation exchanger like Diaion WK40 may be installed to remove potential metal contamination that could interfere with the enzymatic conversion of CPC into 7-ACA. Typically about 180 g CPC (at pH 6) can be applied per litre resin.

Following this purification, CPC can either be isolated as sodium or potassium salt, washed and dried, or the CPC in solution can be directly passed on to the enzymatic conversions. Several options are available at this point (Fig. 6):

- 7′ Side chain removal to yield 7-ACA
- 7′ Side chain removal and addition of new side chain at position 7′ for the synthesis of advanced 7′ intermediates
- Partial removal of the 7′ side chain followed by the modification at position 3′ and complete removal of 7′ chain for the synthesis of advanced 3′ intermediates

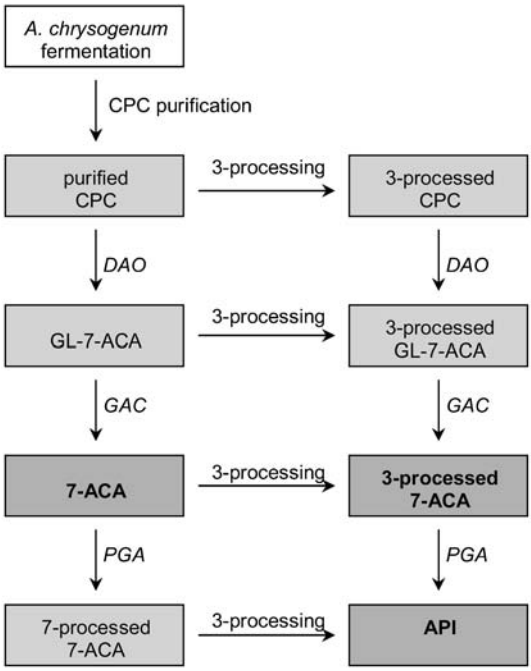

Fig. 6 Biocatalytic routes from CPC to 3′ and 7′ processed 7-ACA derivatives and APIs. *Dark shaded boxes with bold lettering* indicate potential commercial intermediates/products

– 3′ Side chain modification followed by the 7′ side chain removal to result in the synthesis of advanced 3′ intermediates

3.2 Conversion of Cephalosporin C into 7-ACA

Cephalosporins are semisynthetic products that derive from the fermentative product CPC. CPC is initially converted to 7-ACA by either a chemical or an enzymatic removal of the 7-amino adipoyl side chain. 7-ACA represents the key intermediate for the synthesis of the active pharmaceutical ingredient (API) which is obtained after (bio-)chemical derivatization at positions 3′ and 7′ of the β-lactam ring (Fig. 1).

3.2.1 Chemical Cleavage

The nitrosyl chloride cleavage of CPC to 7-ACA developed by Morin et al. 1969 [30] opened the way to 7-ACA production in an industrial scale. This process has now been superseded by processes based on that developed by Ciba [31]. CPC is cleaved using phosphoric pentachloride after protection of the amino

