

# **Stereocomplementary Bioreduction of $\beta$ -Ketonitrile without Ethylated Byproduct**

Guo-Chao Xu, Hui-Lei Yu,\* Zhi-Jun Zhang, and Jian-He Xu\*

Laboratory of Biocatalysis and Synthetic Biotechnology, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, People's Republic of China

Fax: (+86)-21-64250840; e-mail: [huileiyu@ecust.edu.cn](mailto:huileiyu@ecust.edu.cn); [jianhexu@ecust.edu.cn](mailto:jianhexu@ecust.edu.cn).

## **Supporting Information**

|   |           |
|---|-----------|
| <b>General remarks</b> .....  | <b>2</b>  |
| <b>Primers used in this study</b> .....   | <b>3</b>  |
| <b>Construction of co-expression plasmids</b> .....   | <b>4</b>  |
| <b>SDS-PAGE of co-expressed carbonyl reductase and glucose dehydrogenase</b> ..                         | <b>5</b>  |
| <b>Asymmetric reduction of benzoylacetone nitrile with the top six reductases in the tool-box</b> ..... | <b>6</b>  |
| <b>Partition coefficient of benzoylacetone nitrile in a water/organic solvent biphasic system</b> ..... | <b>7</b>  |
| <b>Study of enzyme stability</b> .....  | <b>7</b>  |
| <b>GC spectra</b> .....   | <b>8</b>  |
| <b>Mass and NMR data</b> .....  | <b>11</b> |
| <b>References</b> .....   | <b>15</b> |

## General remarks

All the aromatic benzoylacetone nitriles were from commercial source. Heterogeneous overexpression and purification of carbonyl reductases were based on our previous work.<sup>1,2</sup>

### Activity assay

Reductase activity was determined by monitoring the decrease in absorbance of NADPH at 340 nm using spectrophotometer at 30 °C. The reaction mixture (1 mL) comprised of 1 mM aromatic  $\beta$ -ketone nitriles, 0.1 mM NADPH, 100 mM sodium phosphate buffer (pH 6.5) and an appropriate amount of enzyme (cell lysate). One unit of enzyme activity was defined as the amount of enzyme that catalyzing the oxidation of 1  $\mu$ mol of NADPH per minute.

### Standard bioreduction of aromatic $\beta$ -ketone nitriles

Standard bioreduction of aromatic  $\beta$ -ketone nitriles was carried out in a 10-mL reaction mixture (10 mL aqueous or 5 mL aqueous plus 5 mL organic phase). To 10 mL sodium phosphate buffer (pH 6.5) containing lyophilized cells of *BmGDH-DhCR* (**0.5 g**) or *BmGDH-DhCR* (**0.2 g**), were added 10 g/L  $\beta$ -ketone nitrile and 1.5 *equiv.* glucose, and the resulting mixture was agitated at 25 °C. The pH was maintained at 6.5 by titration of 2.0 M NaOH.

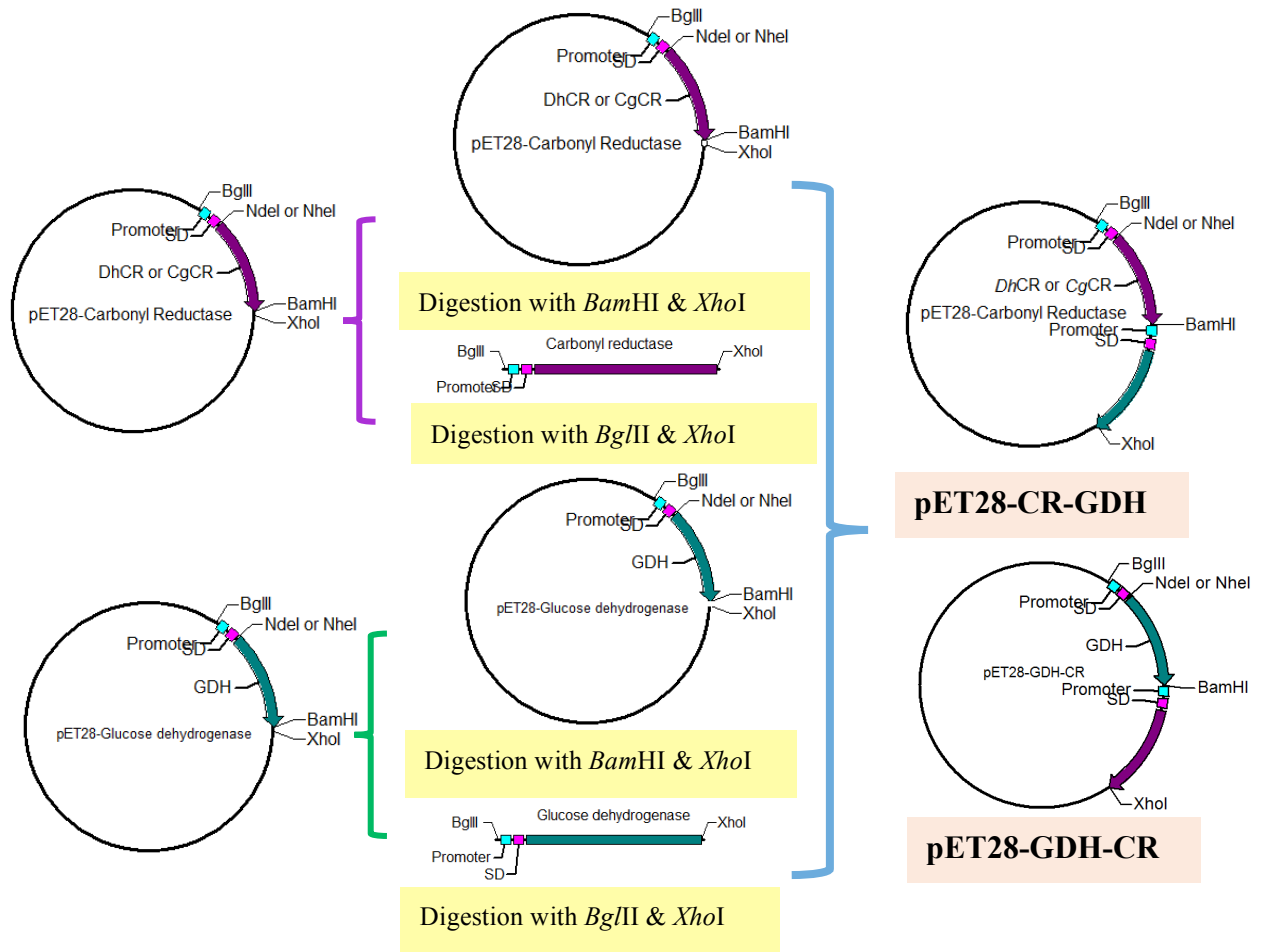
### Semi-preparative preparation of (*R*)-3-hydroxy-3-phenylpropanenitrile

