CHIMIA 1999, 53, No. 12

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Received: October 5, 1999

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Chimia 53 (1999) 596–600
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ISSN 0009–4293

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1. Introduction

FK-506 (**1**) is a relatively new macrolide immunosuppressant [1][2], reported to be 100 times more effective *in vitro* than cyclosporin [3], the extremely effective anti-rejection drug which revolutionized the field of organ-transplant surgery. The macrolide **1** is produced by *Streptomyces tsukubaensis* fermentation and was originally isolated by the *Fujisawa Pharmaceutical Company*. FR-900520 [4][5], a closely related macrolide, is produced by *Streptomyces hygroscopicus* subsp. *yakushimaensis* and was later shown to be identical with ascomycin (**2**) [6–10], an antifungal compound produced by *Streptomyces hygroscopicus* var. *ascomyceticus*, which was discovered by *Bristol-Myers & Co.* in 1960. FK-506 and ascomycin are useful in preventing host rejection of organ transplants, *e.g.*, bone marrow, liver, lung, kidney, and heart transplants. They inhibit interleukin production [11], mixed lymphocyte proliferation, and generation of cytotoxic T-cells [12][13]. Like cyclosporin, FK-506 and ascomycin have undesirable side effects, particularly CNS and renal toxicity [14]. FK-506 and ascomycin may offer some advantages over cyclosporin, but it is unlikely that they will become a safe replacement for cyclosporin in transplantation rejection. Thus, structural modification of **1** and **2** to generate new analogs with reduced side effects and increased bioavailability may provide utility not only in transplantation surgery, but also in other therapies.

The structure and absolute configuration of FK-506 was determined by chemical and spectroscopic techniques, including single-crystal X-ray analysis [2]. It is an unusual macrocyclic lactone possessing several functionalities including three methoxy groups at C(13), C(15), and C(31), two hydroxy groups at C(24) and C(32), one oxo group at C(22), two unsatura-

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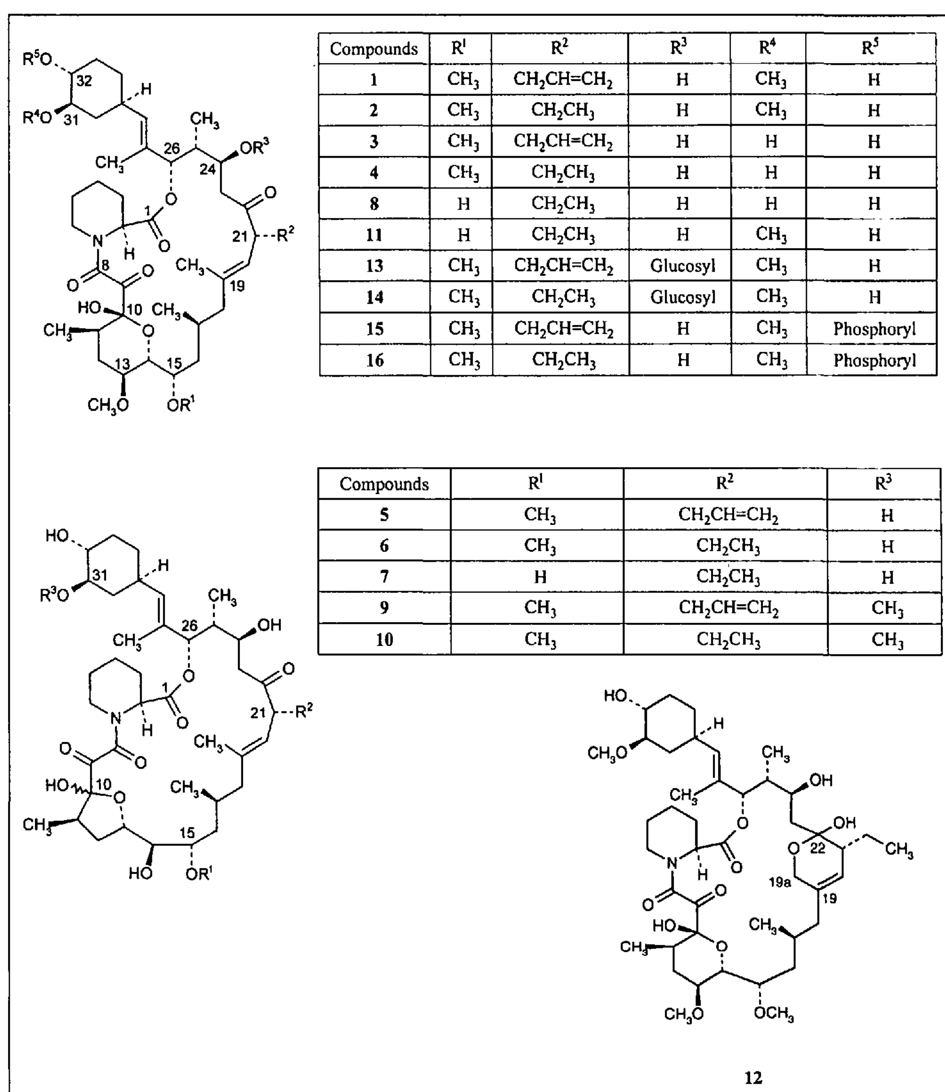
tions, one piperidine ring and a novel dicarbonyl hemiketal moiety from C(8) to C(10). FK 506 differs structurally from ascomycin by substitution of an allyl group for an ethyl group at C(21) of the macrocyclic ring. Both FK-506 and ascomycin (*Fig.*) are water insoluble, but dissolve readily in organic solvents such a methanol, acetone, and DMSO.

