Supporting Information

Highly efficient synthesis of (*R*)-3-quinuclidinol in a space time yield of 916 g L^{-1} d^{-1} using a new bacterial reductase *Ar*QR

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1. General

All commercial reagents were purchased in the highest purity available. *E. coli* DH5α and *E. coli* BL21 (DE3) were used for cloning and expression of reductases, respectively. Other strains used were purchased from China General Microbiological Culture Collection Center (Beijing, China). Cells harboring the recombinant plasmid were grown in Luria-Bertani (LB) medium. This enzyme belongs to short-chain alcohol dehydrogenase/reductase [S1] based on sequence analysis.

2. Cloning and expression of *Ar*QR gene in *Escherichia coli*

After a survey of various microorganisms stocked in our lab, we identified *Agrobacterium radiobacter* ECU2556 which can reduce 3-quinuclidinone. Genomic DNA was extracted from *Agrobacterium radiobacter* ECU2556, using a TIAN amp Bacteria DNA Kit from Tiangen (Shanghai, China). The DNA fragment of *Ar*QR gene was amplified by polymerase chain reaction using primers (**Table S1**) with *Nde* I and *BamH* I restriction sites as shown in Table S1. After double-digested with *Nde* I and *BamH* I, the fragment was inserted into expression vector pET-28a, and then transformed into *E. coli* BL21 (DE3). The cells were cultivated in LB medium containing 50 μg/ml kanamycin till the OD_{600} reached 0.6. Then IPTG was added to a final concentration of 0.2 mM, and incubated at 25 ^{o}C for further 13 h.

Table S1 Sequence of primers used in this research

Primer	Sequence
f*Ar*QR-NdeI	GGAATTCCATATGGAGGCTTCATTGTCGG
f*Ar*QR-BamHI	CGCGGATCCTCAGTCCATGCGAACGCCAC
l*Bm*GDH-BamHI	CGCGGATCCAAGGAGATATAATGTATAAAGATTTAGAAGG
l*Bm*GDH-XhoI	CCGCTCGAGTTATCCGCGTCCTGCTTG
f*Bm*GDH-NdeI	GGAATTCCATATGTATAAAGATTTAGAAGG
f*Bm*GDH-BamHI	CGCGGATCCTTATCCGCGTCCTGCTTGGAAT
l*Ar*QR-BamHI	CGCGGATCCAAGGAGATATAATGGAGGCTTCATTGTCGG
l*Ar*QR-XhoI	CCGCTCGAGTCAGTCCATGCGAACGCCAC

3. Purification of *Ar*QR

The cells were harvested and resuspended in buffer A (20 mM sodium phosphate buffer, pH 7.4, 500 mM NaCl, 20 mM imidazole). Afterwards the cell was broken by sonication, and the cell debris was discarded by centrifugation (10,000× *g* for 10 min). The supernatant was filtered and loaded onto a His trap Ni–NTA FF column (1 ml, GE

Healthcare Corp) which was pre-equilibrated with buffer A. The adsorbed protein was eluted with an increasing gradient of imidazole from 20 to 500 mM in buffer A at a flow rate of 1 ml/min. After assayed by SDS-PAGE, the pure protein was pooled and dialyzed against 20 mM sodium phosphate buffer (pH 7.4) for desalting, finally the protein was condensed by ultrafiltration and stored at -80 ^{o}C with 20% glycerol.

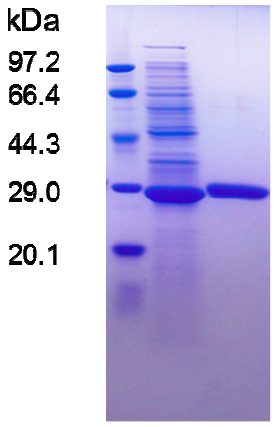


Figure S1. SDS-PAGE analysis of purified ArQR. Lane 1, Marker; Lane 2, cell-free extract of *Ar*QR; Lane 3, purified *Ar*QR.