Benzoylacetone nitrile (0.725 g, 5 mmol) was dissolved in 5.0 mL toluene. 0.5 g *BmGDH-DhCR* dry cells and 1.50 *equiv.* glucose were added into 5.0 mL PBS buffer (100 mM, pH 6.50). The reaction was started by adding the substrate solution into the PBS buffer and carried out at 25 °C until completion. The pH was controlled at 6.5 by titrating 2.0 M NaOH. The reaction mixture was saturated with NaCl, extracted three times with ethyl acetate and centrifuged to promote phase separation. The combined organic layers were dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporated under vacuum to afford the optically pure (*R*)-3-hydroxy-3-phenylpropanenitrile.

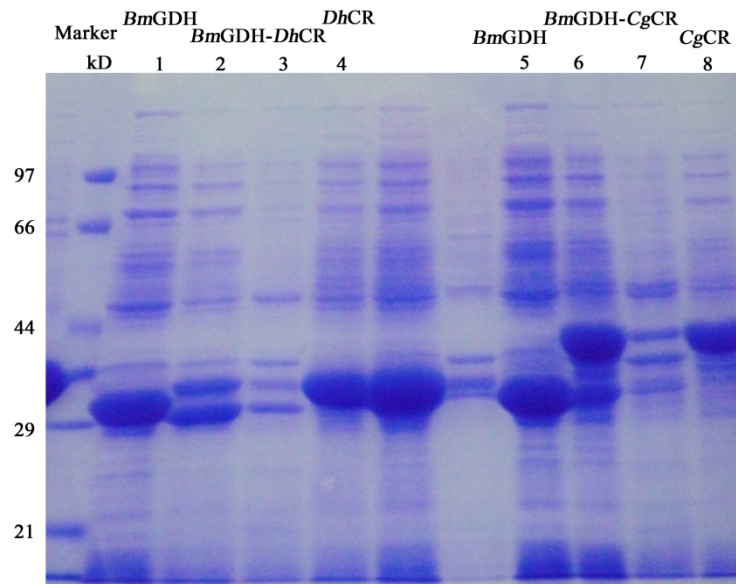
Enantioselectivity was determined by GC equipped with CP-Chirasil-Dex CB capillary column (25 m  $\times$  0.25 mm  $\times$  0.39 mm, Varian). Injector, detector and column temperatures were set at 280 °C, 280 °C and 180 °C, respectively. Retention times:  $R_T$  (benzoylacetone nitrile) = 4.90 min,  $R_T$  ((*R*)-3-hydroxy-3-phenylpropanenitrile) = 7.73 min,  $R_T$  ((*S*)-3-hydroxy-3-phenylpropanenitrile) = 7.97 min. Yield 89%; >99% *ee*;  $[\alpha]_D^{25}$ : +68.9 (*c* 1.00, CHCl<sub>3</sub>) {lit.<sup>3,4</sup>  $[\alpha]_D^{25}$ : +70 (*c* 0.5, CHCl<sub>3</sub>)}; Mass (EI): 147, 121, 107, 105, 91, 79, 77; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400 MHz):  $\delta$  = 2.72 (d, *J* = 6.2 Hz, 2H), 3.41 (s, 1H), 4.99 (t, *J* = 6.2 HZ, 1H), 7.33–7.44 (m, 5H).

**Table S1** Primers used in this study.

| entry        |         | primer sequences                        |
|--------------|---------|---|
| <i>DhCR</i>  | Forward | cta <u>GCTAGCAT</u> GTGCACACCCAACATCAGT |
|              | Reverse | cgc <u>GGATCC</u> TATGGGGCGGAGTAACCA    |
| <i>CgCR</i>  | Forward | ggaattc <u>CATATG</u> CCCAAGACTATCGCCA  |
|              | Reverse | cgc <u>GGATCC</u> TCAGATGCTGCTGTACCC    |
| <i>BmGDH</i> | Forward | ggaattc <u>CATATG</u> TATAAAGATTTAGAAGG |
|              | Reverse | cgc <u>GGATCC</u> TTATCCGCGTCCTGCTTGAAT |



Scheme S1. Construction of co-expression plasmids.



**Figure S1.** SDS-PAGE of co-expressed carbonyl reductase and glucose dehydrogenase. Lane 1: Supernatant of *BmGDH*; Lane 2: Supernatant of *BmGDH-DhCR*; Lane 3: Precipitant of *BmGDH-DhCR*; Lane 4: Supernatant of *DhCR*; Lane 5: Supernatant of *BmGDH*; Lane 6: Supernatant of *BmGDH-CgCR*; Lane 7: Precipitant of *BmGDH-CgCR*; Lane 8: Supernatant of *CgCR*.