Structural modifications can be obtained using either a chemical or a biological approach. The chemical approach, involving total synthesis or chemical modification, has been extensively developed and is extremely successful. The biological approach, however, becomes very attractive when dealing with complex molecules which cannot be easily synthesized or modified using chemical synthetic techniques. Microbial transformation is the most popular biological technique. It utilizes enzymes produced by microorganisms to perform organic reactions on substrates added to the culture medium. Microorganisms are seen as a rich source of diverse biocatalysts; their enzymes can mediate a variety of reactions of interest to organic chemists. They offer attractive alternatives to more conventional chemical methods, because they can attack chemically inert molecular regions and catalyze reactions with high degrees of regio- and/or stereospecificities and -selectivities. Moreover, a single microorganism may contain more than one useful enzyme and thus catalyze several reactions at a time, yielding a simple multi-step conversion process. Other important features are that microbial reactions occur under extremely mild conditions with respect to pH and temperature. However, enzyme binding specificity may require that large numbers of microorganisms be screened to discover cultures yielding a useful transformation.

In this review, FK-506 (**1**) and ascomycin (**2**) have been selected from our various projects to illustrate the potential for microbial transformation to prepare derivatives of biologically important natural products.



Compounds	R¹	R²	R³	R⁴	R⁵
1	CH_3	$CH_2CH{=}CH_2$	H	CH_3	H
2	CH_3	CH_2CH_3	H	CH_3	H
3	CH_3	$CH_2CH{=}CH_2$	H	H	H
4	CH_3	CH_2CH_3	H	H	H
8	H	CH_2CH_3	H	H	H
11	H	CH_2CH_3	H	CH_3	H
13	CH_3	$CH_2CH{=}CH_2$	Glucosyl	CH_3	H
14	CH_3	CH_2CH_3	Glucosyl	CH_3	H
15	CH_3	$CH_2CH{=}CH_2$	H	CH_3	Phosphoryl
16	CH_3	CH_2CH_3	H	CH_3	Phosphoryl

Compounds	R¹	R²	R³
5	CH_3	$CH_2CH{=}CH_2$	H
6	CH_3	CH_2CH_3	H
7	H	CH_2CH_3	H
9	CH_3	$CH_2CH{=}CH_2$	CH_3
10	CH_3	CH_2CH_3	CH_3

Figure. *The structures of FK-506* (**1**), *ascomycin* (**2**), *and their derivatives obtained by biotransformation*

2. Microbial Reactions

2.1. *31-O-Demethylation*

Because of the structural complexity of FK-506 and ascomycin, specific demethylation is very difficult, if not impossible, from a synthetic point of view. Our microbial transformation program discovered that *Actinoplanes* sp. ATCC 53771 [15] can specifically demethylate the methoxy group at C(31) of FK-506 and ascomycin in an overnight incubation to give the 31-*O*-demethyl derivatives **3** and **4**. The structure determination of **3** and **4** was based on a virtually complete analysis by ^{1}H-NMR. The ^{1}H-NMR spectra showed a loss of the MeO–C(31) signals, and the absence of the H–C(31) resonance at its characteristic chemical shift near 3.1 ppm for both **1** and **2**. The FAB mass spectra of **3** and **4** were consistent with the proposed structure revealing $[M+Li]^+$ ions of *m/z* 796 and 784, which correspond to a loss of 14 mass units from **1** and **2**, respectively. The 31-*O*-demethyl derivatives were very helpful in guiding the purification of 31-*O*-methyltransferases from the producing cultures as well as in mutation studies to find cultures producing 31-*O*-demethyl derivatives by fermentation [16][17].

