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A novel resolution of a pharmaceutically important bridged bicyclic ketone intermediate via selective enzymatic reduction with a commercially available ketoreductase

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Abstract

An efficient route to the pharmaceutically important (6*S*,9*R*)-11-oxo-5,6,7,8,9,10-hexahydro-6,9-methanobenzocyclooctene intermediate has been demonstrated via kinetic resolution of 11-oxo-5,6,7,8,9,10-hexahydro-6,9-methanobenzocyclooctene using a commercially available ketoreductase. Biocatalytics KRED 101 has been shown to selectively reduce the (6*R*,9*S*) enantiomer leaving behind the desired (6*S*,9*R*) enantiomer. This novel reaction is the first demonstration of a high yielding (44% versus 50% maximum theoretical yield) highly stereoselective (>99% ee) resolution of a bicyclic ketone, via enzymatic reduction using a commercially available ketoreductase, where the stereochemistry of the substrate is determined by a bridged ring system. Several challenges were overcome, including enhancing the selectivity of the enzyme by controlling temperature and increasing substrate solubility by employing a combination of cyclodextrin and organic co-solvent in the aqueous reaction system.
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Keywords: Ketoreductase; Bicyclic; Stereoselective; Bridged; Ketone

1. Introduction

The reaction discussed is the following (Scheme 1).

The preparation of optically pure compounds is of particular importance to the pharmaceutical industry. Biocatalysts have been routinely used to create optically pure molecules that have been difficult to synthesize using conventional organic chemistry. When the asymmetric synthesis of a desired chiral compound is not feasible, enzymatic resolution of a racemic starting material is often employed to afford the target molecule. One of the most common examples of enzymatic resolution is ester hydrolysis using an esterase or lipase [1]. The preparation of enantiomerically pure substituted β-keto esters has been demonstrated by pig liver esterase catalyzed hydrolysis of their racemic precursors [2]. Steenkamp and Brady [3] have demonstrated the enantiomeric resolution of (*R*,*S*)-naproxen esters using a commercially available lipase that yielded (*S*)-naproxen with >99% ee. Lipases have also been used for the kinetic resolution of cyclohexenones by enantioselective acetylation of the racemic starting material [4]. A few examples exist in which a ketoreductase was used to reduce a prochiral carbonyl group to an optically enhanced alcohol [5] for the kinetic resolution of a racemic substrate. Most of these examples employed whole cell enzyme preparations, such as the kinetic resolution of β-keto esters [6] and α-hydroxy-α-substituted-β-keto esters [7] by baker's yeast reduction to their diol counterparts. Additionally, access to enantiomerically pure bicyclic ketones such as the Wieland–Miescher ketone has also been obtained by kinetic resolution using yeast-mediated reduction [8]. A drawback of whole cell catalysts is that the preparation may contain multiple enzymes that act on both enantiomers of a substrate, leading to decreased stereospecificity [5,9]. For example, Hioki et al. [10] observed that several enzymes participated in their baker's yeast reduction and kinetic resolution of (±)-4-methyl-Hajos–Parrish ketone.

While few examples of kinetic resolution of racemic ketones by enzymatic reduction using a commercially available alcohol

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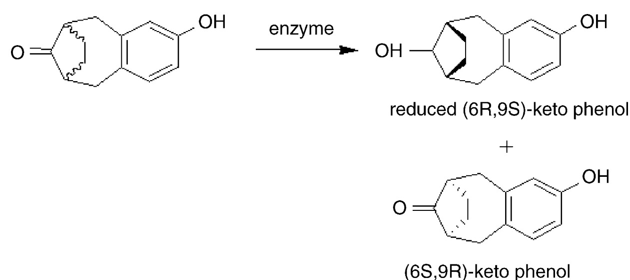

Scheme 1. Enzymatic kinetic resolution of racemic keto-phenol by selective reduction of the ketone.

dehydrogenases/ketoreductases exist [11], even fewer examples exist in which the substrate's chirality is determined by a carbon bridge across a bicyclic ring system. Horse liver alcohol dehydrogenase has been used to selectively resolved cage-shaped molecules [12–14] and some bicyclic bridged ketones [15], but with poor to moderate selectivity.