4. Enzyme assays

*Ar*QR activity was assayed spectrophotometrically at 30 ^{o}C by monitoring the absorbance at 340 nm corresponding to the change in NADH concentration. The reaction mixture consisted of 2 μmol 3-quinuclidinone, 0.1 μmol NADH, 100 μmol potassium phosphate buffer (pH 7.0), and an appropriate amount of enzyme in a total volume of 1 ml. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol NADH per minute under these conditions.

5. Effects of pH and temperature on enzyme activity and stability

The optimum pH was determined by standard activity assay at different pH (4.0–9.0), with sodium citrate buffer for pH 4.0–6.0, sodium phosphate for pH 6.0–8.0, and Tris-HCl for pH 8.0–9.0. The optimum temperature was determined under standard conditions at different temperatures in the range of 20–50 ^{o}C. The thermostability of *Ar*QR was assayed by incubating the pure enzyme at different temperatures (30, 35 and 40 ^{o}C) for 100 h. During this period, samples were withdrawn and the residual activity was measured under standard conditions.

*Ar*QR displayed highest activity at pH 7.0, 40 °C and the half time of *Ar*QR was 89, 8.3, 1.2 h at 30, 35, and 40 °C respectively, indicating it was more stable under moderate reaction conditions.

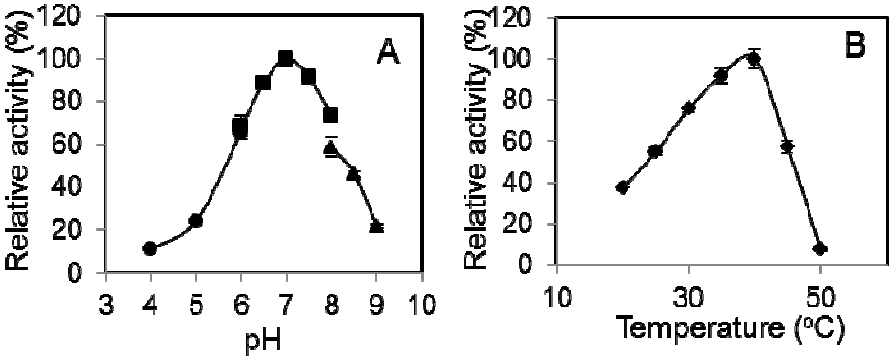


Figure S2. The effects of pH and temperature on the activity of purified *Ar*QR. **A)** The activity was measured in the following buffers of 100 mM: (●) Sodium citrate (pH 4.0–6.0), (■) sodium phosphate (pH 6.0–8.0) and (▲) Tris-HCl (pH 8.0–9.0). Relative activity was expressed as a percentage of the maximum activity under the experimental conditions. **B)** The enzyme activity was determined at different temperatures in the range of 20–50 °C in 100 mM sodium phosphate buffer, pH 7.0.

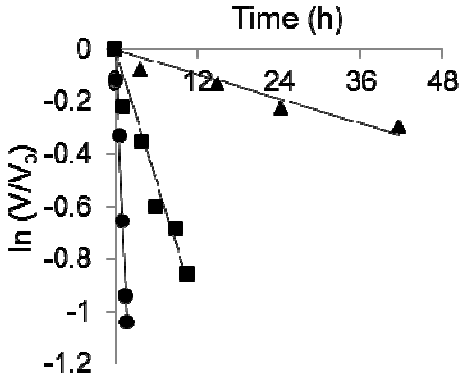


Figure S3. Thermostability of purified *Ar*QR. The purified ArQR was pre-incubated in sodium phosphate buffer (100 mM, pH 7.0) at varied temperatures and the residual activity was measured. (●) 40 °C; (■) 35 °C; (▲) 30 °C.