**Table S2.** Asymmetric reduction of benzoylacetone nitrile with the top six reductases in the tool-box

| entry | catalyst <sup>a)</sup> | Specific activity<br>[nmol·min <sup>-1</sup> ·mg <sup>-1</sup> ] <sup>b)</sup> | conv<br>[%] <sup>c)</sup> | % <i>ee</i> <sup>c)</sup><br>/config. |
|-------|------------------------|--|---------------------------|---------------------------------------|
| 1     | <i>CICR</i>            | 235  | 92.5                      | 91.3 ( <i>R</i> )                     |
| 2     | <i>KtCR</i>            | 82   | 20.2                      | >99 ( <i>R</i> )                      |
| 3     | <i>DhCR</i>            | 60   | >99                       | >99 ( <i>R</i> )                      |
| 4     | <i>PgCR</i>            | 45   | 17.4                      | 97.6 ( <i>R</i> )                     |
| 5     | <i>CgCR</i>            | 186  | >99                       | >99 ( <i>S</i> )                      |
| 6     | <i>KICR</i>            | 40   | 13.4                      | 89.5 ( <i>S</i> )                     |

<sup>a)</sup> *CgCR* (GenBank accession No. CAG60239, *Candida glabrata*), *KICR* (GenBank accession No. CAH02579, *Kluyveromyces lactis*), *CICR* (GenBank accession No. EEQ39616, *Clavispora lusitanae*), *KtCR* (GenBank accession No. CAR23611, *Kluyveromyces thermotolerans*), *PgCR* (GenBank accession No. EDK41368, *Pichia guilliermondii*) and *DhCR* (GenBank accession No. CAG87931, *Debaryomyces hansenii*). <sup>b)</sup> Specific activity was spectrophotometrically measured with 1 mM benzoylacetone nitrile, 0.1 mM NADPH and proper amount of purified enzyme at 30 °C. <sup>c)</sup> Reactions were carried out with 10 mM benzoylacetone nitrile, the same amount of enzymes (3 kU/L) coupled with glucose dehydrogenase in 0.5 mL PBS (pH 6.5, 100 mM) at 30 °C, 900 rpm for 12 h. The conversion and *ee* value were determined by GC analysis.

### Partition of benzoylacetoneitrile in a water/organic solvent biphasic system

10  $\mu\text{L}$  of 0.5 M benzoylacetoneitrile (dissolved in DMSO) was added to 390  $\mu\text{L}$  100 mM PBS buffer (pH 6.5), and shaken with each tested organic solvent (400  $\mu\text{L}$ ) at 30 °C, 900 rpm for 30 min in a closed vessel. Then the mixture was centrifuged at 12000 rpm for 5 min. 100  $\mu\text{L}$  of aqueous phase was carefully transferred and extracted with equal volume of ethyl acetate containing dodecane as internal standard for three times. The organic solution was combined, dried over with anhydrous  $\text{Na}_2\text{SO}_4$ . The concentration of benzoylacetoneitrile in aqueous phase was determined by GC analysis. The amount of benzoylacetoneitrile in organic phase was calculated according to the aqueous phase.

### Study of the enzyme stability

10 U/mL *DhCR*, *CgCR* or *BmGDH* in 100 mM PBS buffer (pH 6.5) was combined with an equal volume of each tested organic solvent in a closed vessel. After the mixture was shaken for 24 h at 30°C, 900 rpm, the remaining enzyme activity in the aqueous phase was assayed according to standard method, <sup>[1]</sup> as shown in Table S3.

**Table S3.** Partition coefficients of organic solvents for benzoylacetoneitrile and stability of *DhCR*, *CgCR* and *BmGDH* in various water/organic solvent biphasic systems.

| Entry | Organic solvent    | Log <i>P</i><br><sup>a)</sup> | Partition<br>coefficient <sup>b)</sup> | Residual activity /% <sup>c)</sup> |             |              |
|-------|--------------------|-------------------------------|--|------------------------------------|-------------|--------------|
|       |                    |                               |  | <i>DhCR</i>                        | <i>CgCR</i> | <i>BmGDH</i> |
| 1     | Control            | –                             | –                                      | 41.1                               | 74.7        | 50.8         |
| 2     | Ethyl butyrate     | 1.7                           | 66.8 $\pm$ 1.5                         | 90.4                               | 78.1        | 101.6        |
| 3     | Butyl acetate      | 1.8                           | 72.5 $\pm$ 2.2                         | 82.9                               | 63.9        | 76.0         |
| 4     | Toluene            | 2.5                           | 34.0 $\pm$ 1.2                         | 74.6                               | 82.4        | 104.9        |
| 5     | Ethyl hexanoate    | 2.8                           | 50.0 $\pm$ 1.3                         | 84.9                               | 79.8        | 88.1         |
| 6     | Cyclohexane        | 3.2                           | 0.1 $\pm$ 0.1                          | 80.1                               | 72.5        | 112.4        |
| 7     | <i>n</i> -Hexane   | 3.5                           | 0.1 $\pm$ 0.1                          | 81.5                               | 68.2        | 105.8        |
| 8     | Octyl acetate      | 3.9                           | 21.2 $\pm$ 2.0                         | 72.6                               | 91.4        | 109.4        |
| 9     | <i>n</i> -Heptane  | 4.0                           | 0.2 $\pm$ 0.1                          | 80.8                               | 82.4        | 113.6        |
| 10    | <i>iso</i> -Octane | 4.5                           | 0 $\pm$ 0.1                            | 62.3                               | 81.5        | 110.3        |
| 11    | Dibutylphthalate   | 4.8                           | 66.9 $\pm$ 0.5                         | 1.4                                | 0.8         | 5.4          |

<sup>a)</sup> Shimizu et al. <sup>[2]</sup> <sup>b)</sup> Partition coefficient is given as the molar ratio of each compound found in the organic phase to that in the aqueous phase. <sup>c)</sup> 0.1 M PBS (pH 6.5) containing 10 U/mL *DhCR*, *CgCR* or *BmGDH* was combined with an equal volume of each tested organic solvent in a closed vessel. The mixture was shaken at 30 °C for 24 h, and the remaining enzyme activities in the aqueous solution were assayed.