The enzyme which catalyzed the 31-*O*-demethylation of **1** and **2** was purified from a cell-free extract by ammonium-sulfate fractionation followed by FPLC on DEAE-Sepharose and on an anion-exchange *Mono Q* column. The purified 31-*O*-FK-506 demethylase is a monomeric protein of 42 kDa apparent molecular weight having a pI of 4.2–4.5. It is a constitutive P450-type protein requiring NADPH, ferredoxin-$NADP^+$-reductase, and ferredoxin for activity [18].

2.2. *13,31-O-Bisdemethylation, 15,31-O-Bisdemethylation and 13,15,31-O-Trisdemethylation*

Actinoplanes sp. ATCC 53771 is useful in that further incubation for 5–6 h produces, in addition to the 31-*O*-demethyl derivatives **3** and **4**, 13,31-*O*-bisdemethyl (**5** and **6**), 13,15,31-*O*-trisdemethyl (**7**), and 15,31-*O*-bisdemethyl (**8**) compounds in a sequential manner. Demethylation of the methoxy group at C(13) can lead to isomers by formation of different hemiketals as shown in the *Scheme*.

Purification of bisdemethyl and trisdemethyl derivatives was accomplished by preparative HPLC. Chromatographic studies demonstrated that two major constituents existed in equilibrium when the methoxy group at C(13) was demethylated. The two major isomers were separated

CHIMIA 1999, 53, No. 12

by more than three minutes retention time. The ^{1}H-NMR spectra of **5**, **6**, and **7** are complicated by the existence of two tautomeric isomers and show major differences from their parent compounds. They all show close similarity with the distinctive features of the downfield displacement of the proton signals at C(2), C(11), C(12), and C(13) relative to their parents. The shifts are attributed to the formation of the new ring which causes the perturbed protons to be either closer to or farther away from a nearby nucleus. From the spectra, the five-membered ring structure is strongly favored as a major component, primarily based on the large vicinal coupling constants of 10.8 and 8.7 Hz between the proton at C(11) and the methylene protons at C(12). The two large vicinal coupling constants are most unusual and, to our knowledge, do not occur in six-membered rings, but are seen in appropriately substituted five-membered ring systems containing oxygen. The FAB mass spectra gave $[M+Li]^+$ ions which correspond to a loss of 28 or 42 mass units for bisdemethylated (**5**, **6**) and trisdemethylated (**7**) derivatives, respectively.

The structure of the 15,31-*O*-bisdemethyl compound **8** was assigned based on the disappearance of MeO–C(15) and MeO–C(31) signals, coupled with a 0.37 ppm downfield shift of the H–C(15) signal, while the H–C(13) signal was unchanged in the ^{1}H-NMR spectrum. The structure was further confirmed by fragmentation analysis of mass spectra.

2.3. *13-O-Demethylation*

Microbial transformation of **1** and **2** was rewarding in that several microorganisms provided 13-*O*-demethyl derivatives **9** and **10**. Four microorganisms were found in our screening program to specifically biotransform **1** and **2** into **9** and **10**, respectively. The actinomycete ATCC 53828 gave a 35% yield of **9** and **10** after incubation with **1** and **2** in shake flasks [19]. The ^{1}H-NMR spectra of **9** and **10** revealed mostly the same major differences from **1** and **2** as the 13,31-*O*-bisdemethyl derivatives **5** and **6**, which have undergone further demethylation. The 13-*O*-demethyl derivative was isolated as major metabolite of **1** from liver microsomal incubation [20]. Therefore, large quantities of **9** and **10** were produced by microbial transformation for further biological evaluation.