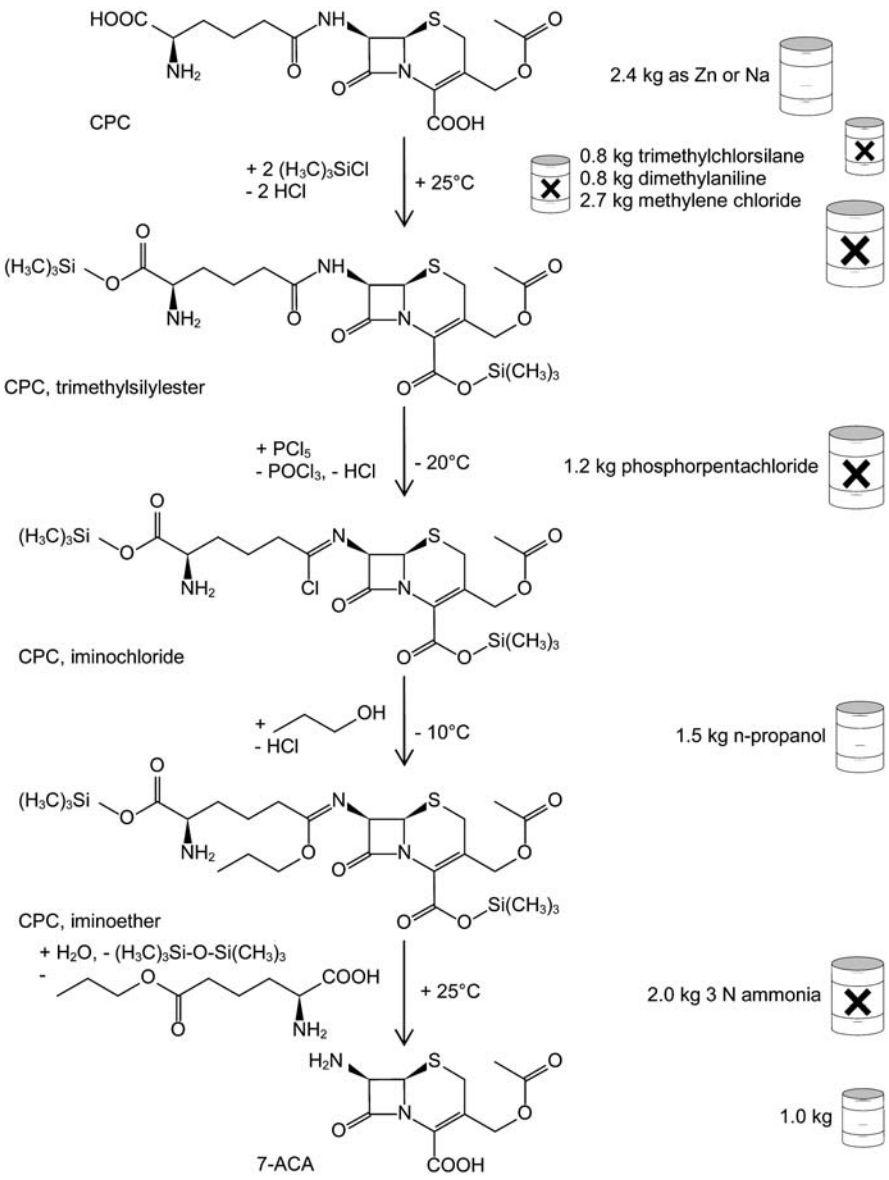

Fig. 7 Reaction scheme of chemical cleavage of CPC into 7-ACA. The material balance on the *left* indicates that 2.4 kg CPC are converted into 1 kg 7-ACA producing 9 kg of waste products. Material requiring special attention is marked with an **X**

and carboxyl functions. The reaction proceeds as far as an imino chloride intermediate in the presence of base, to an imino ether intermediate by addition of alcohol and finally to an ester of 7-ACA by hydrolysis with acid. Further improvements have been introduced by using silyl protection which simultaneously blocks the amino and carboxyl functions of CPC and permits the cleavage with PCl_5 to be carried out in the common solvent methylene chloride [32, 33] (Fig. 7).

About 3000 tons of 7-ACA are produced from CPC annually of which the majority is manufactured by chemical deacylation (China, Japan, Korea). Chemical CPC processing represents the traditional route to the key intermediate.

While yields and product quality produced by the chemical route are excellent, a major drawback is the need for organic solvents and the production of toxic chemical waste. This resulted in a gradual replacement of the chemical route to 7-ACA by the environmentally safer enzymatic cleavage of CPC in Europe. Hence, most of the chemically produced 7-ACA originates form Korea, China and Japan (Table 4).

3.2.2 Enzymatic Cleavage

The biocatalytic conversion for CPC into 7-ACA, the key intermediate to all but the cefalexin-type cephalosporins, was developed in the late 1960s [34]. Two principle enzymatic routes are proposed (Fig. 8):

- One-step hydrolysis of CPC with a CPC acylase (CA)
- Two-step cleavage with D-amino acid oxidase (DAO) and glutaryl acylase (GAC)

The acylase route, however, was never developed into an industrial process. None of the CAs evaluated had satisfactory kinetic properties with CPC as substrate [35–39]. Recently it has been proposed to change the binding site of *Pseudomonas* GAC systematically based on the results of X-ray analysis [40].

In contrast to the one-step cleavage, CPC conversion in two enzymatic steps has become industrial standard for 7-ACA production [34, 41–43]. In the first step of this process, CPC is oxidatively deaminated to keto-adipoyl-7-ACA (KA-7-ACA) by means of a DAO. Peroxide is released in this reaction inducing a spontaneous oxidative de-carboxylation to glutaryl-7-ACA (GL-7-ACA). Addition of small quantities of peroxide after DAO treatment does ensure the quantitative conversion of KA-7-ACA to GL-7-ACA. GL-7-ACA is then hydrolysed by the GAC to 7-ACA and glutarate.

