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SUBSTRATE SPECIFICITY OF IMMOBILIZED PENICILLIN-G ACYLASE

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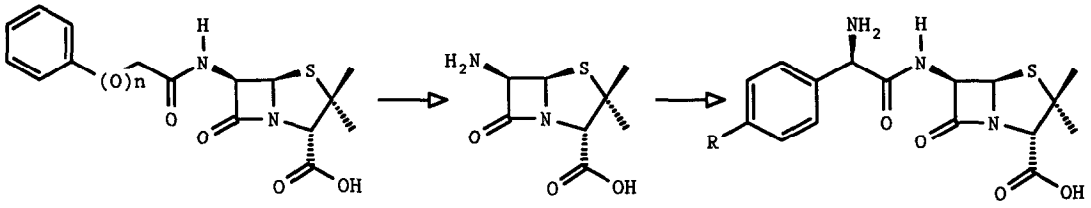
Abstract: The substrate specificity of immobilized penicillin-G acylase towards penicillin and cephalosporin derivatives was studied. The phenylacetyl moiety can be altered at the α-position with several small substituents. Depending on polarity and size of the substituent, enzyme activity decreases or increases. Insertion or deletion of atoms between the aromatic nucleus of the phenylacetyl group and the center of hydrolysis leads to substrates that are no longer recognized by the enzyme.

6-Aminopenicillanic acid (6-APA, 2) is an important intermediate in the production of semi-synthetic β-lactam antibiotics like Amoxicillin (3, R=OH) and Ampicillin (3, R=H). For many years, 6-APA has been produced on an industrial scale starting from penicillin-G (1, n=0) or -V (1, n=1) employing a chemical deacylation procedure[1]. Similarly, 7-amino-desacetoxycephalosporanic acid (7-ADCA, 5) is a valuable precursor in the synthesis of a wide range of cephalosporin antibiotics. Penicillin-G is converted into cephalosporin-G (4) using an oxidative ring-expansion[2] and subsequently converted into 7-ADCA. Recently, these chemical deacylation processes have been replaced by enzymatic procedures using penicillin acylase (EC 3.5.1.11) from bacterial (penicillin-G acylase) or fungal (penicillin-V acylase) origin.

At Gist-brocades, an immobilized penicillin-G acylase was developed to serve the industrial production of 6-APA from penicillin-G and of 7-ADCA from cephalosporin-G. In order to further evaluate scope and limitations of this enzyme, several substrates were tested.



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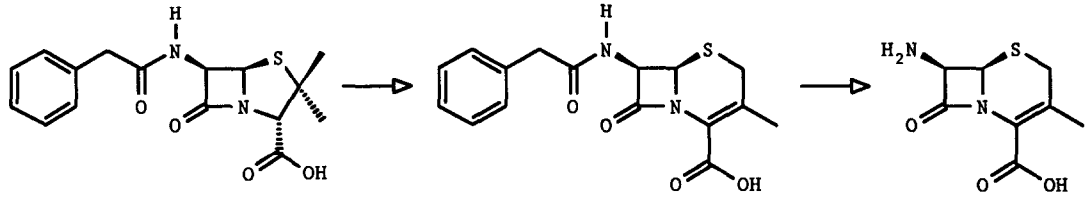

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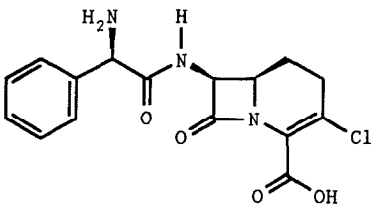
Enzymatic hydrolysis of the phenylacetyl side chain has been known for well over four decades. Microbial conversion of penicillin-G into 6-APA and phenylacetic acid has been reported as early as 1950[3]. The development of enzyme immobilization techniques has made application of enzymatic hydrolysis industrially feasible. The worldwide shift towards enzymatic deacylation represents an important contribution to the suppression of hazardous wastes like dichloromethane and phosphate.

