

# Highly Efficient Synthesis of (*R*)-3-Quinuclidinol in a Space–Time Yield of 916 g L<sup>−1</sup> d<sup>−1</sup> Using a New Bacterial Reductase ArQR

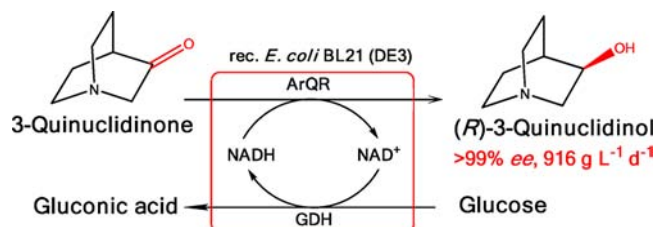
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## ABSTRACT



A new keto reductase (ArQR), identified from *Agrobacterium radiobacter* ECU2556, can efficiently reduce 3-quinuclidinone in excellent enantioselectivity and high space–time yield for the synthesis of (*R*)-3-quinuclidinol, a chiral building block of many antimuscarinic agents. This is the first time that a high yield of (*R*)-3-quinuclidinol up to 916 g L<sup>−1</sup> d<sup>−1</sup> using a bioreduction approach is reported.

Biocatalysis has been growing rapidly as a practical and environmentally friendly alternative to traditional chemical synthesis to access secondary alcohols,<sup>1</sup> which are intermediates of many pharmaceuticals. For example, optically pure (*R*)-3-quinuclidinol is an important chiral building block of many antimuscarinic agents for the treatment of COPD (chronic obstructive pulmonary disease),<sup>2</sup> such as talsaclidine, revatropate, solifenacin, and acridinium bromide.<sup>3</sup>

There are various methods to synthesize (*R*)-3-quinuclidinol, including chemical synthesis, enzymatic kinetic resolution, and asymmetric bioreduction. Chemical synthesis is one of the most

efficient routes to produce (*R*)-3-quinuclidinol. For example, asymmetric hydrogenation of 3-quinuclidinone with RuBr<sub>2</sub>[(*S,S*)-xylskewphos](pica) in a base containing ethanol has afforded (*R*)-3-quinuclidinol in 88–90% ee.<sup>4</sup> A combined catalyst system of RuCl<sub>2</sub>[(*S*)-binap][(R)-iphan] and *t*-C<sub>4</sub>H<sub>9</sub>OK in 2-propanol afforded the chiral alcohol in 97–98% ee,<sup>5</sup> but the trace metal contamination in the product and the incredible fluctuation in the price of rare metals over the past decade quite rightly make a cause for concern in the pharmaceutical industry. Enzymatic resolution of the racemic quinuclidinol ester is a reliable procedure to obtain (*R*)-3-quinuclidinol in high optical purity. *Aspergillus melleus* protease was used for hydrolyzing (±)-3-(butyryloxy)quinuclidinium butyrate, giving (*R*)-3-quinuclidinol in 96% ee and 42% overall yield,<sup>6</sup> despite the necessary modification of the substrate prior to enzymatic reaction and the unsatisfactory ≤50% yield, which limits

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the potential of the enzyme. In recent years, more researchers have been paying attention to the bioreduction approach of producing (*R*)-3-quinuclidinol, since biocatalyzed asymmetric reductions can offer highly selective reactions, environmentally benign processes, and energy-effective operations.<sup>7</sup> *RrQR* from *Rhodotorula rubra*,<sup>8</sup> *QNR* and *BacC* from *Microbacterium luteolum* JCM 9174,<sup>9</sup> and *Nocardia* sp. WY1202<sup>10</sup> can convert 3-quinuclidinone stereospecifically to (*R*)-3-quinuclidinol, but the common disadvantages of all these reductases were their poor substrate tolerance and low volumetric productivity, which limits their industrial application.

Herein, a new reductase *ArQR* (GenBank accession no.: YP\_002542435.1) was identified from *Agrobacterium radiobacter* ECU2556 through the screening of various microorganisms stocked in our laboratory. After heterologous expression in *E. coli*, it can efficiently convert 3-quinuclidinone to (*R*)-3-quinuclidinol with high activity and excellent enantioselectivity.

To construct the cofactor regeneration system, *ArQR* and *BmGDH* (glucose dehydrogenase from *Bacillus megaterium*) were coexpressed in *E. coli* in a tandem mode. Considering that the reduction of 3-quinuclidinone was a rate-limiting step comparing with the oxidation step of glucose, *E. coli* BL21 (DE3) (pET28a-*ArQR-BmGDH*), rather than *E. coli* BL21 (DE3) (pET28a-*BmGDH-ArQR*), was chosen for further research. The lyophilized cells were employed as the biocatalyst to perform the reductive reaction of 3-quinuclidinone.