GAC also accepts KA-7-ACA as substrate but the affinity is low and the reaction is strongly inhibited in the presence of GL-7-ACA. Nevertheless, the possibility of a third process exists: The enzymatic cleavage of CPC by DAO combined with catalase and GAC in a single reaction vessel. DAO catalyses the oxidative deamination to KA-7-ACA and the catalase is immediately removing

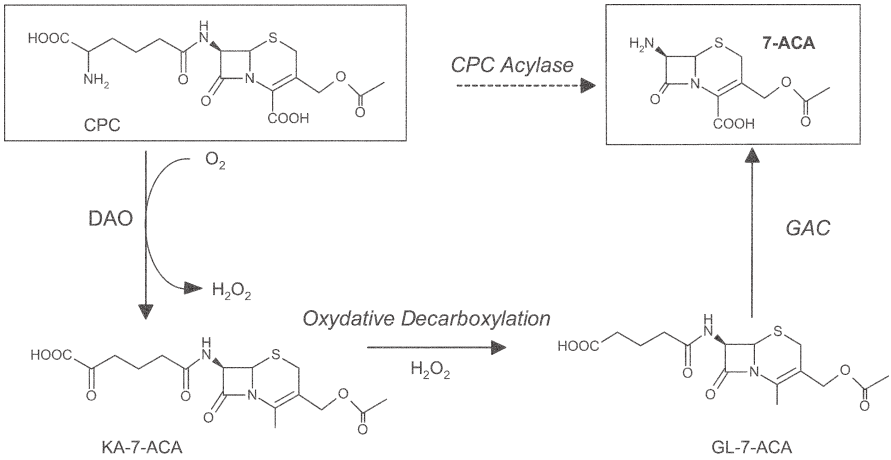

Fig. 8 Enzymatic splitting of CPC into 7-ACA by CPC acylase (*upper, dashed line*) and by the twin enzyme splitting using DAO and GAC (*lower, solid line*)

all the peroxide generated during this reaction. With no GL-7-ACA being formed under these conditions, GAC will convert KA-7-ACA into 7-ACA and ketoglutarate [44].

3.2.3 The Enzymes DAO and GAC

3.2.3.1 DAO

D-Amino acid oxidase (DAO; EC 1.4.3.3) belongs to a group of flavine oxidoreductases and catalyses an oxidative deamination of D-amino acids to produce keto-acids and ammonia [45]; the reaction forms part of the metabolism of D-amino acid.

D-Amino acid oxidases are rather frequent in nature; they are present in mammalian organs, mainly in the kidneys. Different microorganisms too are known to produce DAO, such as the yeasts *Trigonopsis variabilis* [46, 47], *Candida tropicalis* [48] and *Rhodotorula gracillis* [49], the fungi *Neurospora crassa* [50]; *Rhodosporidium* spec. [51], *Fusarium solani* [52], *Penicillium chrysogenum* and *Fusarum oxysporum* [53, 54] to mention only those of microbial origin. Only two enzymes, namely DAO from *Rhodotorula gracilis* and from *Trigonopsis variabilis*, have been developed into an industrial biocatalyst.

The DAO of *Rhodutorula gracilis* and *Trigonopsis var*iabilis is a homodimer (2×38 kDa) of which each subunit contains one iron ion and a non-covalently bound FAD [47, 55].

The biotechnological and biochemical applications for DAO extend from preparations of pure L-amino acids from racemic mixtures [56], the production of keto-acids from D-amino acids or in the quantitative and qualitative analysis of D-amino acids [57, 58] to the by far most important application, the enzymatic cleavage of CPC into GL-7-ACA.

3.2.3.2 GAC

GAC is a metal free heterodimer of 16 kDa+54 kDa subunits without any prosthetic group [59]. GAC has been isolated from many organisms [59, 42], but only the one from *Preudomonas diminuta*, cloned and expressed in a recombinant *E. coli* has been developed into an industrial biocatalyst.

The conversion of CPC to 7-ACA by a single enzymatic reaction has been of great interest. GACs from different sources are able to utilize several substrates and substrate analogs, but their activities on CPC vary from 0 to 4% relative to GL-7-ACA [37, 60, 61]. Site-directed mutagenesis has been carried out to improve its activity on CPC, but only a less than twofold improvement was obtained compared with the wild-type enzyme from *Pseudomonas* N176 [62, 63]. The recently determined three-dimensional structure of the active site conformation of the *Pseudomonas* GAC revealed the detailed interactions of GL-7-ACA with the side chain pocket in the active site. It was suggested that the glutaryl side chain moiety of GL-7-ACA is a dominating factor in substrate binding in the active site [64].