(6*S*,9*R*)-11-oxo-5,6,7,8,9,10-hexahydro-6,9-methanobenzocyclooctene ("(6*S*,9*R*) keto-phenol") has been shown to be an important intermediate in the preparation of pharmaceutically active substances [16,17]. Until recently, racemic 11-oxo-5,6,7,8,9,10-hexahydro-6,9-methanobenzocyclooctene ("keto-phenol") [18] has been employed as an intermediate, with the final compound being resolved chromatographically later in the synthesis, as no efficient route to (6*S*,9*R*) keto-phenol had been determined [16]. This work details the enzymatic reduction and kinetic resolution of racemic keto-phenol (Scheme 1) along with the challenges of optimizing the reaction system conditions, including enhancing the solubility of the keto-phenol substrate using cyclodextrin. The solubility of the substrate in the aqueous hydrolytic reaction system was extremely poor (<0.5 g/L). Cyclodextrins have been shown to serve as carrier molecules for poorly water-soluble compounds by forming a water-soluble inclusion complex, with the hydrophobic substrate molecule contained in an apolar cavity within the cyclodextrin ring structure [19,20]. Various groups have used cyclodextrins to successfully enhance the solubility of their substrates >5-fold [21,22].

2. Experimental

2.1. Whole cells and isolated enzymes

Our in house whole cell yeast culture library containing ∼400 organisms was screened along with a ketoreductase screening kit purchased from Biocatalytics. The kit included isolated enzymes KRED 101–KRED 108. All enzymes (KRED 101–108 and GDH) and cofactor were purchased through Biocatalytics, Inc. (Pasedena, CA).

2.2. Chemicals

Racemic keto-phenol substrate was made in house according to the procedure outlined by Belanger et al. [18]. Unless otherwise noted, all chemicals used were certified as analytical research grade and purchased from Sigma: dimethylsulfoxide (DMSO), dimethylformamide (DMF), methyl ethyl ketone, methanol, acetonitrile, ethanol, hexanes, glucose, potassium phosphate, beta W7 M1.8 cyclodextrin (purchased from Wacker).

2.3. Experimental setup

Small scale reactions (0.15–1.5 g) were run at 10–100 mL scale in temperature controlled reactors equipped with overhead mechanical stirring. Temperature control was variable and could be adjusted to operating temperatures between 5 and 75 °C. Large scale reactions (1 kg) were carried out in a 100 L Buchi reactor with pH control, temperature control and overhead mechanical stirring.

A typical reaction mixture consisted of 200 mM potassium phosphate buffer at pH 7.0 with 10% v/v DMSO. The component concentrations in the reaction mixture were 10 g/L keto-phenol substrate, 0.2 g/L KRED 101, 0.1 g/L NADP, 0.15 g/L GDH, 90 g/L glucose, and 70 g/L cyclodextrin.

The keto-phenol substrate was dissolved in DMSO and added to a solution containing the buffer, cyclodextrin, and glucose at 45 °C to rapidly solubilize the substrate. The GDH, KRED 101 and NADP were then added to the reaction mixture. Next, the reaction temperature was adjusted rapidly down to 10 °C where it was held for 20 h and then assayed for conversion and desired (6*S*,9*R*) keto-phenol product ee.

Because the subsequent step in the synthesis of the final drug product was selective for the keto-phenol and residual alcohol could easily be rejected in downstream purification steps, the desired keto-phenol and alcohol mixture was isolated together. The keto-phenol and alcohol were initially partitioned into methyl ethyl ketone and the extract was washed with DI water. The washed extract was concentrated, solvent switched to methanol, and the keto-phenol and alcohol were then crystallized from the solution using water as an anti-solvent. The >99% ee keto-phenol was isolated with 84% isolation yield.

2.4. Analysis

The concentrations of reactants and products were determined by reverse phase HPLC using a FluoroSep-RP Phenyl/HS (5 cm × 4.6 mm, 5 μm) column at 40 °C and a detection wavelength of 230 nm. The mobile phase consisted of 30% acetonitrile and 70% water.