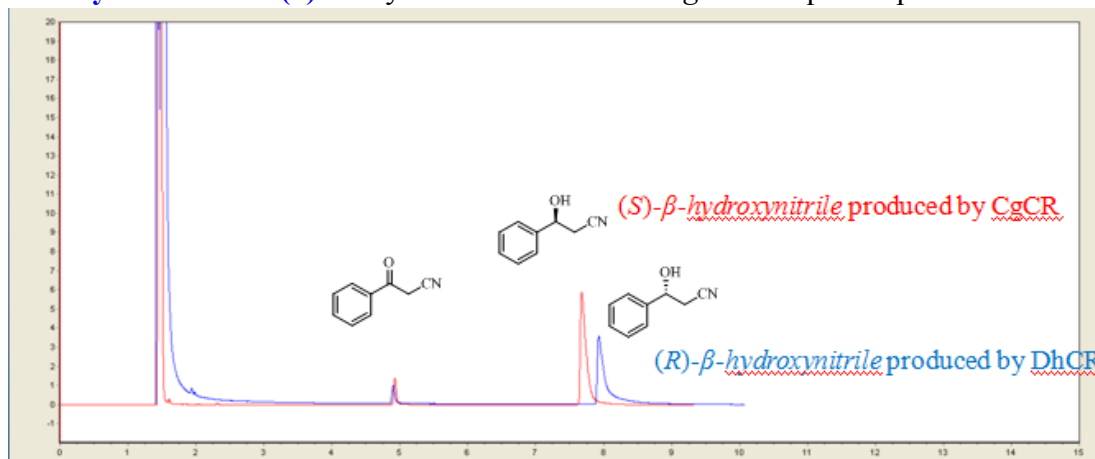
## GC spectra

Enantioselectivity was determined by GC equipped with CP-chirasil-Dex CB Capillary Column (25 m × 0.25 mm × 0.39 mm, Varian). Injector, detector and column temperatures were set at 280 °C, 280 °C and 180 °C respectively. The retention times of substrate and product are listed in **Table S4**.

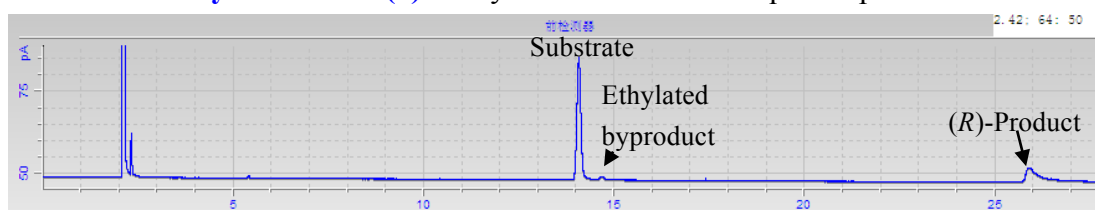
**Table S4.** Retention times ( $R_T$ ) of substrates and products.

| Substrates | $R_T$ (sub.)<br>/min | $R_T$ (byproduct)<br>/min | $R_T$ (( <i>R</i> )-prod.)<br>/min | $R_T$ (( <i>S</i> )-prod.)<br>/min |
|------------|----------------------|---------------------------|------------------------------------|------------------------------------|
| <b>1</b>   | 4.90                 | –                         | 7.97                               | 7.73                               |
| <b>2</b>   | 14.14                | 14.75                     | 25.24                              | 25.95                              |
| <b>3</b>   | 12.15                | 14.82                     | 31.75                              | 30.15                              |
| <b>4</b>   | 13.24                | –                         | 22.30                              | 20.72                              |
| <b>5</b>   | 12.75                | 10.69                     | 17.38                              | 16.50                              |

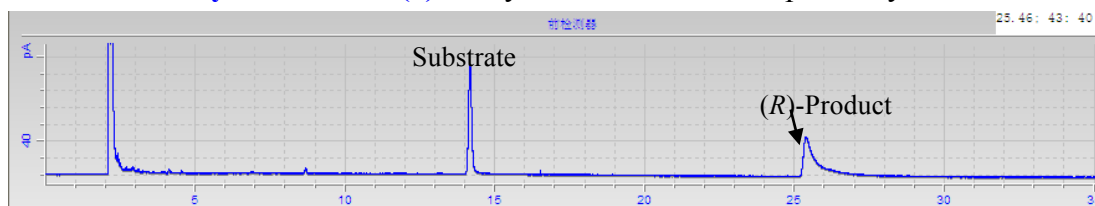
**Benzoylacetoneitrile (1)** catalyzed with *Dh*CR and *Cg*CR in aqueous phase.



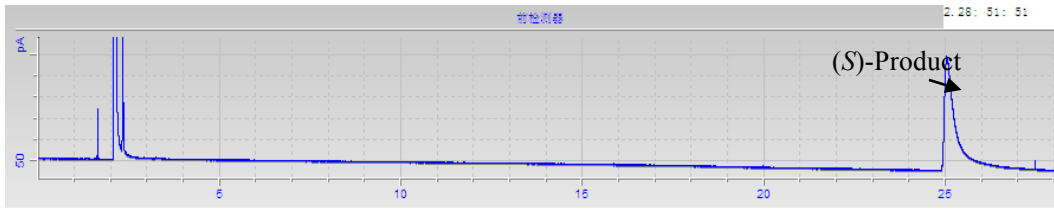
**3-Chlorobenzoylacetoneitrile (2)** catalyzed with *Dh*CR in aqueous phase.



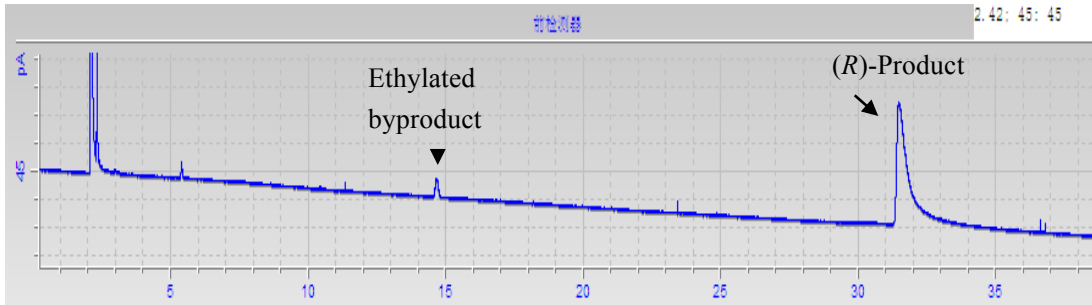
**3-Chlorobenzoylacetoneitrile (2)** catalyzed with *Dh*CR in biphasic system.