The unsuccessful attempts to obtain a direct enzymatic transformation of CPC into 7-ACA by a single GAC have led to the development of a two-enzyme process using DAO and GAC in sequence that was recently described by Tischer and coworker [65].

3.2.3.3 Isolation and Immobilization

Development of DAO and GAC into an industrial biocatalyst includes the fermentative production, the isolation, purification, and immobilization of the enzymes. Enzyme purification is needed to eliminate any unwanted catalytic activities found in cell extracts. In particular the activities of the CAH and catalase need to be removed. CAH increases the content of deacetyl-7-ACA (DA-7-ACA) in the product by cleaving off the acetyl group from position 3′. CAH will react on all CPC derivates throughout the process an can hence cause considerable losses in yields and purity. Catalase will consume the peroxide needed for the spontaneous de-carboxylation of KA-7-ACA into GL-7-ACA. KA-7-ACA itself is unstable and decays to unknown products and the addition of increased amounts of peroxide to drive the conversion to GL-7-ACA will, as result of the interaction of peroxide with the CPC nucleus, lead to more side product [42]. CAH and catalase can either be sep-

arated from DAO and GAC employing standard protein purification technologies [66, 67] or can be inactivated by chemical or physical treatment [68–70]. Alternatively catalase deficient production strains for the two enzymes may be used [71, 72].

DAO as well as GAC are both inactivated by separation of their subunits. Hence the immobilization of the enzymes on a solid support greatly increases their stability. Both enzymes have been immobilized as whole cells or as cell extracts in gels or on prefabricated carriers [69, 71, 73]. Currently only purified enzyme preparations are used for immobilization on commercially available carriers. DAO can be successfully immobilized covalently on epoxy carrier or glutardialdehyde activated amine matrixes, while the GAC is robust enough to be immobilized also on strong ion exchanger with subsequent crosslinking by glutardialdehyde [74]. By using selective immobilization conditions it seems to be possible to bind specific areas of DAO and GAC to the carrier in an "oriented binding" [75] and to provide a multipoint attachment [44]. Both measurements lead to a higher stability for these enzymes.

Latest developments of enzyme immobilization like CLECs or CLEAs have not yet been applied in large scale immobilization of DAO and GAC.

3.2.3.4 Large Scale Enzymation of CPC

Different strategies for the use of the immobilized enzymes have been proposed.

According to the three aforementioned reaction steps the use of three vessels is common: In the first reactor is CPC converted to GL-7-ACA by DAO, the second reactor is used to oxidize the remaining amounts of KA-7-ACA to GL-7-ACA with peroxide, and in the third reactor GAC hydrolyses GL-7-ACA to 7-ACA (www. roche-applied-science.com/indbio/ind/PDF/cc2.pdf) (three pot design).

It is possible to carry out the DAO reaction and addition of peroxide to complete the oxidative de-carboxylation by slowly adding H_2O_2 in the first reactor, however accepting a slight peroxide-induced inactivation of the DAO [41, 65] (two pot design).

The combined use of DAO, GAC and catalase (one pot design) has been developed but has up to now not been implemented at large scale [44].

Several reactor design studies were performed which have not been realized in industrial scale. These include the cascade of reactors performing each enzymatic stage in three consecutive vessels to improve the efficacy of the enzyme use [76], and a packed column reactor which did not fulfil the requirement on oxygen transfer for DAO due to the low solubility of oxygen in the substrate solutions supplied to the column. Thus, reactions are largely performed batchwise in stirred tanks, pressurized with dispersed oxygen or air. In contrast to DAO, GAC can be used in packed columns as well as in stirred batch reactors.

Important parameters for any enzyme reaction are substrate concentration, pH, and temperature.

The DAO has no back reaction and hence no product inhibition. Therefore substrate concentration can be as high as 180 mmol/l CPC and the pH has no impact on the reaction yield as long as the enzyme, product and substrate are stable. Furthermore, the DAO catalysed oxidation shows linear substrate consumption and slows down as a concentration of 3 mmol/l CPC is approached due to the K_m-value of the immobilized DAO. Under industrial conditions the reaction is usually performed until the residual CPC concentration is below 1% of the starting substrate solution.