The ee of the keto-phenol was determined by normal phase HPLC using a ChiralPak AD (25 cm × 4.6 mm, 10 μm) column at 25 °C and a detection wavelength of 230 nm. The mobile phase consisted of 10% ethanol and 90% hexanes.

3. Results and discussion

3.1. Catalyst screen

Our in house whole cell yeast culture library containing ∼400 organisms along with eight commercially available isolated ketoreductase enzymes were screened in a reaction system comprised of potassium phosphate buffer at pH 7.0 with 2 g/L

keto-phenol charged in 5% v/v DMSO at room temperature. The commercially available isolated enzyme KRED 101 proved to be the most selective catalyst, producing the desired (6*S*,9*R*) keto-phenol product with 98% ee at 60% conversion ($E=23$). Twenty-two whole cell cultures resolved the compound with some selectivity. The best whole cell catalyst identified was a strain from *Candida rhagii* that showed poorer selectivity than the isolated enzyme, with 90% ee of (6*S*,9*R*) keto-phenol at 65% conversion ($E=8$).

3.2. Substrate solubility and effect of cyclodextrin

Keto-phenol substrate solubility in the aqueous reaction buffer was shown to be extremely poor (<0.5 g/L). Reaction rate was greatly limited by this poor solubility. Typically, miscible organic co-solvents can be used to increase substrate solubility. With 20% DMSO added to the reaction buffer, keto-phenol solubility increased to 5 g/L. However, KRED 101 was shown to be intolerant of high concentrations (>15%) of organic co-solvents (MeOH, DMSO, DMF, THF). The rapid deactivation of KRED 101 in the presence of high concentrations of organic co-solvent led us to examine another approach for increasing substrate solubility. Cyclodextrin molecules are extremely soluble in aqueous systems (>2000 g/L) and have a chemical structure that includes a non-polar interior cavity that can form complexes with hydrophobic molecules. Cyclodextrin beta W7 M1.8 (MW = 1310 g/mol) at a concentration of 100 g/L in buffer was able to solubilize 5 g/L keto-phenol. The combination of 100 g/L cyclodextrin with 10% DMSO in buffer increased keto-phenol solubility to 10 g/L with no negative impact on KRED 101 stability.

A range of cyclodextrin concentrations was studied from 50 to 300 g/L. At concentrations below 70 g/L the keto-phenol substrate was not soluble in the reaction system at 10 g/L. Reactions were run at cyclodextrin concentrations ranging from 70 to 300 g/L. The initial rate of keto-phenol reduction was measured for each reaction. The rate of reaction decreased at increasing concentrations of cyclodextrin (Fig. 1). This effect can be explained by the low concentration of free substrate in solution that is available to the enzyme. Although the system is fully soluble, most of the substrate is held tightly in the apolar cyclodextrin ring at high cyclodextrin concentrations. This affect has also been observed by others [23,24]. Dunnwald et al. observed a 50% decrease in enzyme activity in reactions run with a cyclodextrin additive [24]. Based on this data, the cyclodextrin concentration was set at 70 g/L as it provided for complete solubility of the keto-phenol substrate (10 g/L) and it offered the maximum rate of reaction. This concentration of cyclodextrin equates to a 1:1 molar ratio of cyclodextrin to substrate.

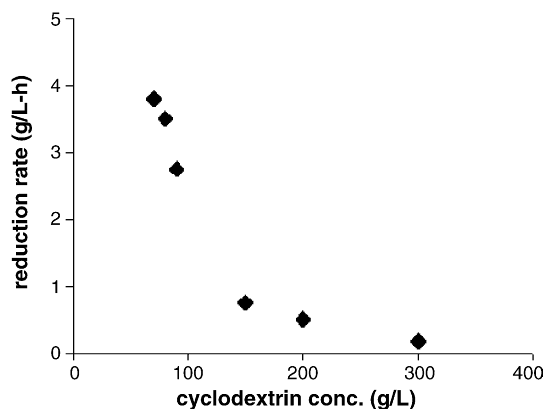

Fig. 1. Effect of cyclodextrin concentration on reaction rate. Reaction conditions: 10% DMSO, 10 g/L keto-phenol, 0.1 g/L KRED 101, 0.5 g/L NADP, 0.15 g/L GDH, 90 g/L glucose in 200 mM potassium phosphate buffer at pH 7 and 35 °C.

