Complexants for the clathration mediated synthesis of the antibiotic cephradine

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Enzymatic synthesis of cephalosporins is hampered by secondary hydrolysis and by complicated down-stream processing. Instantaneous removal of cephalosporin product by clathration, using an efficient and selective complexing agent, offers an attractive opportunity to tackle these problems. A series of benzene derivatives that form clathrate-type complexes with the cephalosporin antibiotics was subjected to efficiency measurements with Cephradine and enzyme inhibition studies. The best results for the antibiotic Cephradine were obtained with methyl 2-aminobenzoate, 2-hydroxybiphenyl and methyl 4-hydroxybenzoate. These three compounds are environmentally and toxicologically fully acceptable for application in a 'green' process.

Introduction

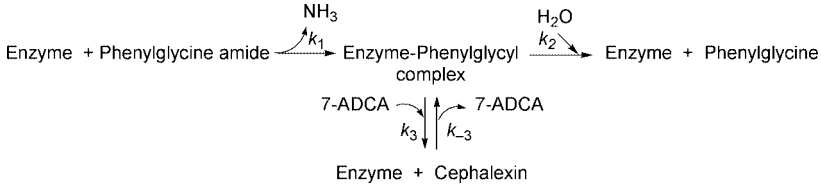
The enzymatic synthesis of cephalosporins forms a challenging new development for the manufacture of these antibiotics from a β-lactam nucleus and a D-amino acid side chain.[1] The enzymatic synthesis offers interesting possibilities for improvement of the processes used for the preparation of the cephalosporins. Another, attractive aspect of the enzymatic synthesis is the possibility to reduce the environmental impact of the processes for the manufacture of these antibiotics.[2] Introduction of biocatalysis will contribute enormously to the greening of the chemistry needed for the production of these important antibiotics. This development is fully in line with the global trend in the fine-chemical industry.[3]

A suitable biocatalyst for the coupling of nucleus and side chain is penicillin G acylase. Since the equilibrium constant of this thermodynamic coupling is extremely low,[4] the coupling has to be performed in a kinetic fashion using an activated precursor of the side chain in order to obtain a reasonable conversion to the product. A severe problem of this kinetic enzymatic synthesis of cephalosporins from the β-lactam nucleus and the ester or amide derivative of the side chain is hydrolysis of side chain and product, as is depicted for Cephalexin in Scheme 1.[5,6] In the first step phenylglycine amide is converted into its complex with the enzyme (k_1), which is accompanied by liberation of ammonia. The enzyme–phenylglycyl complex can undergo either hydrolysis with water (k_2) resulting in the formation of phenylglycine or reaction with the β-lactam nucleus 7-ADCA (k_3) to give Cephalexin. The cleavage of Cephalexin (k_{-3}) reverts to the enzyme–phenylglycyl complex and 7-ADCA. The competing reaction of the thus obtained enzyme–phenylglycyl complex with water leads to a loss of product and is often referred to as *secondary hydrolysis*.

Due to hydrolysis reactions the yield of the kinetic enzymatic coupling of nucleus and side chain is very low.[7] The unwanted hydrolysis can in principle be suppressed using a solvent other than water, which is, however, neither possible in real practice, nor desirable from an environmental point of view. In an

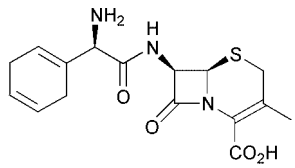
Green Context

The enzymatic synthesis of cephalosporins is an important process which is hampered by hydrolysis of the side chain in the aqueous environment. This paper describes the use of a series of benign clathrates, which efficiently remove the product from the aqueous phase as it is produced, minimising the extent of the hydrolysis, and making significant improvements in the overall process. The best clathrates remove the product very efficiently, and are of low toxicological impact. This work helps to make feasible a low energy, simple synthesis of an important group of compounds. ***DJM***



Scheme 1 Enzymatic synthesis of Cephalexin.