To optimize the reaction conditions, the biocatalyst dosage, substrate loading, and external  $\text{NAD}^+$  concentration have to be assessed. Because both the substrate and product are water-miscible, all the reactions were performed in aqueous solution. Initial experiments were performed in 10 mL of potassium phosphate buffer (200 mM, pH 7.0) with 5 mmol of substrate (81 g/L), 1  $\mu\text{mol}$  of  $\text{NAD}^+$ , 7.5 mmol of glucose, and 0.1 g of lyophilized cells of *E. coli* (pET28a-*ArQR-BmGDH*), and the pH of the reaction mixture was controlled at 7.0 by titrating 2 M NaOH. Surprisingly, the substrate was completely transformed to the desired product within merely 0.5 h (Table 1, entry 1). Clearly it was possible to transform more substrate, so the substrate loading was raised to 162 g  $\text{L}^{-1}$  without changing the other conditions, and the reaction was completed easily within 1.5 h (Table 1, entry 2). In a stepwise manner, a further attempt was made to convert 162 g  $\text{L}^{-1}$  substrate without external supplement of  $\text{NAD}^+$ . It was found that the conversion also reached 100% in less than 4 h (Table 1, entry 3). When the substrate was further increased up to 242 g  $\text{L}^{-1}$ , without any external  $\text{NAD}^+$ , only 44% substrate was converted to product even if the reaction time was extended to 20 h (Table 1, entry 4). The

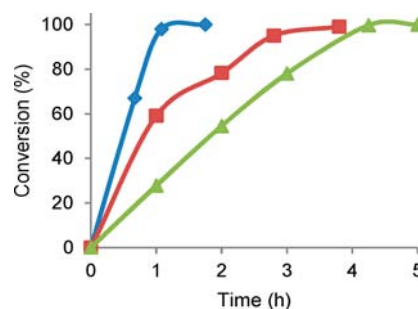
**Table 1.** Asymmetric Reduction of 3-Quinuclidinone HCl with Coexpressed Cells of *E. coli* (pET28a-*ArQR-BmGDH*)<sup>a</sup>

entry	substrate (g/L)	cell (g/L)	$\text{NAD}^+$ (mM)	time (h)	conv <sup>c</sup> (%)	ee <sup>c</sup> (%)
1	81	10	0.1	0.5	100	>99
2	162	10	0.1	1.5	100	>99
3	162	10	0	4.0	100	>99
4	242	10	0	20	54	>99
5	242	10	0.1	4.5	100 (90) <sup>b</sup>	>99
6	242	5	0.1	10	>99 (90) <sup>b</sup>	>99

<sup>a</sup> Reaction conditions: 3-quinuclidinone HCl (0.8–2.4 g), dry cells of *E. coli* BL21 harboring pET28a-*ArQR-BmGDH* (0.05–0.1 g), D-glucose (1.5 equivalent),  $\text{NAD}^+$  (0–1  $\mu\text{mol}$ ), 10 mL phosphate buffer (200 mM, pH 7.0), 30 °C. pH was kept at 7.0 with 2 M NaOH. <sup>b</sup> Isolated yield of (*R*)-3-quinuclidinol. <sup>c</sup> Determined by GC analysis.

internal cofactor of the *E. coli* cells could probably not meet the need of high substrate loading.

Further reaction was tried using 242 g  $\text{L}^{-1}$  substrate and 0.1 mM of external  $\text{NAD}^+$ . As expected, the substrate conversion reached 99% in 4.5 h (Table 1, entry 5), and the space–time yield of (*R*)-3-quinuclidinol reached 916 g  $\text{L}^{-1} \text{d}^{-1}$ . Considering the internal content (ca. 9.7  $\mu\text{mol/g}$  dry cell) of  $\text{NAD}(\text{H})^+$  in *E. coli* cells,<sup>11</sup> the total turnover number (TTN) was estimated to be around 7500, which represents the highest among all the 3-quinuclidinone reductases reported to date. The time course of bioreduction showed that increasing the amount of  $\text{NAD}^+$  gave faster bioreduction (Figure 1).



**Figure 1.** Reduction of 3-quinuclidinone-HCl by *E. coli* transformants harboring pET28a-*ArQR-BmGDH*: (◆) 162 g/L substrate, 0.1 mM  $\text{NAD}^+$  was added; (■) 162 g/L substrate without addition of  $\text{NAD}^+$ ; (▲) 242 g/L substrate, 0.1 mM  $\text{NAD}^+$  added.