6. Kinetic parameters

To determine the kinetic parameters, various concentrations of substrate 3-quinuclidinone (0.1–5.0 mM), and cofactor NADH (0.01–0.25 mM) in 100 mM phosphate buffer (pH 7.0) were used. The activity for each concentration was assayed in triplicate, resulting in a mean value for each substrate concentration. All the mean

activities were plotted versus the substrate concentration and adjusted to a Michaelis–Menten model using Origin 8.

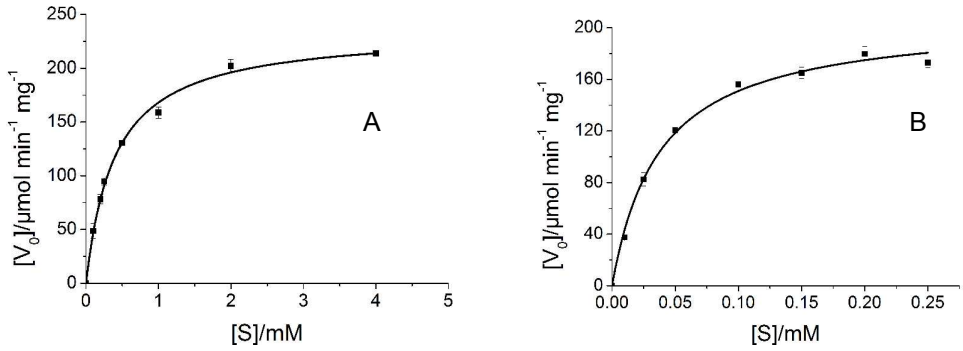


Figure S4. Kinetic curves of asymmetric reduction of 3-quinuclidinone. **A)** Concentrations of 3-quinuclidinone as variants; **B)** Concentrations of NADH as variants.

7. Coexpression of *Ar*QR and *Bm*GDH genes in *E. coli* cells

NADH is rather expensive, and this challenge can be overcome by cofactor regeneration system. BmGDH [S2] was used for the regeneration of cofactor NADH. The PCR primers used for the construction of expression vector were shown in **Table S1**.

E. coli BL21(DE3) (pET28-*Ar*QR-*Bm*GDH): 3-quinuclidinone reductase gene *Ar*QR was amplified by PCR with the primer pair of f*Ar*QR-NdeI and f*Ar*QR-BamHI. After digested with *Nde*I and *Bam*HI, the DNA fragment was ligated into the *Nde*I and *Bam*HI sites of pET28a. The primer pairs l*Bm*GDH-BamHI and l*Bm*GDH-XhoI were used for the amplification of *Bm*GDH and digested with *Bam*HI and *Xho*I, then ligated into the *Bam*HI and *Xho*I sites of pET28a which contained *Ar*QR to achieve the construction of pET28-*Ar*QR-*Bm*GDH. Similarly, pET28-*Bm*GDH-*Ar*QR was constructed with primer pairs f*Bm*GDH-NdeI and f*Bm*GDH-BamHI, l*Ar*QR-BamHI and l*Ar*QR-XhoI.

In our construction of coexpressed plasmid, both *Ar*QR and GDH were inserted into pET28a with independent terminal codons. So they were expressed as separate proteins instead of a fused protein, as shown in SDS-PAGE (**Fig. S5**). Considering the different restriction sites, the molecular weight of both enzymes in different expression vector was shown in Table S2. For *E. coli* BL21(DE3) (pET28-*Ar*QR-*Bm*GDH), the activity of *Ar*QR and *Bm*GDH were 1700 U and 520 U

per gram wet cells respectively. In contrast, lower activity (470 U/g wet cell) of *Ar*QR (which might be due to the low expression level) and relatively higher activity (720 U/g wet cell) of *Bm*GDH were observed in *E. coli* BL21(DE3) harboring plasmid pET28-*Bm*GDH-*Ar*QR. Considering the rate-limiting step was the reduction of 3-quinuclidinone, *E. coli* BL21(DE3) (pET28-*Ar*QR-*Bm*GDH) was chosen for the reduction of 3-quinuclidinone.