alternative manner to avoid secondary hydrolysis the cephalosporin product can be removed from the aqueous reaction mixture. Two techniques for the removal of Cephalexin from aqueous solutions are reported, *viz.* adsorption using amberlite XAD[8] and extraction using an aqueous two-phase system.[9] However, neither of these techniques is selective and consequently the starting materials are also withdrawn from the reaction mixture. In contrast to the aforementioned techniques, clathration of cephalosporins with a complexing agent such as β-naphthol is highly selective.[10] Hence, clathration of cephalosporins during the enzymatic synthesis may be suitable to tackle the problem of secondary hydrolysis. In addition, clathration of cephalosporins may be an elegant method to facilitate down-stream processing as the precipitated clathrate can be readily filtered off from the rather complex reaction mixture. The application of *in situ* clathration during the enzymatic synthesis of cephalosporins has been reported previously.[11] The beneficial effect of clathration with naphthalene-derived complexants on the product yield during the enzymatic synthesis of Cephalexin, has also been described in a quantitative manner.[12] Several requirements have to be fulfilled by a complexing agent before application during the enzymatic synthesis of cephalosporins becomes feasible. First, the clathration process must be sufficiently effective under the conditions used for the enzymatic coupling. Second, the complexing agent used must not (irreversibly) inactivate the enzyme. And third, preferably the complexing agent must be non-toxic. Despite the fact that by decomplexation, which is done by acidic or basic hydrolysis of the complex, extraction of the complexing agent followed by neutralisation of the resulting concentrated aqueous solution of cephalosporin, the complexing agent can be completely removed from the final product, the use of a toxic compound, *e.g.* β-naphthol, in a 'green' enzymatic process is not desirable. This paper describes a study of several non-toxic complexants for the cephalosporin antibiotic Cephradine with regard to complexation efficiency and enzyme inhibition.



Cephradine

Results

The beneficial effect of *in situ* clathration has been clearly demonstrated for the enzymatic synthesis of Cephalexin.[12] However, the toxicity of the complexing agent has not been taken into account yet. Although the complexing agent can be completely removed from the final product it is desirable to use a non-toxic complexing agent in a 'green' enzymatic process. The naphthalene-derived complexants do not meet this requirement. The benzene derivatives that were previously identified as complexing agents for Cephradine,[13] offer interesting prospects as several of them are used as preservatives in various food products. Such complexants are environmentally and toxicologically fully acceptable and according to their R classification, *i.e.* the risk of a substance in case of a particular exposure such as *via* skin, eyes, inhalation or swallowing, they have a toxicity index similar to other commonly used preservatives. Taking into account the complexing capacity and the favourable toxicity index the series of benzene derivatives listed in Table 1 were selected for the efficiency study in the enzymatic synthesis of Cephradine. These compounds were subjected to efficiency measurements under conditions that resemble those of the enzymatic synthesis of Cephradine, *viz.* pH = 7.2, T = 5 °C. The residual concentration of Cephradine was measured 90 min after the addition of the complexing agent. The results obtained for the most effective complexants under the just mentioned conditions are collected in Table 2. The other compounds of Table 1 were not effective at all under the desired conditions and are therefore of no interest for the removal of the antibiotic from aqueous reaction mixtures. For an acceptable complexant the residual concentration of antibiotic should be below 10 mM, as below this residual concentration the impact of complexation on the yield of the enzymatic synthesis becomes significant.[12] Five compounds listed in Table 2 met this criterion, *viz*. entries 1, 2, 6, 7, and 8. These five compounds were investigated in enzyme inhibition experiments using Assemblase[14] as the biocatalyst.[16]