2.4. *15-O-Demethylation*

None of the cultures screened produced the 15-*O*-demethyl derivative of **2**. A solution to this problem might be using *Actinoplanes* sp. ATCC 53771 to produce the 15,31-*O*-bisdemethyl compound **8**, then incubate it with a cell-free extract from *Streptomyces* sp. *ascomyceticus* MA 6475 containing 31-*O*-methyltransferase, which catalyzes the specific methylation of the hydroxy group at C(31) of **2** [21][22], to produce the 15-*O*-demethyl derivative of **2**. Using **8** as substrate, an enzymatic reaction with 31-*O*-demethylimmunomycin *O*-methyltransferase and *S*-adenosylmethionine as the methyl donor produced **11** [23]. The structure of **11** was completely characterized by a combination of MS and ^{1}H-NMR spectroscopy. Briefly, the FAB-MS analysis showed a molecular ion which was 14 mass units heavier than **8** and 14 mass units lighter than **2**. ^{1}H-NMR showed a new methyl signal at 3.41 ppm, which was assigned to MeO–C(31). The methyl signal of MeO–C(15) apperars at 3.30 ppm. In addition, the chemical shift of H–C(31) in **11** was shown to be 0.1 ppm upfield, as expected. This is an elegant example of how microbial transformation can be combined with an enzymatic reaction to quickly produce all the *O*-demethyl derivatives of **1** and **2** for biological evaluation.

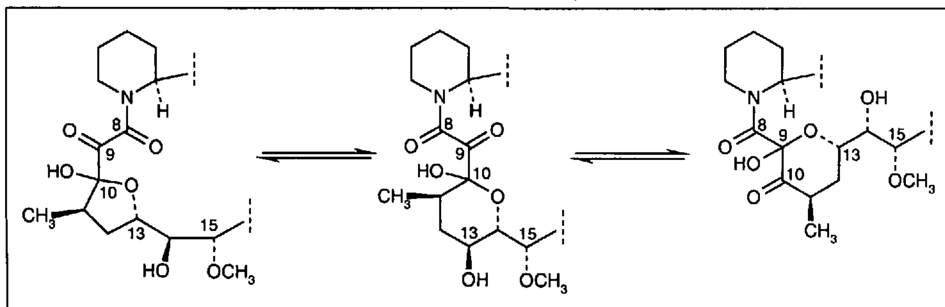
2.5. *19-Hydroxylation and 19a-O-22 Hemiketal Formation*

Compounds **1** and **2** have six allylic carbon atoms: C(18), C(19a), C(21), C(26), C(27a), and C(29). In our microbial transformation study, four microorganisms, *Streptomyces* sp. ATCC 55279, ATCC 55280, ATCC 55281, and ATCC 55282, specifically catalyzed the hydroxylation at C(19a) of **2**, leading to a novel hemiketal structure **12**, formed by attack of the 19a-hydroxy group at the C(22)=O group [24]. The compound was idenified as a cyclized hemiketal structure based on the following distinctive ^{1}H- and ^{13}C-NMR features: *1*) absence of C(19a) methyl proton signal at 1.59 ppm; 2) absence of typical C(23) methylene signals at 2.78 ppm, *3*) the H–C(20) signal was shifted downfield by about 0.6 ppm and its vicinal coupling constant reduced from 10.1 Hz to 6.0 Hz, *4*) absence of the C(19a) methyl carbon signal at 15.8 ppm, *5*) disappearance of the C(22)=O signal at 212 ppm, *6*) appearance of a new signal at 98.7 ppm, consistent with a hemiketal involving C(22), and *7*) a new carbon signal at 74.8 ppm that is assigned to C(19). Evidence of bioconversion was observed as early as 24 h after substrate addition, and the yield of the hemiketal derivative reached 70% within 54 h. The formation of the hemiketal involves two steps: hydroxylation at C(19a) and ring closure to the hemiketal. The microbial enzyme appears to facilitate the attack of the new HO–C(19a) group at C(22)=O.

2.6. *24-Glucosylation*

Both **1** and **2** are highly lipophilic molecules. It would be beneficial if structural modification could increase water solubility and, hopefully, their bioavailability. *Bacillus subtilis* ATCC 55060 is the only microorganism discovered in our study that can conduct the bioconversion of **1** and **2** to their corresponding C(24)-*O*-glucosylated derivatives **13** and **14** [25]. A resting cells system was also developed to carry out this glucosylation reaction. The structure was determined by MS, NMR, and chemical degradation studies. The positive-ion FAB-MS of **13** and **14** gave $[M+Na]^+$ signals of *m/z* 988 and 976, respectively, which corresponds to a 162 mass-unit increase compared to the parent compounds, suggesting that they are indeed glucosylated derivatives. Acid hydrolysis of **13** and **14** yielded a sugar moiety identified as glucose using ^{1}H-NMR analysis. The ^{1}H-NMR spectra of **13** and **14** showed the presence of at least four novel methine signals in the region characteristic for H–C–O group. The spectral perturbations relative to the parent compounds are most apparent in the C(22)–C(26) region. This is evidenced by an increase in the H–C(26) and H–C(25) coupling constant from 2.5 Hz to 6.0 Hz and a 0.3–0.4 ppm downfield displacement of the higher-field signal of one of the protons attached to C(23). The changes can be rationalized by alterations in the C(25)–