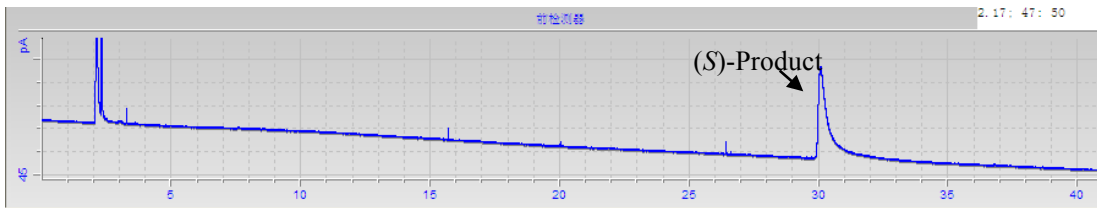
**3-Chlorobenzoylacetoneitrile (2)** catalyzed with *Cg*CR in aqueous phase.



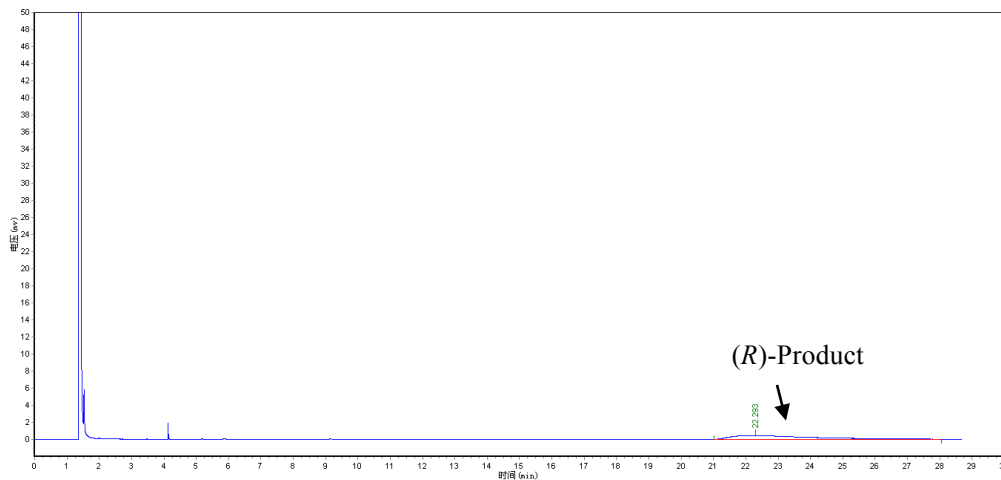
**3-Methoxybenzoylacetonitrile (3)** catalyzed with *DhCR* in aqueous phase.



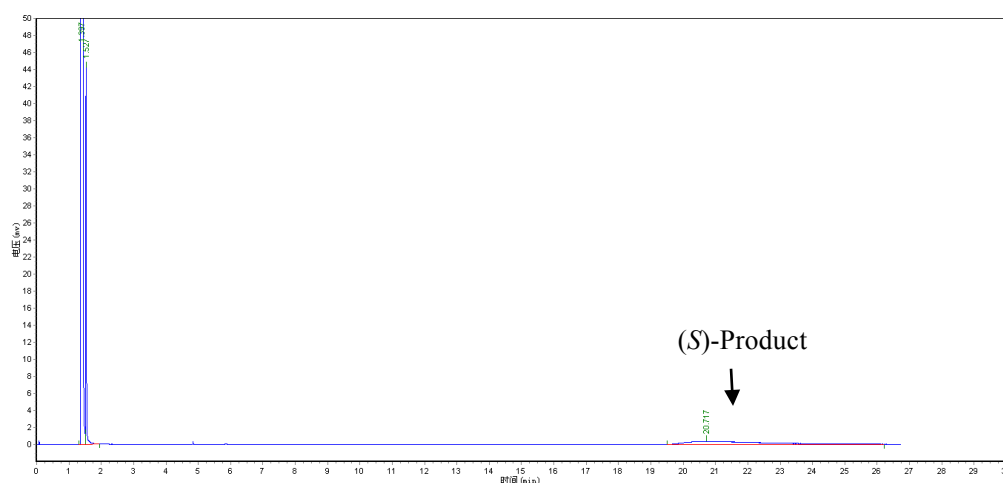
**3-Methoxybenzoylacetonitrile (3)** catalyzed with *CgCR* in aqueous phase



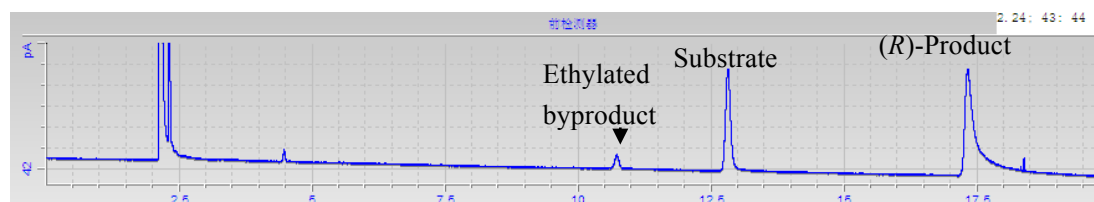
**4-Chlorobenzoylactonitrile (4)** catalyzed with *DhCR* in aqueous phase.



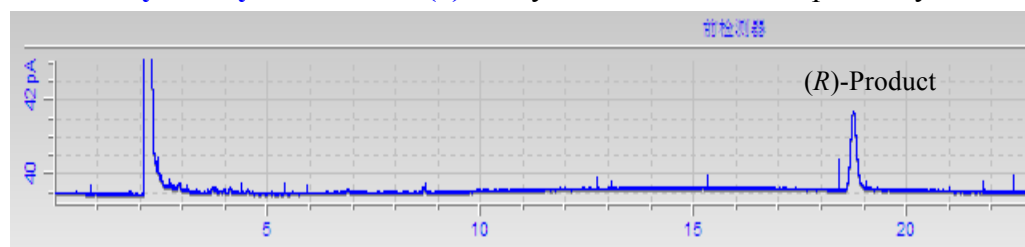
**4-Chlorobenzoylacetonitrile (4)** catalyzed with *CgCR* in aqueous phase.