Table 1 The compounds tested in efficiency measurements with Cephradine

o-Toluic acid	3,5-Dihydroxybenzoic acid
4-Aminoacetophenone	Methyl *m*-toluate
Methyl 2,4-dihydroxybenzoate	Catechol
Methyl *p*-toluate	Methyl 3,5-dihydroxybenzoate
Recorcinol	2-Aminobenzoic acid
3,4,5-Trihydroxybenzoic acid	Hydroquinone
2-Aminobenzamide	Methyl 3,4,5-trihydroxybenzoate
Pyrogallol	Methyl 2-aminobenzoate
Acetophenone	Phloroglucinol
Methyl 3-aminobenzoate	2-Hydroxyacetophenone
4-Methoxyphenol	Methyl 4-aminobenzoate
2-Methoxyacetophenone	2-Hydroxybiphenyl
2,4-Dihydroxybenzoic acid	2-Methylacetophenone
Benzoic acid	3,4-Dihydroxybenzoic acid
4-Methylacetophenone	Vanillin

In these studies the activity of the enzyme in the presence of a complexing agent was compared with that in the absence of complexing agent. The rate of hydrolysis of D-phenylglycine amide (the side chain precursor of Cephalexin) was taken as a measure for the enzyme activity. Three types of experiments were performed in order to obtain information about both reversible inhibition and inactivation (irreversible inhibition) of a complexing agent.

(i) Activity measurement in the presence of complexing agent.

(ii) Incubation of the enzyme with a complexing agent for 16.5 h, then the Assemblase was thoroughly washed with water and the activity was measured in the absence of complexing agent.

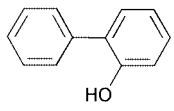
(iii) Incubation of enzyme with complexing agent for 16.5 h, after washing of the Assemblase with water the activity was measured in the presence of complexing agent.

The activity measured in experiment (i) was in all cases lower than the activity of Assemblase in the absence of complexant. However, from experiment (i) it cannot be ascertained whether the lower activity is due to reversible inhibition or inactivation (irreversible inhibition) by the complexant. Experiment (ii) indicates whether a complexant inactivates Assemblase. When after thorough washing the activity of Assemblase in the

Table 2 The residual concentration of Cephradine (pH = 7.2 and T = 5 °C) using benzene derivatives as complexants

Entry	Complexant	[Cephradine]/mM
1	2-Aminobenzoic acid	6.6
2	Methyl 2-aminobenzoate	3.7
3	2-Aminobenzamide	16
4	2-Methoxyacetophenone	15
5	4-Aminoacetophenone	16
6	2-Hydroxybiphenyl	1.3
7	Methyl 4-hydroxybenzoate	5.5
8	Methyl 3-hydroxybenzoate	6.9
9	Methyl 3,5-dihydroxybenzoate	14

absence of the complexant is not equal to the activity of fresh Assemblase this points to irreversible inhibition by the complexant. If this is the case then the complexing agent is not suitable for use during the enzymatic synthesis. In case no irreversible inhibition takes place, the activity of Assemblase after long-lasting (16.5 h) exposure to a complexant should not further diminish but remain constant at the value measured in experiment (i). This is verified by experiment (iii), which in case of reversible inhibition only, should show the same activity in the presence of a complexing agent as was found in experiment (i). The latter was observed for compounds **1**–**3**



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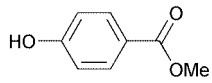

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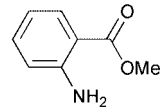

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shown in Table 3.These three compounds only show reversible inhibition and do not permanently inactivate the enzyme. This implies that the enzyme can be reused for next batches when exposed to either of these three compounds. This is an important observation as the enzyme accounts for a substantial part of the total cost of enzymatically prepared cephalosporins. It should be noted that compounds **1** and **3** are also effective in the complexation with Cephalexin and accordingly also suitable for the clathration mediated enzymatic synthesis of this antibiotic. In Table 3 the residual activity of Assemblase when exposed to either of the three complexing agents as a percentage of the enzyme activity in the absence of complexing agent is listed. These residual activities are similar to those measured after exposure to β-naphthol, a naphthalene derivative that has a significant beneficial effect on the yield of the enzymatic synthesis of Cephalexin.[12] Although a lower enzyme activity on itself is not desirable, it has the advantageous effect that it diminishes the diffusion limitations of Assemblase. This effect was observed for the enzymatic synthesis of Cephalexin using *in situ* clathration with β-naphthol and has been described in a quantitative manner using models developed for Assemblase and free Penicillin G acylase.[12]

