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Unexpected enantioselectivity and activity of penicillin acylase in the resolution of methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate

Yiming Yao*, Jim J. Lalonde

Altus Biologics, Inc., 625 Putnam Avenue, Cambridge, MA 02139-4807, USA

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Abstract

The penicillin acylase (PA) from *E. coli* catalyzes the hydrolytic kinetic resolution of methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate with remarkably high enantioselectivity and catalytic efficiency. This result is highly unusual as this ester does not contain the phenylacetic acid residue, normally considered to be a prerequisite for high activity and enantioselectivity in PA catalyzed resolutions. The apparent enantioselectivity (E_{app}) was found to be high (>50) at neutral or slightly acidic pH and to decrease at more alkaline pH (>7.5) due to significant non-specific chemical hydrolysis. Similarly, enantioselectivity increased with decreasing temperature. The substrate concentration had only a slight effect on enantioselectivity and activity. The rate of hydrolysis of ester **1** is comparable to that for PA's "natural" substrate, penicillin G.

Keywords: Penicillin acylase; Enantioselective kinetic resolution; Methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate; Dioxalane; C3 chiral synthon

1. Introduction

Penicillin acylase (PA) has been used in industry for decades for the cleavage of penicillin G to 6-amino-penicillanic acid (6-APA) and phenylacetic acid [1] and more recently for the synthesis of semi-synthetic antibiotics [2]. It has also been used for the synthesis of peptides [3], and for the resolution of amino acids [4,5], amino alcohols [6], 1-aminoalkylphosphonic [7], 1-aminoalkylphosphonous acid [8] and amines [9]. In a few instances, penicillin acylase has also been used for the resolution of chiral alcohols via the enantioselective hydrolysis of phenacetyl esters [10] and the resolution of α-substituted phenylacetic acid derivatives [11], although the enantioselectivity is typically low to moderate. A common feature of these substrates is that the large preponderance are based on those containing phenylacetic acid as the acid residue. Crystal structure [12] and previous substrate specificity studies [13] reveal that penicillin acylase has a unique bonding preference towards phenylacetic acid derivatives.

Penicillin acylase is readily available and inexpensive and is available in soluble, immobilized and cross-linked enzyme crystal (CLEC®) forms. Industrial use of penicillin acylase however has been limited to 6-APA and 6-ADCA production, antibiotic synthesis and the resolution of unnatural amino acids—processes based on PA's selectivity for the phenylacetic acid group.

In our routine enzyme screening we have found that penicillin acylase accepts a diversity of substrates.

* Corresponding author. Tel.: +1-617-2992945; fax: +1-617-2992950.
E-mail address: yao@altus.com (Y. Yao).

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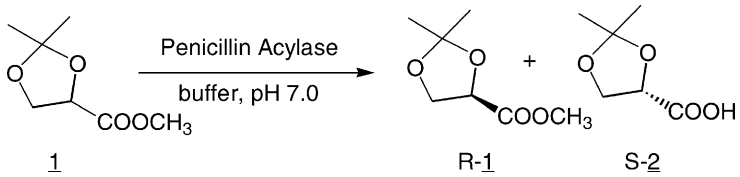

Scheme 1. Penicillin acylase catalyzed enantioselective hydrolysis of methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate.

Our strategy of screening enzymes for use in kinetic resolutions has been to not bias these screens with the results of previous work, but to include many commercially available, inexpensive hydrolases. Here, we report the surprising discovery that penicillin acylase catalyzes the resolution of methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate with high enantioselectivity, an ester that has no apparent structural homology with known PA substrates. The resulting optically pure (4*R*)-methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate is a particularly important intermediate for the preparation of optically active dioxolane nucleosides [14]. It has also served as a C3-chiral building block in a number of natural products and drug syntheses [15]. Conventional methods for synthesis of optically pure (4*R*)-methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate based on D-serine [14] or D-mannitol [16] involve multiple steps and suffer from low yield. The process reported here is efficient with respect to enzyme usage and so can be easily adapted for large-scale production using commercially available penicillin acylase as immobilized or soluble forms (Scheme 1).

2. Experimental

2.1. Materials

Penicillin acylase from *E. coli* was purchased from Roche Bioscience (Penzberg, Germany), 29.4 U/mg. Activity units (U) are reported based on the manufacturers determination of activity using the hydrolysis of a 5% solution of penicillin G potassium salt at pH 8.0, 28 °C. Methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate and its *R* and *S* enantiomers were purchased from Sigma–Aldrich (St. Louis, MO, USA). The enzyme library used for screening was the ChiroScreen-EH from Altus Biologics (Cambridge, MA, USA).