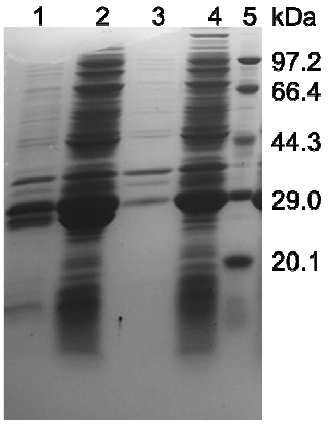


Figure S5 SDS-PAGE analysis of proteins which were coexpressed. Lanes 1 & 2 are the precipitate and supernatant of *E. coli* BL21(DE3) (pET28a-*Ar*QR-*Bm*GDH) respectively. Lanes 3 & 4 are the precipitate and supernatant of *E. coli* BL21(DE3) (pET28a-*Bm*GDH-*Ar*QR) respectively. Lane 5 is Marker.

Table S2 The molecular weights of *Ar*QR and *Bm*GDH in *E. coli* BL21(DE3) harboring different expression vectors

Protein	*E. coli* BL21(DE3) (pET28a-*Ar*QR-*Bm*GDH)	*E. coli* BL21(DE3) (pET28a-*Bm*GDH-*Ar*QR)
*Ar*QR	29.612 kDa	27.449 kDa
*Bm*GDH	28.085 kDa	30.248 kDa

8. Typical Procedure for Asymmetric Reduction to Produce (*R*)-3-Quinuclidinol

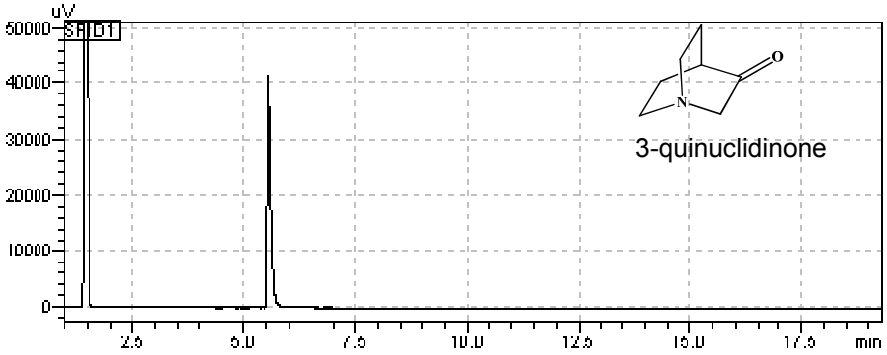
Lyophilized cells of *E. coli* (pET28-*Ar*QR-*Bm*GDH) (0.1 g) were added to 10 ml sodium phosphate buffer (pH 7.0, 200 mM) containing 0.8 g 3-quinuclidinone, 1.5 equivalent glucose and 0.1 mM NAD^+. The mixture was agitated at 30 ^oC, with pH

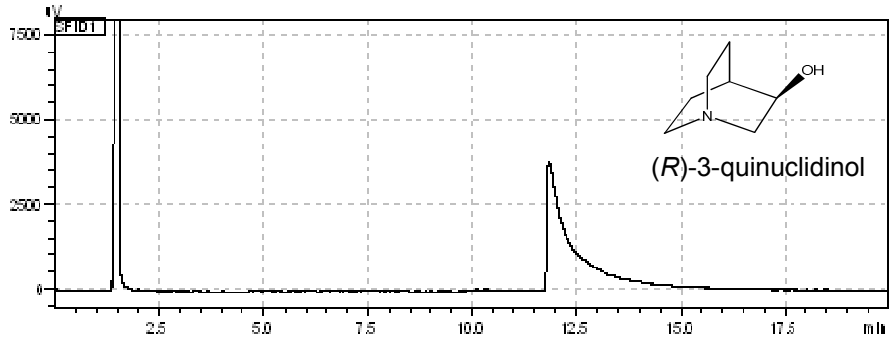
maintained at 7.0 by titrating 2 M NaOH. After stirred for 2 h, the reaction mixture was alkalified with NaOH and extracted twice with chloroform. Then the collected organic phases were combined and dried over anhydrous Na_2SO_4, filtered and evaporated under vacuum, affording (*R*)-3-quinuclidinol in 90% isolated yield. The enantiomeric excess (*ee*) of the product and the level of conversion were determined by GC analysis. GC analyses were performed using a CP-Chirasil-DEX CB (Varian, USA), with injector and detector at 220 ^{o}C, and column temperature at 140 ^{o}C.