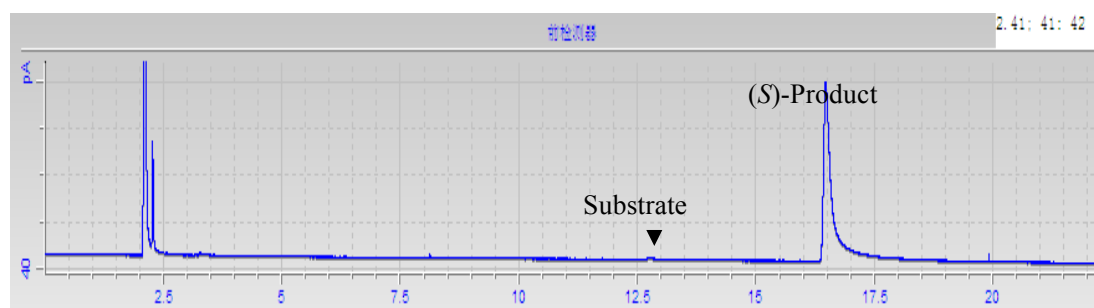
**4-Methoxybenzoylacetonitrile (5)** catalyzed with *DhCR* in aqueous phase.



**4-Methoxybenzoylacetonitrile (5)** catalyzed with *DhCR* in biphasic system.

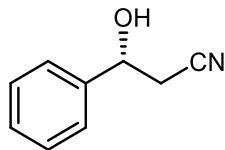


**4-Methoxybenzoylacetonitrile (5)** catalyzed with *CgCR* in aqueous phase.

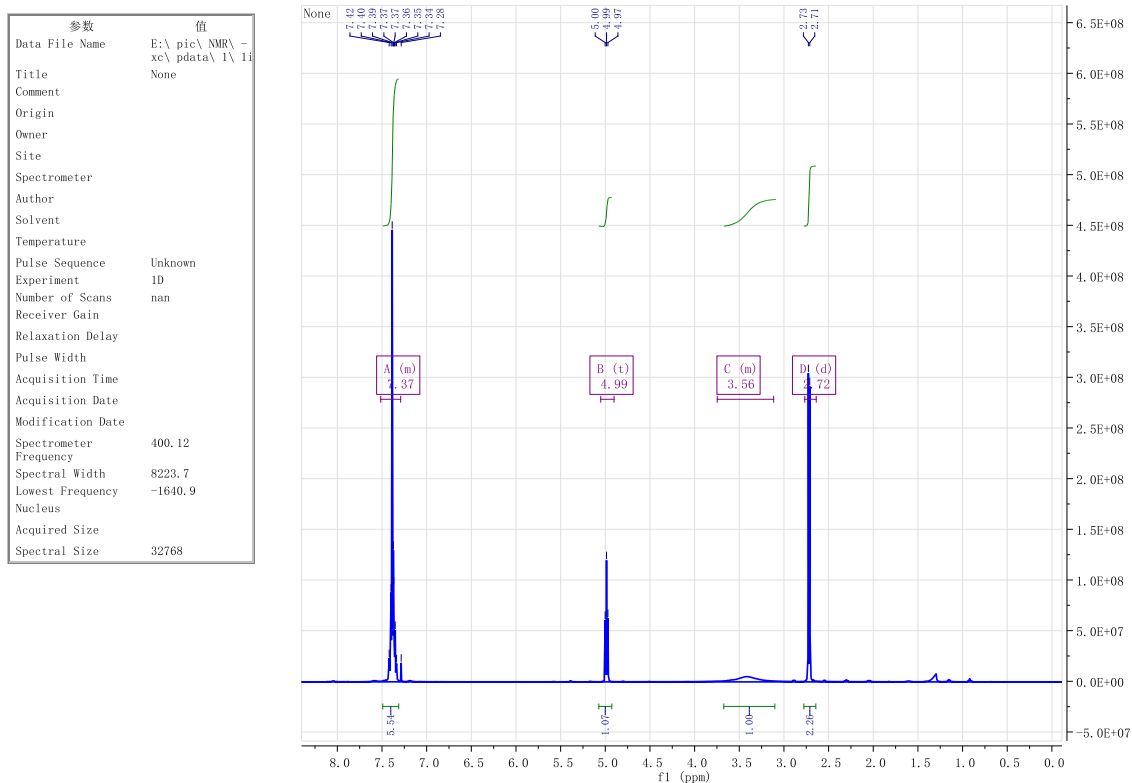


## Mass and $^1\text{H}$ NMR data

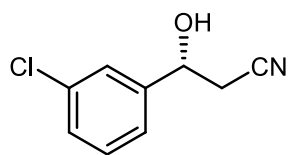
### (*R*)-3-Hydroxy-3-phenylpropanenitrile (product of substrate 1)



$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  = 2.72 (d,  $J$  = 6.2 Hz, 2H), 4.99 (t,  $J$  = 6.2 Hz, 1H), 7.33–7.44 (m, 5H); Mass (EI): 147, 121, 107, 105, 91, 79, and 77.