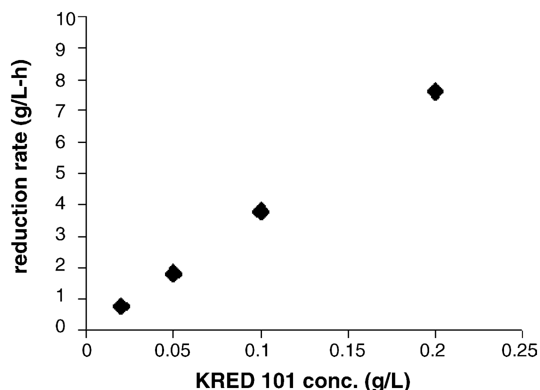

Fig. 2. Effect of catalyst loading on reaction rate. Reaction conditions: 10% DMSO, 10 g/L keto-phenol, 70 g/L cyclodextrin, 0.5 g/L NADP, 0.15 g/L GDH, 90 g/L glucose in 200 mM potassium phosphate buffer at pH 7 and 35 °C.

3.3. Effect of catalyst loading

KRED 101 catalyst loading was varied from 0.02 to 0.2 g/L. Around 0.2 g/L was determined to be the upper limit for economic viability of the process at scale. The reaction rate was determined to be linearly proportional to the catalyst loading (Fig. 2), demonstrating that the reaction was not in a mass transfer limited regime at the enzyme concentrations studied. The KRED 101 loading was set at 0.2 g/L as this would provide for the fastest reaction rate and was economically feasible.

3.4. Effect of NADP cofactor concentration

The effect of NADP cofactor concentration was studied to determine the lowest amount of cofactor that could be used without cofactor recycling limiting the reaction rate. Reactions were run with NADP concentrations ranging from 0.001 to 1 g/L. Samples were taken and the initial rate of reduction was determined. The reaction rate increased at increasing NADP concentrations ranging from 0.001 to 0.1 g/L, demonstrating that the reaction was limited by cofactor recycling at NADP concentrations <0.1 g/L. No significant increase in reaction rate was observed from 0.1 to 1 g/L NADP, therefore 0.1 g/L was chosen as the optimal NADP cofactor concentration for this reaction system (Fig. 3).

3.5. Effect of GDH concentration

Reactions were run with GDH concentrations ranging from 0.05 to 0.5 g/L to determine the minimum amount of GDH that

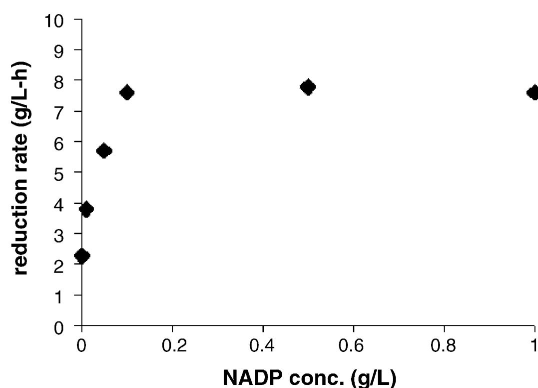

Fig. 3. Effect of NADP concentration on reaction rate. Reaction conditions: 10% DMSO, 10 g/L keto-phenol, 70 g/L cyclodextrin, 0.2 g/L KRED 101, 0.15 g/L GDH, 90 g/L glucose in 200 mM potassium phosphate buffer at pH 7 and 35 °C.

can be used while ensuring that the reaction is not limited by cofactor recycling rate. The reaction rate improved with increasing GDH concentration up to 0.15 g/L GDH (Fig. 4). No increase in reaction rate was observed in reactions run with >0.15 g/L GDH, so 0.15 g/L GDH was chosen as the optimal GDH concentration.

3.6. Effects of pH and temperature

pH was optimized by running reactions at a range of pH values from 6 to 8. pH 7.0 was determined to be the optimal pH for this reaction system.