$[\alpha]_D^{27}$ -45^{o} (*c* 2.0, 1 M HCl) {lit.[S3]: $[\alpha]_D^{25}$ -44.9^{o} (*c* 2.0, 1 M HCl)} ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.34 (m, 1H), 1.45 (m, 1H), 1.67 (m, 1H), 1.79 (m, 1H), 1.95 (m, 1H), 2.61 (m, 2H), 2.75 (m, 2H), 2.89 (m, 1H), 3.09 (m, 1H), 3.80 (m, 1H), 4.26 (br s, 1H). ^{13}C NMR (CDCl3, 400 MHz) δ 67.2 (CH), 57.9 (CH_2), 47.3 (CH_2), 46.3 (CH_2), 28.3 (CH), 24.7 (CH_2), 18.9 (CH_2).

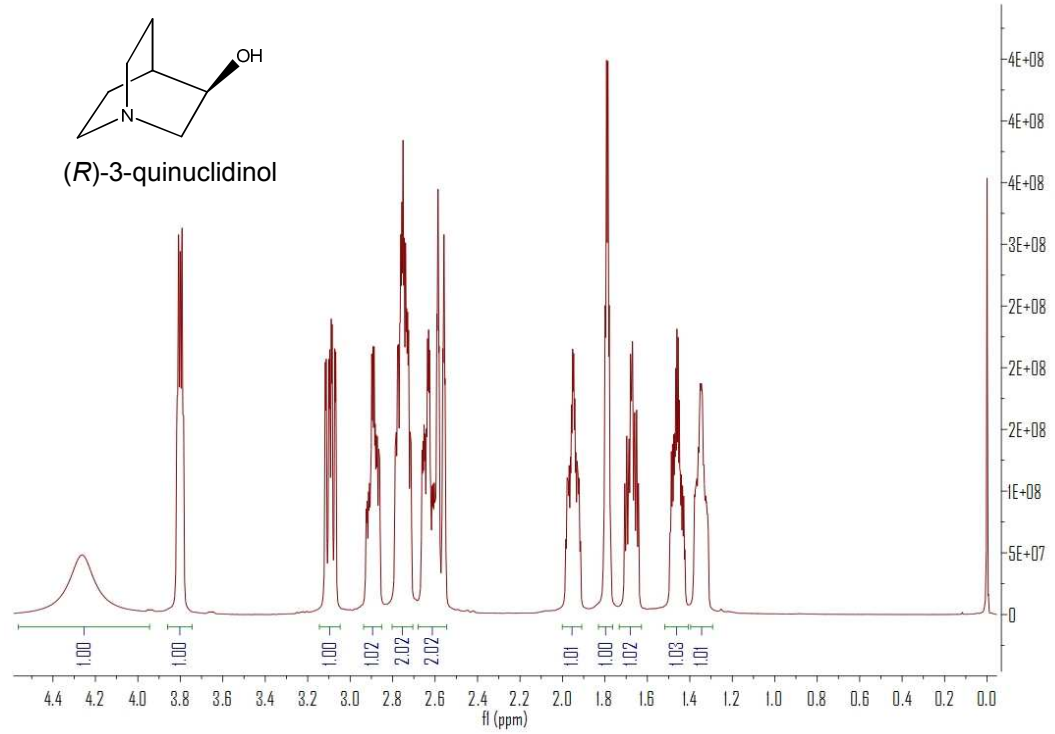
9. GC spectra