2.2. Analysis

The enantiomeric purity was analyzed by chiral gas chromatography and the extent of hydrolysis was determined by reversed phase HPLC. For accurate determination of the E_{app} value, the conversion was calculated based on the enantiomeric excess of the substrate (ee_S) and product (ee_P). E_{app} was then calculated using ee and conversion, assuming a simple first-order kinetic resolution using the method of Sih and co-workers [17]. The enantiomeric excess of the substrate was determined by periodically removing aliquots (30 μl) of the well-stirred reaction mixture and adding this to a 2 ml vial containing 1.0 ml of methyl *t*-butyl ether and 300 mg of anhydrous $MgSO_4$. The dried solvent mixture was transferred to a vial for GC analysis.

The enantiomeric excess of the product was determined by extracting a 2 ml aliquot of the reaction mixture with methyl *t*-butyl ether until no unreacted ester substrate was detected by GC. Then the aqueous phase was acidified to pH 3.0 by the addition of 1 N hydrochloric acid, extracted with methyl *t*-butyl ether, and then 1 ml of the organic solvent mixture was dried over anhydrous $MgSO_4$ and transferred to a GC vial. One drop of trimethylsilyldiazomethane solution (2 M in hexane) was added to convert the carboxylic acid product to its methyl ester for GC analysis.

2.2.1. Chiral GC conditions

A J&W Scientific Cyclodex B column 0.25 μm, 15 m × 0.25 mm column was used. After 10 min at 100 °C, the temperature of the oven was increased at a rate of 10 °C/min to 140 °C. Authentic samples of the *R* and *S* enantiomers were used for assignment of

absolute configuration. Retention times: *S*-methyl ester, 9.2 min; *R*-methyl ester, 10.9 min.

2.2.2. Reversed phase HPLC conditions

Varian Microsorb MV^{TM} 100 Å, C18, 0.46 cm × 15 cm HPLC column, mobile phase = water:acetonitrile:trifluoroacetic acid (90:10:0.1), flow-rate = 1 ml/min, UV detection at 220 nm. Retention times: methyl ester, 13.31 min; acid, 5.75 min.

2.3. Enzymatic reactions

Racemic methyl 2,2-dimethyl-1,3-dioxolane-4-carboxylate (0.32 g, 2.0 mmol) was dissolved in 30 ml of 0.01 M pH 7.0 phosphate buffer. Penicillin acylase (168 U) was then added to the mixture and the resulting reaction mixture was stirred with gentle agitation at the desired temperature. The pH was maintained at 7.0 by the addition of 0.05 M aqueous sodium hydroxide as necessary. The progress of the hydrolysis reaction was monitored by GC.

2.3.1. Concentration study

Racemic methyl 2,2-dimethyl-1,3-dioxolane-4-carboxylate (0.30 g, 1.88 mmol for 1% substrate concentration; 1.5 g, 9.38 mmol for 5% substrate concentration; 3 g, 18.75 mmol for 10% substrate concentration) was dissolved in 0.3 M pH 7.0 phosphate buffer (30, 28.5, and 27 ml, respectively). The reaction mixture was cooled to 10 °C, and penicillin acylase (168, 840, and 1680 U, respectively) was added and the resulting reaction mixture was stirred with gentle agitation. The pH was maintained at 7.0 by the addition of 3.0 M aqueous sodium hydroxide as necessary. The progress of the reaction was monitored by GC.

3. Results and discussion

A qualitative screen using the Altus enzyme library against the substrate showed that penicillin acylase catalyzes the enantioselective hydrolysis of methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate with apparent high activity and selectivity. It is known that penicillin acylase has esterase-like activity, although penicillin acylase is primarily used for the hydrolysis of amides. The use of penicillin acylase for the enantioselective resolution of esters compound is relatively rare.

To date, resolutions involving chiral acids using penicillin acylase have been limited to those based on phenylacetic acid derivatives. This is not surprising since penicillin acylase has long been regarded to be highly active and selective only in substrates containing the phenylacetic acid group. Despite its ability to accept alcohols and amides of great structural diversity, early research on the substrate specificity of PA showed that enzyme tolerates only very minor changes in the structure of the acid residue, limiting substrates to simple derivatives of the phenacetyl group [4,18]. Thus, despite the ready availability of penicillin acylase and its low cost, PA is rarely screened for activity towards non-phenylacetic acid esters or amides.