Table 3 The residual activity of Assemblase in the presence of the complexants

Complexing agent	Residual activity (%)
2-Hydroxybiphenyl **1**	30
Methyl 4-hydroxybenzoate[17] **2**	30
Methyl 2-aminobenzoate **3**	40

Concluding remarks

From a series of benzene-derived complexants, three are effective in the clathration-mediated synthesis of Cephradine, *viz* methyl 2-aminobenzoate, 2-hydroxybiphenyl, and methyl 4-hydroxybenzoate.[17] These agents showed a reversible inhibitive effect on Assemblase resulting in a residual activity of approximately 30% when exposed to these compounds. Methyl 2-aminobenzoate and 2-hydroxybiphenyl are also effective in the clathration of Cephalexin. It is highly relevant to note that these three compounds have a low toxicity, and that two thereof are commonly used as preservatives of food products. Application of these toxicologically and environmentally acceptable complexing agents in the clathration mediated synthesis of Cephradine or other cephalosporin antibiotics has interesting prospects, which deserve further elaboration.

Experimental

For the analysis of the complexation experiments a Pharmacia LKB.LCC 2252 HPLC was used, with a reversed phase column (Merck 50983 LiChrospher 100RP18, 5 μm, 250 × 4 mm) and a UV detector (λ = 254 nm) of Farmacia LKB.UV-MII. An appropriate eluent for the analysis was a mixture of acetonitrile (HPLC grade) and a 50 mM phosphoric acid buffer with pH = 2.7. The pH stat apparatus used was a Schot Geräte Titrator TR154. Cephradine was a generous gift from DSM Research, Geleen, The Netherlands.

Efficiency measurements

Cephradine (524 mg) was dissolved in water (50 ml). The pH was adjusted to 7.2 and the solution was cooled to 5 °C. Complexing agent (1.5 mmol) was added as such to the stirred solution. The pH was maintained constant during the experiment by adding 5% HCl using a pH stat apparatus. After 90 min a sample was taken in duplo. The samples were filtered in order to remove the precipitated complex and subsequently analysed by HPLC. The residual Cephradine concentration was determined using standard solutions.

The compounds that have been tested in efficiency measurements are collected in Table 3. In the case of 2-phenylphenol, 4-methylacetophenone and methyl 4-hydroxybenzoate, 2 equivalents of complexant (3 mmol) were added as these compounds form 1 : 1 complexes with Cephradine, whereas the other compounds form 2 : 1 complexes.

Assemblase inhibition experiments[16]

The rate of phenylglycine amide hydrolysis is taken as a measure for the activity of Assemblase. Each experiment was performed under the following conditions:
0.2% (w/w) of Assemblase relative to phenylglycine amide, starting concentration of phenylglycine amide 80 mM, pH = 8, and T = 20 °C.

The activity measured during a blank experiment (in the absence of complexant) was taken as a reference, which was set as 100% activity. The residual activity of Assemblase in the presence of a complexing agent was expressed as a percentage of the activity measured during the blank experiment. The rate of the phenylglycine amide hydrolysis was followed by titration with 80 mM hydrochloric acid.

For the inhibition studies the complexant was added in an amount equimolar to phenylglycine amide. For each complexant three activity measurements where performed. First the activity of fresh Assemblase was determined in the presence of the complexing agent. In a second experiment fresh Assemblase was incubated for 16.5 h in the presence of the complexing agent. Next, the Assemblase was thoroughly washed with water and its activity was measured in the absence of complexing agent. For the third experiment fresh Assemblase was incubated during 16.5 h in the presence of complexing agent. After washing with water the activity of the biocatalyst was measured in the presence of the complexing agent.

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