Scheme. *The Possible Isomers after MeO–C(13) Demethylation*



C(26) and C(23)–C(24) bond angles, suggesting that the site of attachment of glucose is probably HO–C(24). The attachment of glucose to the C(24) hydroxy group is further underpinned by 2D-NOESY spectra. Cross peaks show the proximity between H–C(24) and the anomeric proton of glucose. The glycosidic bond of C(24)–O–glucose was determined to be β-configured based on the proton-proton coupling constant of $J = 7.5$ Hz for the anomeric proton.

Enzymatic glucosylation was demonstrated using cell-free extracts derived from *Bacillus subtilis* ATCC 55060. The 24-glucosyltransferase was solubilized from cell membranes by treatment with 0.1% *Nonidet P-40* detergent. The enzyme appears to be UDP-glucose-dependent and has a temperature optimum at 40°. Addition of magnesium ions resulted in a tenfold increase of activity, while potassium ions were inhibitory.

2.7. *32-Phosphorylation*

32-*O*-Phosphorylated FK-506 (**15**) and ascomycin (**16**) are other water-soluble derivatives discovered in our biotransformation program. *Rhizopus oryzae* ATCC 11145 efficiently carries out the phosphorylation of **1** and **2**. The phosphorylation reaction was performed using resting cells. The substrate was mixed with mycelium suspended in a 100 mM phosphate buffer at pH 7.0 containing 1% glycerol. After overnight incubation, the resting cells had converted **1** and **2** to the 32-*O*-phosphorylated derivatives **15** and **16** quantitatively. The structures of **15** and **16** were established using spectroscopic techniques including MS and NMR. The FAB mass spectra of **15** and **16** gave $[M+Na]^+$ signals of 906 and 894, respectively, corresponding to a 80 mass-unit difference to the parent compounds, suggesting either a phosphate or sulfate conjugate. The ^{1}H-NMR spectra are characterized by two distinctive features: a broad, structureless peak close to 4 ppm identified as a downfield-displaced H–C(32), and a broadened methoxy-proton signal near 3.5 ppm assigned to MeO–C(32). The distinction between phosphate or sulfate was made by the ^{31}P-NMR spectrum which revealed two peaks at 14.6 and 19.2 ppm in a ratio of 4.5:1, corresponding to the major and minor rotamers. In addition to **1** and **2**, *Rhizopus oryzae* can also phosphorylate **3**, **4**, and **12** at the HO–C(32) position.

Table. *Inhibition of T-Cell Proliferation by Derivatives Obtained by Biotransformation*

Compound	IC_{50} [nM]
FK-506 (1)	0.4
Ascomycin (2)	0.8
3	0.9
4	1.7
5	37.0
6	50.0
7	>1,000
8	>1,000
9	2.2
10	5.0
11	>1,000
12	3.0
13	>1,000
14	>1,000
15	4.0
16	16.0

3. Biological Activity and Structure-Activity Relationship

Schreiber et al. first reported on the FK-506 mechanism of action. FK-506 binds competitively and with high affinity to FK-506 binding protein (FKBP) [26]. The immunosuppressive activity is not due to affinity of ligands for FKBP alone. They found that the FKBP·FK-506 complex binds in a Ca^{2+}-dependent manner to the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin and demonstrated a strong correlation between the ability of FK-506-analogue·FKBP complexes to inhibit the phosphatase activity of calcineurin and their ability to inhibit T-cell signalling [27]. These studies suggest that calcineurin may be the common biological target of FK-506. Analysis of high-resolution structures derived from crystallographic and NMR studies [28–30] of FKBP·FK-506-analogue complexes suggest that roughly half of the FK-506 molecule is in contact with the FKBP, while the other half remains exposed on the complex's surface, available for interaction with calcineurin. The portion of the FK-506 molecule in contact with FKBP includes the piperidine and pyran rings, the dicarbonyl region, and one edge of the cyclohexane ring.