Further examination of the unexpected ability of penicillin acylase to catalyze the hydrolysis of methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate showed that this enzyme is remarkably active and selective in its hydrolysis of **1**. At room temperature, penicillin acylase catalyzed the hydrolysis of the (*S*)-enantiomer with an E_{app} value of 41 and a rate of hydrolysis comparable to that for its "natural" substrate, penicillin G (Table 1, first entry). Due to our interests in large-scale production of (4*R*)-methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate, we further examined the effects of pH, temperature and substrate concentration on rate and selectivity. We found that control of pH is critical for the resolution since the substrate is unstable at both low pH (<6) and at high pH (>7.5), presumably because of its acid labile acetonide and base labile ester functionality. About 20% non-enzyme catalyzed hydrolysis was observed over 5 h at pH > 7.5. The background chemical hydrolysis can have a significant affect on E_{app} value. For example, the E_{app} value is 41 at 20 °C, however, when corrected for the degree of non-enzymatic hydrolysis the true E_{app} value is calculated to be around 70. At lower temperature, the enantioselectivity was significantly higher while the activity decreased. Since the enzyme is extremely active toward this substrate, the activity loss at low temperature is largely compensated for by the significant increase in enantioselectivity and reduction of chemical hydrolysis. However, when the reaction temperature is too low, the degree of chemical hydrolysis becomes significant due to the extended reaction time. Therefore,

Table 1
Effect of temperature on the hydrolysis of methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate

Temperature (°C)	Initial rate (μmol U^{-1} min^{-1})[a]	Time (min)	Conversion (%)	$ee_{Substrate}$ (%)	E_{app}
20	0.29	120	55.0	98.0	41
10	0.16	190	51.7	95.1	63
0	0.08	300	50.1	92.1	78

[a] The reaction rate was measured over the first 5 min, in μmol U^{-1} min^{-1}. Reactions run in 0.01 M, pH 7.0 phosphate buffer at substrate concentration of 66.7 mM.

Table 2
Effect of substrate concentration on the hydrolysis of methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate

Substrate concentration (mM)	Initial rate (μmol U^{-1} min^{-1})[a]	Time (min)	Conversion (%)	$ee_{Substrate}$ (%)	E_{app}
63	0.35	120	54.59	99.30	58
313	0.65	60	57.41	99.91	49
625	0.70	45	56.77	99.58	43

[a] The reaction rate was measured over the first 5 min, in μmol U^{-1} min^{-1}. Reactions run in 0.3 M, pH 7.0 phosphate buffer at 10 °C.

the optimized conditions for the resolution of methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate is pH 7.0, 10 °C using sufficient PA to achieve a short reaction time (<5 h) (Table 1).

The effect of substrate concentration on selectivity and rate was investigated at 10 °C and a fixed enzyme:substrate ratio. The rate of hydrolysis at three different substrate concentrations is reported relative to that for penicillin G hydrolysis (Table 2) using the standard unit definition.[1] Standard conditions for the determination of penicillin acylase activity employ a 5% solution of penicillin G at pH 8, 28 °C. At equivalent substrate concentrations, the rate of hydrolysis of **1** is 65% that of penicillin G hydrolysis, although the latter is conducted at a higher pH and temperature. The resolution can be carried out at substrate concentration over 20% without significant loss of enantioselectivity, allowing for high reactor throughput. The enantioselectivity suffered only a minor decrease as the substrate concentration increased from 1–10%.

4. Conclusions

In this report, we describe the first example of a highly enantioselective penicillin acylase catalyzed resolution of a non phenylacetic acid derivative, methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate. Penicillin acylase selectively catalyzes the hydrolysis of (4*S*)-methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate with activity rivaling that for its "natural" substrate, penicillin G. The remaining (4*R*)-methyl 2,2-dimethyl-1,3-dioxane-4-carboxylate is a versatile building block for chiral synthesis. Under optimized conditions of pH, temperature and ester concentration, the resolution is highly efficient. Based on the high activity and selectivity of penicillin acylase and its relatively low cost, this resolution represents an attractive method to resolve this important three carbon chiral synthon. Investigations concerning the scope of this unexpected substrate diversity of penicillin acylase are in progress.

[1] One unit (U) of penicillin acylase catalyzes the hydrolysis of 1 μmol of penicillin G potassium salt per minute at pH 8.0, 28 °C.

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