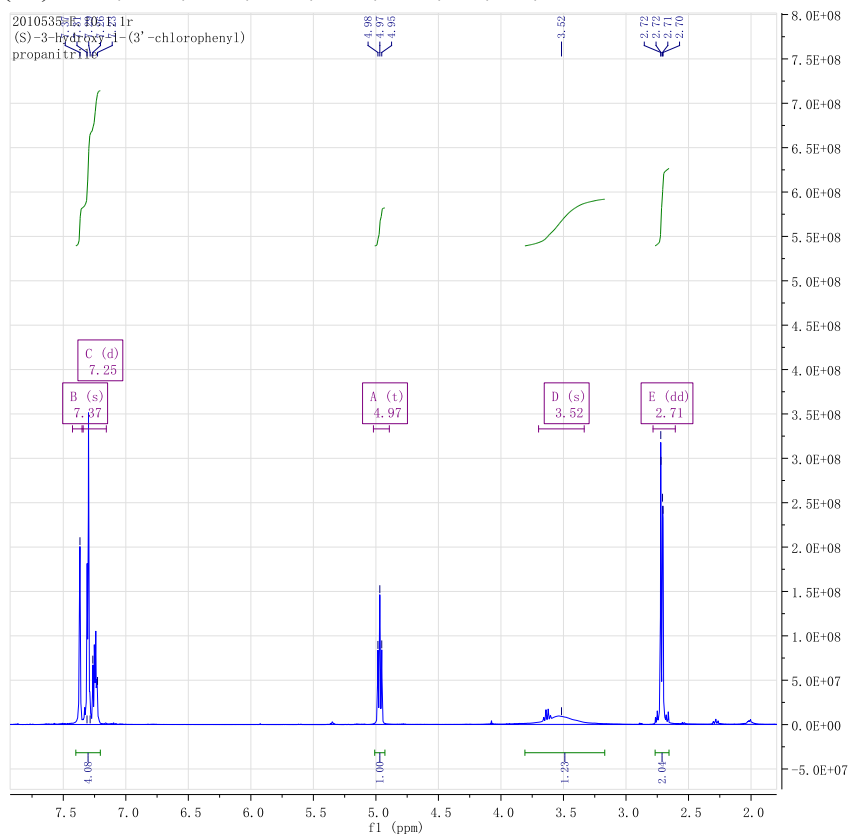


**(R)-3-Hydroxy-3-(3'-chlorophenyl)propanenitrile (product of substrate 2)**

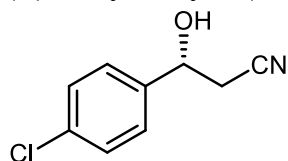


$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta = 2.71$  (d,  $J = 6.2$  Hz, 2H), 4.97 (t,  $J = 6.1$  Hz, 1H), 7.25–7.37 (m, 4H); Mass (EI): 181, 163, 143, 141, 115, 113, 91, 77, and 75.

| 参数                | 值   |
|-------------------|---|
| Data File Name    | E:/ e/ Datas/ NMR/ 20130424/ -E/ pdata/ 1/ 1r   |
| Title             | 2010535-E. 10. 1. 1r                            |
| Comment           | (S)-3-hydroxy-1-(3'-chlorophenyl)propanenitrile |
| Origin            | Bruker BioSpin GmbH                             |
| Owner             | nmr   |
| Site              |   |
| Spectrometer      | spect   |
| Author            |   |
| Solvent           | $\text{CDCl}_3$                                 |
| Temperature       | 294. 9  |
| Pulse Sequence    | zg30  |
| Experiment        | 1D  |
| Probe             | 5 mm PADUL 13C-1H/ D Z-GRD Z108621/ 0008        |
| Number of Scans   | 16  |
| Receiver Gain     | 40  |
| Relaxation Delay  | 1. 0000   |
| Pulse Width       | 9. 3000   |
| Acquisition Time  | 3. 9846   |
| Acquisition Date  | 2013-04-24T12:58:00                             |
| Modification Date | 2013-04-24T12:58:00                             |
| Class             |   |
| Spectrometer      | 400. 12   |
| Frequency         |   |
| Spectral Width    | 8223. 7   |
| Lowest Frequency  | -1649. 4  |
| Nucleus           | $^1\text{H}$                                    |
| Acquired Size     | 32768   |
| Spectral Size     | 32768   |

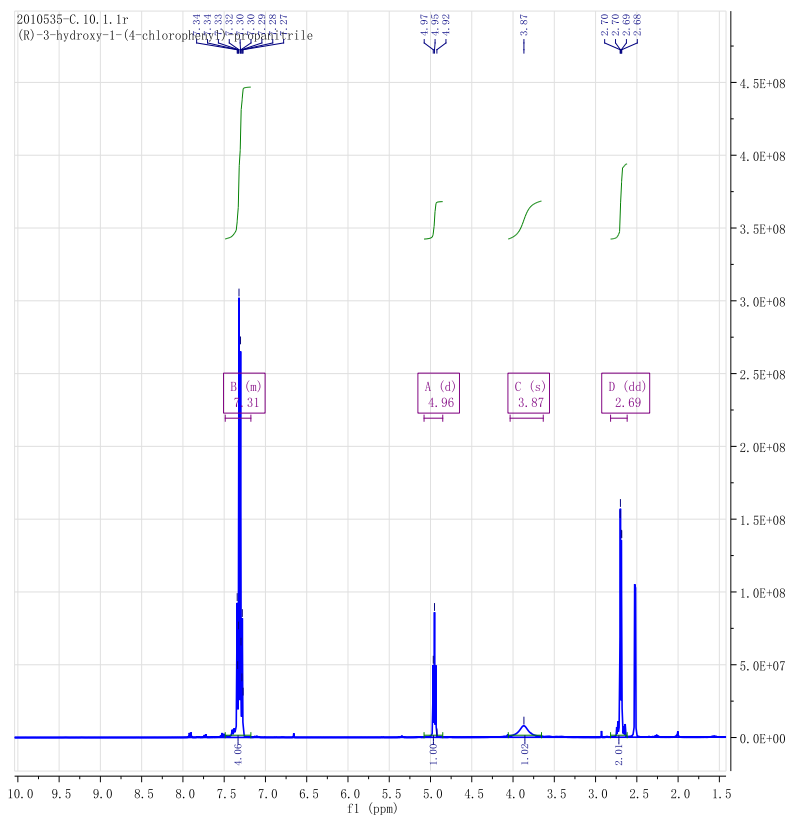


**(R)-3-Hydroxy-3-(4'-chlorophenyl)-propanenitrile (product of substrate 4)**

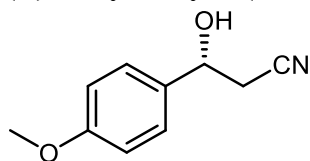


$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  = 2.69 (d,  $J$  = 6.1 Hz, 2H), 4.96 (t,  $J$  = 6.1 Hz, 1H), 7.18–7.49 (m, 4H); Mass (EI): 181, 163, 143, 141, 115, 113, 91, 77, and 75.