In contrast the GAC catalysed reaction is reversible and depends on pH and product concentrations. The reaction between glutarate and 7-ACA reach an equilibrium, and even at pH 8 and initial 75 mmol/l GL-7-ACA, up to 5% of the substrate remains unconverted. This equilibrium can be lowered by increasing the pH up to 8.5 or by decreasing the substrate concentration. However, at this high pH GL-7-ACA and 7-ACA is unstable. Hence exposure to these unfavourable conditions needs to be minimized. Due to their thermodynamic instability CPC, GL-7-ACA and 7-ACA are suffering a considerable decay during down streaming. In average, the β-lactam nucleus remains in aqueous solution for 16 h, from after harvesting the fermentation to the recovery as 7-ACA in the crystallization. Whenever possible, low temperature, 8–10 °C, and the suitable pH of 4.5–6 must be maintained to minimize this decay.

In addition to yields, product quality will decide over commercial success of production. To reach an acceptable 7-ACA, additional product purification may be needed. However, any additional step will reduce the yield and increase considerably running and investment costs.

3.2.4 Deacetyl-7-ACA by CAH

A niche product among the CPC based β-lactam intermediates is the DA-7-ACA with a worldwide annual demand for approximately 100 t. DA-7-ACA is used for the production of 3-vinyl substituted intermediates (Fig. 5).

CPC acetyl hydrolase (CAH) catalyses the hydrolysis of the ester bondage on position 3′ of a CPC nucleus (Fig. 9). The kinetics of this enzyme resembles that of the GAC [77]. The enzyme is widely distributed in nature. It has been isolated from different fungal and bacterial sources [78–82] and been used as either whole cells preparation or as carrier immobilized cell free enzyme preparations.

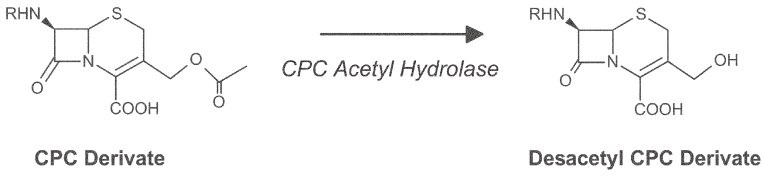

Fig. 9 CPC acetyl Hydrolase (CAH) reaction scheme

DA-7-ACA is normally produced by hydrolysis of 7-ACA, but can also be obtained from DAC, a side product (7–15%) of the CPC fermentation which is normally wasted. Converting CPC quantitatively into DAC using CAH followed by the enzymatic splitting with DAO and GAC has been proposed [41] but is pending implementation in technical scale.

4 Process Economics of 7-ACA Production

As indicated earlier, competition from India, and the Far East with their ability to produce pharmaceutical intermediates and ingredients has put the prices for β-lactam intermediates like 7-ACA under pressure.

Most producers of 7-ACA are back-integrated to the CPC fermentation so that they could apply economical and technical optimisations throughout the whole product value chain. In this respect the fermentation titre of CPC has an important effect on costs since it determines directly the level of 7-ACA output.

Hence, state of the art fermentation equipment, constant strain and process improvement will significantly contribute to cost reduction.

The introduction of the enzymatic CPC cleavage provides another important contribution to cost reduction as CPC from the purified fermentation broth can be directly processed. Crystallization and drying of CPC as sodium or potassium salt is no longer required. Elimination of these steps again increases the yield reducing production costs, in particular for energy, water, waste treatment, equipment, and labour. Finally the application of suitable and appropriate recovery systems for water, solvents, caustic soda, and others can further reduce production cost. Apart from principal measures to increase the yield of every step and to minimize losses, in particular product decay during downstreaming, the cost structure of a company also affects the process economics.

The production costs of 7-ACA are approximately four to five times higher compared to 6-APA which are estimated to be around 15–25 $/kg. Three factors contribute to this discrepancy. First, the CPC yield in fermentation is eight- to tenfold lower than that of penicillin. Second, CPC recovery requires filtration-dialysis and a column chromatography which is very much increasing need of

Table 6 Cost structure for the production of 7-ACA

Raw and running materials	22±4%
Enzymes	15±8%
Utilities and technical services	22±4%
Labor and overheads	18±4%
Capital costs, insurances, depreciation, maintenance	23±4%

utilities (steam, electricity, water) and running materials (filtration membranes, resins) whereas penicillin G or V is extracted directly from the broth and easily purified by crystallization. Third, the conversion of penicillin into 6-APA catalysed by a penicillin G or V acylase is more efficient then the CPC conversion to 7-ACA by DAO and GAC. While on the commercial scale penicillin G acylase (PGA) consumption can be as low as 0.06 kU/kg 6-APA [18], 1 kg 7-ACA requires about 1 kU of DAO and 1 kU of GAC (www. roche-applied-science.com/indbio/ind/PDF/cc2.pdf). In addition, the production costs for DAO and GAC are higher than those for PGA.