The effect of temperature on reaction rate and enzyme selectivity was studied by running reactions across a range of temperatures from 10 to 45 °C. The initial reaction rate was observed to increase logarithmically with increasing temperature (Fig. 5). Enzyme selectivity was calculated using the following equation:

$$E(\text{selectivity}) = \frac{\ln[(1 - \text{conversion})(1 - \text{ee}_{\text{substrate}})]}{\ln[(1 - \text{conversion})(1 + \text{ee}_{\text{substrate}})]}$$

Selectivity of the enzyme improved at decreasing temperatures (Fig. 6). The enzyme selectivity values were used to calculate the yield of >99% ee (6*S*,9*R*) keto-phenol that could

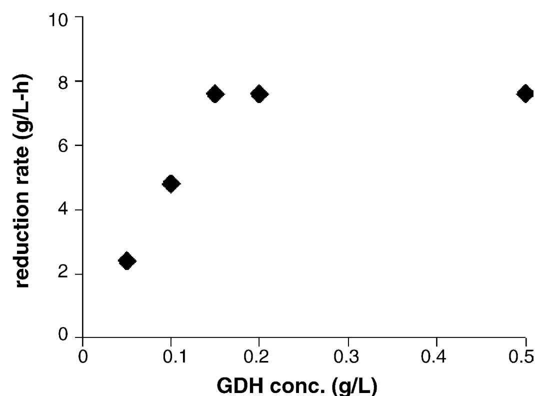

Fig. 4. Effect of GDH concentration on reaction rate. Reaction conditions: 10% DMSO, 10 g/L keto-phenol, 70 g/L cyclodextrin, 0.2 g/L KRED 101, 0.1 g/L NADP, 90 g/L glucose in 200 mM potassium phosphate buffer at pH 7 and 35 °C.

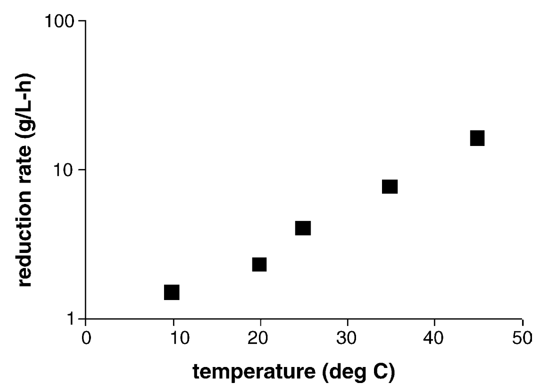

Fig. 5. Effect of temperature on reaction rate (note log scale on *y*-axis). Reaction conditions: 10% DMSO, 10 g/L keto-phenol, 70 g/L cyclodextrin, 0.2 g/L KRED 101, 0.1 g/L NADP, 0.15 g/L GDH, 90 g/L glucose in 200 mM potassium phosphate buffer at pH 7.0.

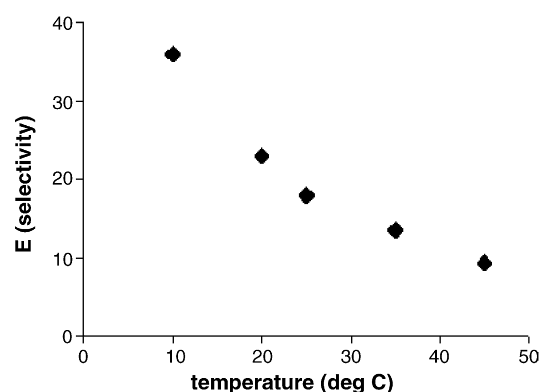

Fig. 6. Effect of temperature on enzyme selectivity. Reaction conditions: 10% DMSO, 10 g/L keto-phenol, 70 g/L cyclodextrin, 0.2 g/L KRED 101, 0.1 g/L NADP, 0.15 g/L GDH, 90 g/L glucose in 200 mM potassium phosphate buffer at pH 7.0.

be obtained at each temperature (Table 1). The maximum theoretical yield for the kinetic resolution is 50%. Based on this data, the optimal reaction temperature was determined to be 10 °C. Although higher temperatures provided for greater reaction rates, product yield at 10 °C was greatly increased over running at elevated temperatures. Running the reaction below 10 °C would only give a marginal increase in yield at the unacceptable expense of reaction rate.