| 参数                | 值   |
|-------------------|---|
| Data File Name    | E:/ e/ Datas/ MMR/ 20130424/ -C/ pdata/ 1/ 1r |
| Title             | 2010535-C.10.1.1r                             |
| Comment           | (R)-3-hydroxy-1-(4-chlorophenyl)-propanitrile |
| Origin            | Bruker BioSpin GmbH                           |
| Owner             | nmr   |
| Site              |   |
| Spectrometer      | spect   |
| Author            |   |
| Solvent           | CDCl3   |
| Temperature       | 295.1   |
| Pulse Sequence    | zg30  |
| Experiment        | 1D  |
| Probe             | 5 mm PADUL 13C-1H/ D Z-GRD Z108621/ 0008      |
| Number of Scans   | 16  |
| Receiver Gain     | 36  |
| Relaxation Delay  | 1.0000  |
| Pulse Width       | 9.3000  |
| Acquisition Time  | 3.9846  |
| Acquisition Date  | 2013-04-24T12:46:00                           |
| Modification Date | 2013-04-24T12:46:00                           |
| Class             |   |
| Spectrometer      | 400.12  |
| Frequency         |   |
| Spectral Width    | 8223.7  |
| Lowest Frequency  | -1646.6                                       |
| Nucleus           | 1H  |
| Acquired Size     | 32768   |
| Spectral Size     | 32768   |

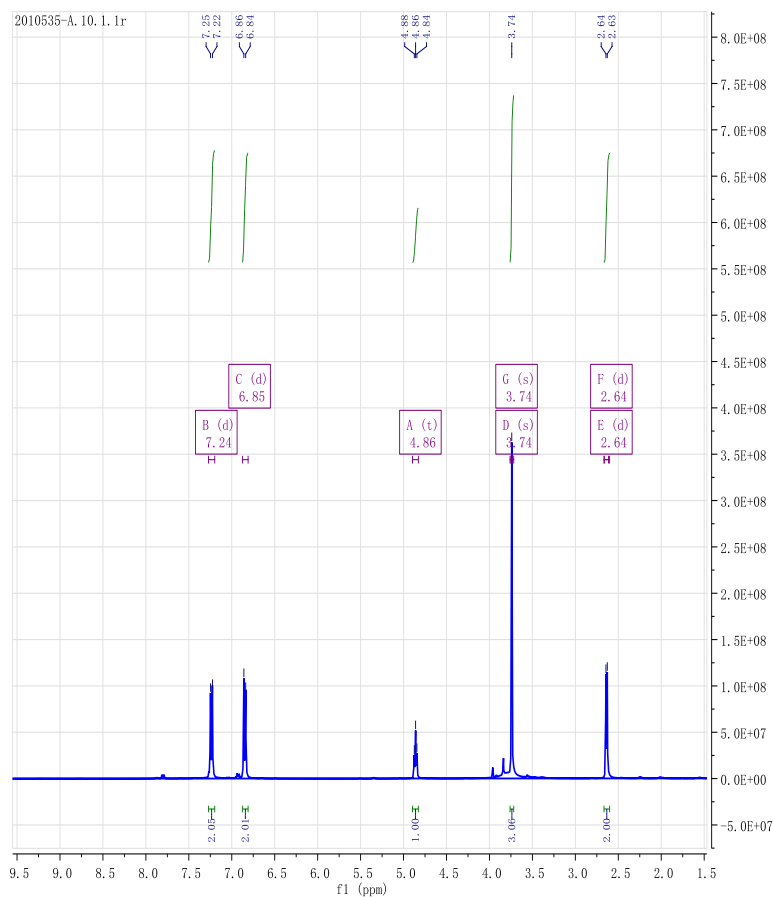


**(R)-3-Hydroxy-3-(4'-methoxyphenyl)-propanenitrile (product of substrate 5)**



$^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400 MHz):  $\delta = 2.64$  (d,  $J = 6.2$  Hz, 2H), 3.874 (m, 3H), 4.86 (t,  $J = 6.1$  Hz, 1H), 6.86 (d,  $J = 8.5$  Hz, 2H), 7.25 (d,  $J = 8.8$  Hz, 2H); Mass (EI): 177, 159, 138, 137, 109, 94, 78, and 77.

| 参数                | 值   |
|-------------------|---|
| Data File Name    | E:/ e/ Datas/ NMR/ 20130424/ -A/ pdata/ 1/ 1r |
| Title             | 2010535-A.10.1.1r                             |
| Comment           |   |
| Origin            | Bruker BioSpin GmbH                           |
| Owner             | nmr   |
| Site              |   |
| Spectrometer      | spect   |
| Author            |   |
| Solvent           | $\text{CDCl}_3$                               |
| Temperature       | 294.9   |
| Pulse Sequence    | zg30  |
| Experiment        | 1D  |
| Probe             | 5 mm PADUL 13C-1H/ D 2-GRD Z108621/ 0008      |
| Number of Scans   | 16  |
| Receiver Gain     | 20  |
| Relaxation Delay  | 1.0000  |
| Pulse Width       | 9.3000  |
| Acquisition Time  | 3.9846  |
| Acquisition Date  | 2013-04-24T12:27:00                           |
| Modification Date | 2013-04-24T12:27:00                           |
| Class             |   |
| Spectrometer      | 400.12  |
| Frequency         |   |
| Spectral Width    | 8223.7  |
| Lowest Frequency  | -1648.5                                       |
| Nucleus           | $^1\text{H}$                                  |
| Acquired Size     | 32768   |
| Spectral Size     | 32768   |



## References

1. Y. Ni, C. X. Li, L. J. Wang, J. Zhang, J. H. Xu, *Org. Biomol. Chem.* **2011**, *9*, 5463–5468.
2. Y. Ni, C. X. Li, H. M. Ma, J. Zhang, J. H. Xu, *Appl. Microbiol. Biotechnol.* **2011**, *89*, 1111–1118.
3. D. M. Hodgson, S. Man, *Chemistry* **2011**, *35*, 9131–9137.
4. T. Touge, T. Hakamata, H. Nara, T. Kobayashi, N. Sayo, T. Saito, Y. Kayaki, T. Ikariya, *J. Am. Chem. Soc.* **2011**, *38*, 14960–14963.
5. S. Shimizu, M. Kataoka, M. Katoh, T. Morikawa, T. Miyoshi, H. Yamada, *Appl. Environ. Microbiol.* **1990**, *56*, 2374–2377.