An estimation of the contribution of the various cost centres for 7-ACA, such as fermentation (mostly raw and running material) downstreaming (mostly enzymes), utilities, labour and company overhead are summarized in Table 6. The variability at each position is indicative for regional and structural differences among companies.

5
Advanced Intermediates

Active semi-synthetic cephalosporins are mostly derived from 7-ACA (Fig. 1). Most often also the 3′ position is changed. Addition of a new side chain in position 7′ or alteration of the 3′ side chain will lead to advanced intermediates which are subsequently converted into the API. Traditionally 7-ACA is a key intermediate for the synthesis of semisynthetic cephalosporins; however, as indicated in Fig. 6, early processing of the 3′ position is possible, resulting in the synthesis of 3′ position advanced intermediates. For example, processing the 3′ position with MMTD or MTZ (Fig. 1) will yield TDA or TTA, the precursors for cefazolin and ceftriaxone, respectively. Both products are traded products.

5.1
3′ Position

The acetyl group in the 3′ position can be substituted in an S_N1 mechanism by heterocyclic mercapto groups or ring bound nitrogen.

Following the traditional chemical route, modifications at the 3′ position will be done by water-free chemistry: 7-ACA is 3-processed by heterocyclic thiols in organic solvents in presence of toxic BF_3 gas, which catalyses the reaction as Lewis acid in 85% yield [83]. The yields of this process are generally high and the end products are of good quality.

Alternatively aqueous chemistry is possible for 3′ processing, e.g. with heterocyclic thiols or pyridine in aqueous solution. The substitution reaction takes place at neutral or slightly acidic pH at a moderately elevated temperature of between 50–75 °C and excess of side chain. While a higher temperature positively influences the conversion rate, the stability of the β-lactam is decreased.

As a consequence, yields in aqueous solution are lower (about 65%) and the isolated derivatives can have an undesired yellow or brownish colour. The colour formation can be reduced or eliminated by the addition of reducing agents, such as sodium sulphite or sodium dithionite [84].

Alternatively, protection of the 7′ amino group of 7-ACA can reduce colour formation. This protection step may not be necessary, if 3′ processing occurs on GA-7-ACA [85] or directly on CPC [86, 87]. Compared to 7-ACA, conversion rate is slower, which can be compensated by a higher temperature with still negligible colour formation. 3′ Processed CPC can be processed by DAO via the keto intermediate to the glutaryl derivative, which is finally converted by GAC to the 3′ processed 7-ACA product. It is recommended to remove unreacted heterocyclic thiols prior to biocatalytic cleavage of the 7′ side chain. In particular the DAO is sensitive to deactivation by thiols.

Finally, the modification in 3′ position may be done after a 7′ derivatization of 7-ACA using the same aqueous chemistry. However, no unreacted 7-ACA should be present in the reaction which would result in colour formation. Conditions, reaction rate and yields are similar to those of CPC or GL-7-ACA conversion. Recovery of unreacted surplus side chain before isolation of the final product can be advantageous, contributing to both, improved product quality and process economy.

5.2
7′ Position

The synthesis on semi-synthetic β-lactams includes the modification of the free amino group of the β-lactam nucleus (7′ position for cephalosporins, and 6′ position for penicillins).

5.2.1
Chemical Route

Traditional chemical synthesis requires that the carboxylic group of the β-lactam nucleus is protected by trimethylsilylation as the formation of acid chlorides is used for activation. For example D-phenylglycyl chloride is formed in halogenated organic solvents from D-phenylglycine and PCl_5, which is formed in situ from PCl_3 and Cl_2.

Sometimes this approach fails e.g. for *p*-hydroxy-D-phenylglyine, where the activated side chain is not available in desired purity and at an attractive price. Then, the so-called Dane salt method [88] was adopted for the activation of amino acids. The Dane salt is prepared from the side chain and methyl acetoacetate for protection of its amino group. It is subsequently converted in situ to a mixed anhydride, e.g. with pivaloyl chloride in presence of a base at low temperature (–60 to –50 °C). After coupling with the unprotected β-lactam nucleus the amino protecting group of the coupled side chain is removed at pH 1.

Although both ways result in nearly quantitative yields, solvents and auxiliary reagents used generate up to 40 kg of non biodegradable waste per 1 kg cefalexin produced [89].

5.2.2 Biocatalytic Route

In particular the environmental issues and associated costs have resulted in the development of economically and environmentally viable enzymatic alternatives for the 7′ derivatization of the β-lactam nucleus. These highly selective conversions are generally performed in aqueous environment and ambient temperatures.