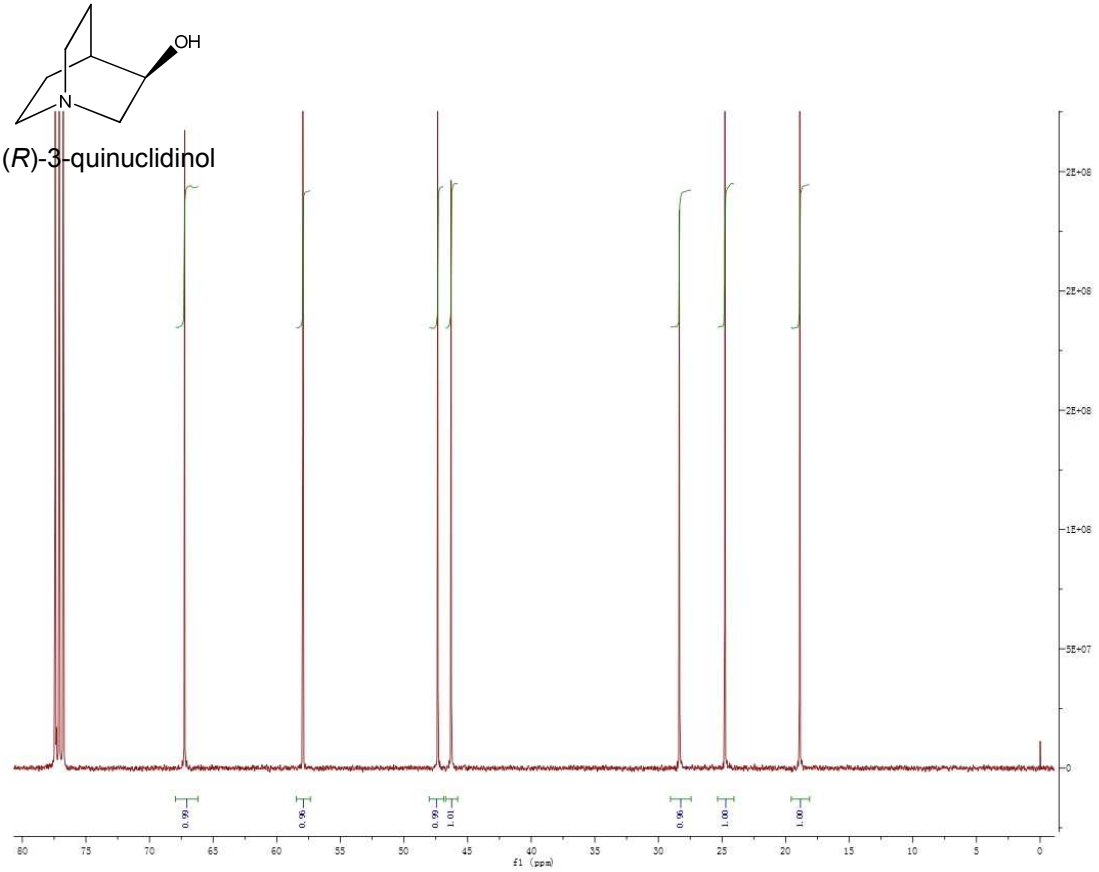
Substrate, 5.4 min; product, (*S*)-form, 11.5 min; prod., (*R*)-form, 11.9 min.





10. NMR spectra





11. References

[S1] Kavanagh, K. L.; Jörnvall, H.; Persson, B.; Oppermann, U., *Cell. Mol. Life Sci.* 2008, *65*, 3895–3906.

[S2] Zhang, J. D.; Li, A. T.; Yu, H. L.; Imanaka, T.; Xu, J. H. *J. Ind. Microbiol. Biotechnol.* 2011, *38*, 633–641.

[S3] Nomoto, F.; Hirayama, Y.; Ikunaka, M.; Inoue, T.; Otsuka, K., Tetrahedron Asymmetry 2003, *14*, 1871-1877.