Otherwise, to be a good catalyst to render a chemical manufacturing process feasible, it needs to meet the requirement of  $\geq 100 \text{ g L}^{-1}$  substrate loading,  $\leq 5 \text{ g L}^{-1}$  biocatalyst loading,  $\leq 24 \text{ h}$  reaction time,  $\geq 98\%$  conversion, and  $\geq 99\%$  ee.<sup>13</sup>

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**Table 2.** Comparison of *ArQR* with Other Reductase Biocatalysts

biocatalyst	coenzyme	$K_m$ (mM)	$k_{cat}/K_m$ ( $s^{-1} mM^{-1}$ )	substrate (g/L)	% conversion (time/h)	% ee (config)	space–time yield ( $g L^{-1} d^{-1}$ )	ref
<i>RrQR</i> <sup>a</sup>	NADPH	145	n.d. <sup>g</sup>	100	100 (21)	>99.9 ( <i>R</i> )	90 <sup>h</sup>	Uzura et al. <sup>8</sup>
<i>DnTR1</i> <sup>b</sup>	NADPH	8.713	0.0032	n.d. <sup>g</sup>	n.d. <sup>g</sup>	n.d. <sup>g</sup>	n.d. <sup>g</sup>	Chen et al. <sup>12</sup>
WY1202 cell <sup>c</sup>	n.d. <sup>g</sup>	n.d. <sup>g</sup>	n.d. <sup>g</sup>	16	95.3 (48)	>99 ( <i>R</i> )	6 <sup>h</sup>	Wang et al. <sup>10</sup>
WY1406 cell <sup>d</sup>	n.d. <sup>g</sup>	n.d. <sup>g</sup>	n.d. <sup>g</sup>	10	95 (48)	>99 ( <i>S</i> )	4 <sup>h</sup>	Wang et al. <sup>10</sup>
QNR <sup>e</sup>	NADH	6.5	5.6 <sup>h</sup>	51	100 (12)	>99.9 ( <i>R</i> )	80 <sup>h</sup>	Isotani et al. <sup>9</sup>
BacC <sup>e</sup>	NADH	13.8	0.083 <sup>h</sup>	51	94 (12)	>99.9 ( <i>R</i> )	75 <sup>h</sup>	Isotani et al. <sup>9</sup>
<b><i>ArQR</i><sup>f</sup></b>	<b>NADH</b>	<b>0.4</b>	<b>290</b>	<b>242</b>	<b>100 (4.5)</b>	<b>&gt;99.9 (<i>R</i>)</b>	<b>916</b>	<b>This work</b>

<sup>a</sup>Quinuclidinone reductase from *Rhodotorula rubra*. <sup>b</sup>Tropinone reductase from *Dendrobium nobile* Lindl. <sup>c</sup>*Nocardia* sp. WY1202 cell. <sup>d</sup>*R. erythropolis* WY1406 cell. <sup>e</sup>Quinuclidinone reductase from *Microbacterium luteolum* JCM 9174. <sup>f</sup>Lyophilized cells of *E. coli* (pET28a-*ArQR*-*BmGDH*). <sup>g</sup>n.d. = not determined. <sup>h</sup>Calculated according to the references.

Satisfactorily, the substrate was completely reduced to give >99% conversion and >99% ee within 10 h even if the cell dosage was cut down to 5 g L<sup>-1</sup> (Table 1, entry 6). In the practical case, the space time yield of (*R*)-3-quinuclidinol still reached 408 g L<sup>-1</sup> d<sup>-1</sup>, indicating the great potential of *ArQR* for industrial usage.

To investigate its intrinsic properties, the recombinant reductase *ArQR* with an *N*-terminal His-tag was purified to electrophoretic homogeneity by nickel affinity chromatography. The specific activity of the crude cell-free extract was 78 U mg<sup>-1</sup> powder, and it reached 198 U mg<sup>-1</sup> protein with an increase of 2.5 folds after purification. Kinetic parameters were measured using varied concentrations of 3-quinuclidinone and NADH. The  $K_m$  measured of *ArQR* for 3-quinuclidinone was about 0.4 mM, which is one to 2 orders of magnitudes lower than those of all the quinuclidinone reductases ever reported. It indicates that the enzyme has excellent affinity to substrate 3-quinuclidinone, implying that the reaction velocity will be high even with a low substrate concentration, while for the enzymes with high  $K_m$ , such as *RrQR*, only 46% of the maximum velocity was exhibited when the concentration of substrate was 120 mM. The catalytic efficiency ( $k_{cat}/K_m$ ) of *ArQR* is

290 s<sup>-1</sup> mM<sup>-1</sup>, as listed in Table 2, which is also outstanding as compared to other quinuclidinone reductases, explaining the higher reaction velocity and shorter reaction time of *ArQR* than the others.

To make the biocatalysis-based processes scalable, one has to fulfill a certain requirement for throughput (volume efficiency) which is industrially acceptable.<sup>14</sup> Among all the enzymes or cells that can reduce 3-quinuclidinone for the synthesis of (*R*)-3-quinuclidinol (Table 2), *ArQR* is the only one that can reduce >200 g L<sup>-1</sup> substrate in a space time yield as high as 916 g L<sup>-1</sup> d<sup>-1</sup>, demonstrating its great potential in industrial manufacturing.

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**Supporting Information Available.** Experimental procedures and compound characterization data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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The authors declare no competing financial interest.