Penicillin G amidase (PGA, EC 3.5.1.11), which is normally used for the hydrolysis of penicillin G or cephalosporin G, is also a powerful tool for synthesis of semi synthetic β-lactam antibiotics. Two approaches have to be distinguished (for review [90]).

The free acids of the side chains are used in the *thermodynamically* controlled synthesis. The equilibrium follows the degree of not dissociated side chain, which depends on the k_s of the acid and the pH value. At the required low pH PGA is not very active and the conversion is slow. The dissociation constant can be shifted to more favourable values by water soluble solvents as ions are less well hydrated. In contrast the k_s is reduced by high ionic strength as ions are better stabilised. Water soluble solvents have also a direct influence on the equilibrium, since they reduce the water activity and therefore water, a by-product of synthesis, is apparently removed. Solvents, however, may cause fast deactivation of PGA. Following the law of mass action, the yield increases with higher concentrations of β-lactam nucleus and side chain. Temperature and enzyme input influence mainly reaction time, but less yields.

The *kinetically* controlled synthesis with esters or amides of the side chains is much faster, since the Gibb's energy of the activated side chains is improved and more alkaline pH values may be applied at which the PGA is more active. The higher Gibb's energy is also the reason for better yields compared to the thermodynamic equilibrium. However, the kinetically controlled formation of the β-lactam antibiotic reaches a maximum and is accompanied by the consecutive β-lactam hydrolysis. Hence, kinetically controlled condensations have to be monitored carefully and terminated in time to minimize loss by hydrolysis. There are often conflicting effects of, e.g. higher pH, higher temperature or more enzyme input increasing the activity for both, hydrolysis and synthesis. In practice a pH around 6 to 7.5, low temperatures, e.g. 10 °C and an enzyme input for 4–8 h reaction time are considered optimal for good yields. In addition, high substrate concentrations will be advantageously for yields. Water soluble solvents and ionic strength will have some influence as detailed earlier.

The substrate specificity of PGA requires that the side chains have the following similarities to the native substrate, phenylacetic acid:

– A residue with π-electrons (phenyl-, pyridyl-, thienyl-, tetrazolyl-, CN- etc.)
– A short spacer (-CH_2-, -CHOH-, $CHNH_2$-, -OCH_2-, -SCH_2-)
– A carboxylic function or derivative (-COOH, COOR, CONHR)

Since the enzyme will convert only dissolved reactants their solubility has a great influence on reaction rate and yields. Solubility problems can be addressed by choosing between the thermodynamically or the kinetically controlled synthesis as shown in the following examples.

For cefalotin synthesis the thermodynamically controlled synthesis is advantageous as the thienylacetic acid side chain is water soluble, while its esters, e.g. methylester, dissolve only partly at optimal reaction conditions (Table 7). The thienyl acetic hydroxyethylester is not soluble under the conditions needed for the reaction.

Yields of the thermodynamically controlled synthesis and the kinetically controlled approach are very similar if a water soluble ester can be chosen for activation [91]. Nevertheless, the latter approach has clear advantages such as significantly lower enzyme consumption for the same conversion time. The 7′

Table 7 PGA mediated cefalotin synthesis from 7-ACA and thienyl acetic acid. Comparison between the thermodynamically controlled reaction using the free acid and the kinetically controlled reaction with the thienyl acetic acid methylester

	No activation (free acid)	Methylester (partly soluble)
7-ACA	150 mmol/l	100 mmol/l
Thienylacetic acid	600 mmol/l	175 mmol/l
Isopropanol	No	10%
PGA-450	16–20 kU/l	4–5 kU/l
Temperature	20 °C	10 °C
pH	Shift 7.0–5.3	6.75
Reaction time	8–10 h	3–5 h
Yield in solution	93–94%	81–86%

Table 8 PGA mediated mandoyl-7-ACA synthesis from 7-ACA and mandelic acid. Comparison between the thermodynamically controlled reaction using the free acid and the kinetically controlled reaction with the methyl- and hydroxyethylester

	Free acid (no activation)	Methylester (partly soluble)	Hydroxyethylester (soluble)
7-ACA	150 mmol/l	150 mmol/l	150 mmol/l
D-Mandelic acid	600 mmol/l	300 mmol/l	288 mmol/l
PGA-450	13 kU/l	2.5 kU/l	1.8 kU/l
Temperature	20 °C	10 °C	10 °C
pH	shift 7.0–5.3	6.5	7.0
Reaction time	5.1 h	4 h	1.5 h
Yield in solution	90.6%	72.8%	91.5%

side chain of cefamandole, mandelic acid, is an example where either the free acid, D-mandelic acid, or its esters may be used to convert 7-ACA into mandoyl-7-ACA. The approaches are compared in Table 8.