The immunosuppressive activities of the biotransformed derivatives of **1** and **2** were determined using an *in vitro* T-cell proliferation assay [31]. The concentration of compounds **3**–**16** inhibiting T-cell proliferation by 50% (IC_{50}) is summarized in the *Table*. The 13,15,31-trisdemethyl (**7**), 15,31-bisdemethyl (**8**), and 15-demethyl (**11**) compounds were inactive up to 1 mM, but act as FK-506 antagonists. The C(24)–O–glucose conjugates **13** and **14** serve as neither agonists nor antagonists. The 13,31-bisdemethyl compounds **5** and **6** retained modest activity. In contrast, the activities of 13-demethyl compounds **9** and **10**, 31-demethyl (**3**, **4**), 32-phosphate conjugates (**15**, **16**), and 19a-22-hemiketal (**12**) derivatives did not decrease significantly. These results agree with observations from three-dimensional conformation studies on complexes of FK-506 with FKBP and calcineurin. The 31-methoxy or 32-hydroxy group does not participate in either FKBP binding or subsequent inactivation of calcineurin. The 13-methoxy group has some contact with FKBP. Removing the 15-methoxy group may hinder productive binding to calcineurin, but does not affect the FKBP binding. It is intriguing that, in spite of structural and conformational changes caused by an oxygenated 19-methyl functionality attached to C(22) to form a cyclic hemiketal structure, compound **12** still retained 35% of T-cell-inhibiting activity. The answer may be that the conformation of **12**, in complex with FKBP, is similar to the one of **1** and **2**; however, in solution, a profoundly different conformation is observed. The loss of T-cell-inhibiting activity of **13** and **14** may suggest that the additional steric bulk of the glucose moiety at C(24) causes a conformational change in the entire macrocycle.

Received: September 10, 1999

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Chimia 53 (1999) 600–607
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ISSN 0009–4293

An Industrial View on Enzymes for the Cleavage of Cephalosporin C

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Abstract. The enzymatic cleavage of cephalosporin C (CephC) into 7-aminocephalosporanic acid (7-ACA) and deacetyl-7-aminocephalosporanic acid (HACA), both key intermediates for cephalosporin antibiotics, has now been commercialized on an industrial scale. This article illustrates economic, technical, and regulatory aspects of the process, with special focus on the enzymes involved.
Due to the compensation for low operational stability by low costs of preparation, cell immobilization of *Trigonopsis variabilis* seems an economically attractive and technically feasible way to prepare D-amino acid oxidase (*EC 1.4.3.3*). However, the application of immobilized cells is restricted to large-volume products, since it involves extensive development and characterization work.
For glutaryl-7-ACA acylase (*EC 3.5.1.3*), expressed in *Escherichia coli*, isolation and immobilization of the enzyme on a commercial carrier seems more attractive from a regulatory point of view. The immobilized enzyme shows very high operational stability, which may compensate for the costs of the carrier.
Despite its lower stability, cephalosporin C acetylesterase (*EC 3.1.1.41*), expressed in *E. coli*, was also immobilized on a commercial carrier for regulatory reasons. Moreover, extensive development of immobilized whole cells seemed economically not acceptable for this low-volume product.
A mathematical model for the enzymatic cleavage showed limitations of a combined application of two biocatalysts in a stirred tank reactor, *e.g.*, in terms of product yield.

1. Introduction

7-Aminocephalosporanic acid (7-ACA) is a key intermediate in the production of more than 50 semi-synthetic cephalosporin antibiotics, such as cefotaxime, cefpodoxime, cefazolin, ceftazidime, and ceftriaxone. The ever-increasing market volume of 7-ACA was estimated to reach almost 2,000 tons p.a. by the year 2000, representing a market value of approximately USD 400 million [1].

Starting with the fermentation, isolation and subsequent chemical cleavage of CephC in organic systems, solid 7-ACA can finally be obtained in *ca.* 95% purity by precipitation, filtration, and drying. The enzymatic cleavage can avoid the use of hazardous chemicals and solvents, and thus may have a positive impact on both the economics of the process and the environment. In addition, CephC does not necessarily have to be precipitated and dried, as it is typically required for the chemical cleavage in organic solvents. Finally, it opens a more economic way to prepare deacetyl-7-aminocephalosporanic acid (HACA), which is another key intermediate in cephalosporin derivatization.

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