Over the past few years, it has been demonstrated that penicillin acylases can be successfully applied in a number of conversions other than hydrolysis of β-lactam side chains. Of particular interest is the hydrolytic use of penicillin-G acylase in the synthesis of the dipeptide sweetener Aspartame[4,5]. The stereospecificity of the enzyme can be employed as was elegantly demonstrated by workers from Eli Lilly[6] who developed an enantioselective synthesis of the carbacephalosporin antibiotic Loracarbef (6). Similarly, a study by Margolin showed that the same enzyme could be applied for the resolution of inhibitors of γ-aminobutyric acid aminotransferase[7].



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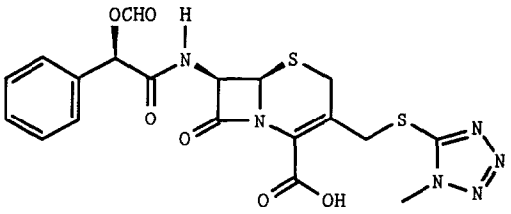

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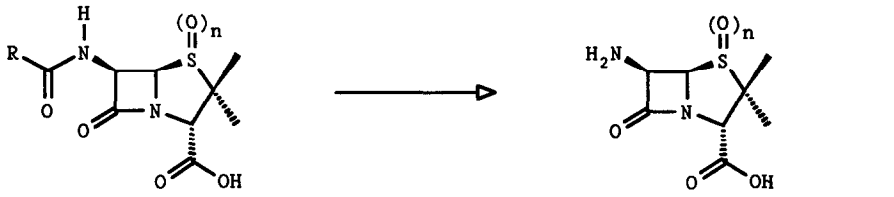
Stereospecificity with regard to the carboxylic constituent in amide bond formation was established by Baldaro *et al.*[8] who synthesized Cephamandole (7) from racemic methyl O-formyl mandelate. Removal of phenylacetyl protective groups in peptide[9] and carbohydrate[10,11] chemistry, from Insulin[12], Glutathione[13] and a Vasopressin derivative[14] has also been achieved using penicillin-G acylase. A survey of applications of penicillin acylases in hydrolysis, optical resolution and synthesis has been published by Shewale *et al.*[15]

The substrate specificity of immobilized penicillin-G acylase as used in the industrial production of 6-APA and 7-ADCA at Gist-brocades[16] has been studied using various substrates as outlined in Tables 1 and 2. As can be expected from previously published investigations, the enzyme is active with penicillin derivatives as well as cephalosporin derivatives, provided the phenylacetyl side chain is unmodified.

Oxidation of the sulfur atom leads to remarkable results. Penicillin-G (*S*)-sulfoxide (Table 1, entry 5) is surprisingly hydrolyzed in view of observations made by Alvaro *et al.*[17] who found that penicillin-G acylases from *E. coli* and *K. citrophila* were inhibited by penicillin-G (*S*)-sulfoxide. Hydrolysis is relatively slow in comparison with the parent penicillin-G (entry 3), whereas this effect is subdued upon hydrolysis of penicillin-G sulfone (entry 4). It is our believe that hydrogen bonding between the *S*-orientated oxygen atom and the amide function accommodates for this phenomenon. In sulfones, the electrondensity at oxygen is sufficiently reduced to prevent hydrogen bonding. As expected, the effect is not observed when cephalosporins are used as substrate (Table 2, entries 4 and 5). The prolonged $t_{1/2}$

Table 1 Hydrolysis of 6-substituted penicillins using immobilized penicillin-G acylase.