3.7. Effect of cyclodextrin on keto-phenol isolation

The strategy for isolating high purity keto-phenol and alcohol required that a procedure be developed that rejects the cyclodex-

Table 1
Yield of >99% ee (6*S*,9*R*) keto-phenol

Temperature (°C)	>99% ee (6*S*,9*R*) keto-phenol (% yield)
45	26
35	30
25	32
20	39
10	44

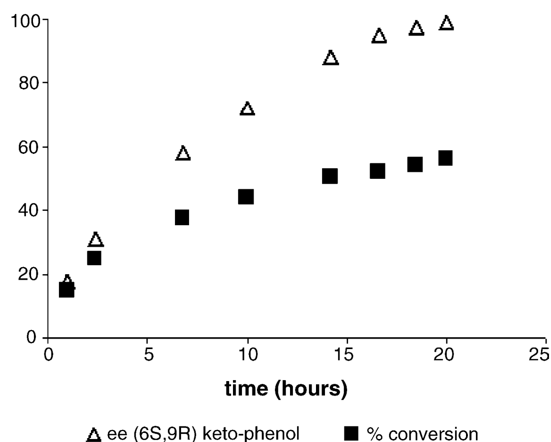

Fig. 7. Kinetic resolution reaction profile for 1 kg scale reaction. Reaction conditions: 10% DMSO, 10 g/L keto-phenol, 70 g/L cyclodextrin, 0.2 g/L KRED 101, 0.1 g/L NADP, 0.15 g/L GDH, 90 g/L glucose in 200 mM potassium phosphate buffer at pH 7.0 and 10 °C.

trin employed in the bioconversion. Solubility studies for the ketone, alcohol and cyclodextrin were conducted using a wide range of solvents including ethyl acetate, methyl ethyl ketone (MEK), acetonitrile, isopropanol, toluene, *n*-heptane, methanol, and water. MEK was found to have one of the highest extraction efficiencies of any of the solvents tested while also rejecting the cyclodextrin. The rejection of the solubility enhancing cyclodextrin in the extraction was crucial to developing an efficient crystallization procedure.

A water wash of this MEK organic phase was necessary to remove polar impurities that were found to complicate the crystallization. The washed MEK extract was then concentrated to 150 g/L keto-phenol and solvent switched to methanol. Water (75% v/v) was added to the concentrate as an anti-solvent, and >98% pure crystals containing >99% ee keto-phenol and alcohol were obtained.

3.8. Process demonstration

The optimized reaction process has been demonstrated at 1 kg (100 L) scale. The reaction produced >99% ee (6*S*,9*R*) keto-phenol with 44% assay yield in 20 h (Fig. 7). A total of 815 g of crystalline product was isolated (82% isolated yield).

4. Conclusions

(6*S*,9*R*) keto-phenol is an important intermediate in pharmaceutical organic synthesis. The compound was typically resolved by chromatography. This work has demonstrated an efficient enzymatic route to arrive at (6*S*,9*R*) keto-phenol through the kinetic resolution of racemic keto-phenol via the selective reduction of the (6*R*,9*S*) enantiomer. Although kinetic resolution via selective enzymatic reduction of racemic substrates has been demonstrated, this is the first time that a compound whose stereochemistry is set by a bridged bicyclic ring system has been resolved in such a fashion with a commercially available enzyme in an optimized high yielding and highly stereoselective system. The challenges of poor substrate solubility in aqueous reaction systems and the lack of tolerance of KRED 101 for high concentrations of organic solvents were overcome through the combined use of cyclodextrin and DMSO to solubilize the substrate. Finally, selectivity of the enzyme was improved by running the reaction at low temperature (10 °C), and the optimized process was demonstrated at 1 kg scale producing the desired (6*S*,9*R*) keto-phenol product with >99% ee and 44% yield (50% maximum theoretical yield for the resolution).

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