The yields obtained with the free acid and the hydroxyethyl-activated side chain are similar. While the use of the free acid may be cheaper, this advantage is offset by the higher concentration of side chain in the reaction, a seven-times higher enzyme input, an over three times longer reaction time, and a more complicated process control (pH ramp).

6 APIs by 7′ and 3′ Modified 7-ACA

As mentioned earlier, the synthesis of an active cephalosporin in most cases requires the derivatization of both 3′ and 7′ positions. Based on the classical chemical synthesis starting from 7-ACA, the 3′ position is modified prior to the 7′ position. In contrast, biocatalytic synthesis combined with aqueous chemistry opens up the possibility to use alternative starting material such as CPC or GL-7-ACA (Fig. 6). Nevertheless, the decision on which route to follow is driven by two parameters: step yields (≈costs) and regulatory issues. The latter point is relevant only if an existing process is to be modified, which then will require a set of actions to verify that the specifications of the final product are not adversely affected by the changes in the process.

Starting with 7-ACA as substrate PGA can be used to perform the 7′ derivatization. Generally, acidic conditions are favoured, to minimize hydrolysis and to keep the side chain in its undissociated form. However, the solubility of the 3′ processed 7-ACA derivatives often requires a more alkaline pH and/or a lower substrate concentration. At higher pH, however, the rate of hydrolysis increases, thus lowering yields, while lower substrate concentrations reduce the space time yield.

As detailed earlier, the 3′ coupling in aqueous conditions is done at elevated temperature causing some decomposition of the substrate. In particular when 7-ACA is used as substrate this decomposition results in a strong colour formation. Hence, if a process is designed to firstly add a side chain at 7′ position, care must be taken to remove all remaining traces of unreacted 7-ACA prior to 3′ processing to avoid excess colour formation. This may require that the 7′ derivative has to be isolated.

With cefazolin synthesis as an example, the two possible routes are compared and summarized in Table 9.

The synthesis of cefazolin via TDA is done at higher pH and with a reduced substrate concentration to overcome the poor solubility of TDA at acidic pH. Consequently, more enzyme (PGA) and side chain are needed and the space-time yields are lower as compared to the route via TZ-7-ACA.

Besides yields, the decision to use either route is also influenced by the product quality obtained and other industrial process considerations, such as costs

Table 9 Synthesis of cefazolin using aqueous chemistry combined at position 3′ and enzymatic synthesis at position 7′. Comparison of the two possible routes starting the synthesis at the 3′ position or at the 7′ position, respectively

	3′→7′ Processing	7′→3′ Processing
3-Processing:	75 mmol/l CPC	
MMTD	75 mmol/l	
Isolated step yield	~50% TDA	
7-Processing:	62.5 mmol/l TDA	150 mmol/l 7-ACA from CPC
TZM	125 mmol/l	265.5 mmol/l
PGA	5 kU/l	1.5 kU/l
pH	Ramp: 7.5–6.7	7.0
Isolated step yield	>80% cefazolin	57% TZ-7-ACA
3-Processing:		150 mmol TZ-7-ACA
MMTD		150 mmol/l
Isolated step yield		60% cefazolin
Total cefazolin yield	35–40%	34%

for the side chain and its recovery or disposal which have not been addressed here.

7 Outlook

The semi-synthetic β-lactam antibiotics will retain a significant market share. Their broad spectrum of activity, high potency, low toxicity, and stability toward hydrolysis makes them a class preferred by clinicians.

New semisynthetic cephalosporins continue to be developed in the pharmacology programs of different companies and new generations of cephalosporins will be available in the future. As the pressure on the price remains high, new developments are fuelled by the advances in knowledge on the molecular biology of CPC biosynthesis. Molecular biology has entered the strain improvement programs of most companies and combined with a more careful control of the fermentation process will lead to more efficient processes. Whether titres similar to those obtained in penicillin fermentation can be reached remains to be seen.

The introduction of 'green' routes to semi-synthetic β-lactams has started in Europe and has much room for improvement and expansion. Still most of the 7-ACA is produced by the traditional route as are most if not all of the APIs. Changes will only gradually be implemented as margins are low and products long off patent. Hence, most production facilities are long depreciated and investment into upgrading a facility is difficult to justify. However, emerging new compounds and new companies have the opportunity to step forward and implement new technologies.

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