Entry	R	n	$t_{1/2}$(min)
1	D-(-)-$C_6H_5CH(NH_2)$-	0	5
2	D-(-)-4-$HOC_6H_4CH(NH_2)$-	0	12
3	$C_6H_5CH_2$-	0	25
4	$C_6H_5CH_2$-	2	50
5	$C_6H_5CH_2$-	1 (*S*)	90
6	$C_6H_5CH(Br)$-	0	80
7	$C_6H_5CH(CH_2CH_3)$-	0	3400
8	$C_6H_5CH(CH_2C_6H_5)$-	0	7000

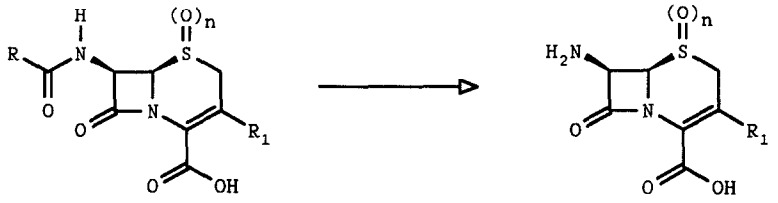
Substrates (2 mmol) were dissolved in water (20 mL) at 32 °C and pH 7.5 and treated with immobilized penicillin-G acylase (2 g, 150 U/g). The pH was kept at 7.5 by the addition of a 0.1 M solution of NaOH using an automated titration apparatus. When NaOH-consumption stopped, the immobilized enzyme was removed by filtration. Crude reaction mixtures were analyzed for free acid and/or deprotected β-lactam nucleus by HPLC.

found for (*R*)-oxidized cephalosporins (entry 9) is due to poor solubility of the substrate. In cephalosporin antibiotics, 3-substitution is common. As can be seen in Table 2, various substituents were tested, none of which appear to influence the activity of the enzyme. It may therefore be concluded that the acylase investigated is suitable for the synthesis of a variety of β-lactam antibiotics.

Substrate specificity is targeted mainly towards the phenylacetyl part of the amide linkage. Aromaticity seems to be of vital importance for recognition by the active site of the enzyme. Furthermore (Table 2, entries 12-14) the distance between the aromatic nucleus and the site of hydrolysis appears to be crucial. It is in this respect that penicillin acylases from fungal origin differ from bacterial acylases. Substitution of the phenyl moiety at the *para*-position with a hydroxy group is tolerated (Table 1, entry 2) and other aromatic groups seem to function as well as phenyl (Table 2, entry 10).

Finally, substitution patterns at the α-position of the phenylacetyl group were investigated. Polar substituents (Table 1, entries 1 and 2; Table 2, entries 1 and 2) dramatically accelerate enzymatic hydrolysis. Bromine substitution is tolerated albeit that the rate of hydrolysis decreases (Table 1, entry 6 *vs* entry 3; Table 2 entry 11 *vs* entry 4). As substituents are bulkier (Table 1 entries 7 and 8), enzyme activity rapidly decreases. Recently, penicillin-G acylase developed by Gist-brocades was applied for the kinetic resolution of *p*-hydroxyphenyl acetamides giving optically pure amines and aminoalcohols[18]. Together with the results presented here this demonstrates that the enzyme is suitable for a wide variety of applications in organic chemistry.

Table 2 Hydrolysis of 7-substituted cephalosporins using immobilized penicillin-G acylase.



Entry	R	R_1	n	$t_{1/2}$(min)
1	D-(-)-$C_6H_5CH(NH_2)$-	CH_3	0	5
2	D-(-)-$C_6H_5CH(OH)$-	CH_3	0	5
3	$C_6H_5CH_2$-	H	0	25
4	$C_6H_5CH_2$-	CH_3	1 (*S*)	33
5	$C_6H_5CH_2$-	CH_3	0	45
6	$C_6H_5CH_2$-	$CH=CHCH_3$	0	50
7	$C_6H_5CH_2$-	CH_2S-2-(1-Me-tetrazolyl)	0	50
8	$C_6H_5CH_2$-	$CH=CH_2$	0	55
9	$C_6H_5CH_2$-	CH_3	1 (*R*)	200
10	(3-Thienyl)CH_2-	CH_3	0	65
11	$C_6H_5CH(Br)$-	CH_3	1 (*S*)	370
12	$C_6H_5OCH_2$-	CH_3	0	1285
13	$C_6H_5OCH_2$-	CH_3	1 (*S*)	1420
14	C_6H_5-	CH_3	0	>10000
15	H-	CH_3	1 (*S*)	>10000

Experimentals as in Table 1.

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