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Enantioselective biotransformation of α,α-disubstituted dinitriles to the corresponding 2-cyanoacetamides using *Rhodococcus* sp. CGMCC 0497

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Abstract—A new application of nitrile-converting enzymes in the synthesis of optically active α,α-disubstituted-α-cyanoacetamides from α,α-disubstituted-malononitriles with whole cells of *Rhodococcus* sp. CGMCC 0497 is described. The products were obtained with enantiomeric excesses of up to >99%, and yields of up to 53%. They are very useful chiral intermediates especially for the synthesis of chiral α,α-disubstituted amino acids but have never been synthesized directly by chemical or enzymatic methods.
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1. Introduction

The enantioselective biotransformation of nitriles has attracted increasing interest in recent years due to the easy availability of nitrile compounds and the versatile utility of the optically active products, amides and acids.[1] So far, the kinetic resolutions of several classes of racemic nitriles using nitrile-converting enzymes have been studied in detail, such as α-alkyl nitriles,[2] α-hydroxy nitriles,[3] α-acyloxy nitriles,[4] α-amino nitriles,[5] and β-acetoxy nitriles.[6] There are also several reports on the desymmetrization of 3-substituted glutaronitriles.[3b,7] However, the hydrolysis of prochiral malononitriles has been somewhat neglected except that Sugai and Ohta et al. have reported the successful hydrolysis of α-butyl-α-methylmalononitrile to α-butyl-α-methylmalonamic acid.[8]

Our previous study has demonstrated that *Rhodococcus* sp. CGMCC 0497, a strain isolated from soil by our group,[9] was able to transform a variety of α-substituted arylacetonitriles into optically active α-substituted arylacetamides and α-substituted arylacetic acids with excellent enantiomeric excesses,[10] and β-hydroxy nitriles into β-hydroxy amides and β-hydroxy acids with high activity and moderate enantioselectivity.[11] Quite recently, we have focused our attention on the asymmetric hydrolysis of prochiral α,α-disubstituted dinitriles, which, after asymmetric desymmetrization, could afford important precursors of many physiologically active compounds and chiral building blocks, such as β-lactams[12] and substituted succinic acids,[13] especially enantiopure α,α-dialkylated α-amino acids. This group of amino acids induces dramatic conformational changes when incorporated into peptides. They can act as enzyme inhibitors and also as chiral building blocks for the synthesis of pharmaceuticals and other biological agents.[14,15]

We have made some progress in the production of optically active malonamic acids by hydrolysis of α,α-disubstituted dinitriles using *Rhodococcus* sp. CGMCC 0497,[16] but the reaction time is very long, which prompted us to try to find an alternative way. Clearly the most important thing in the reaction is to enantioselectively distinguish the two cyano groups by enzymes, no matter that the products are cyanoamides, cyanoacids or amideacids, because all can be converted to amino acids. If cyanoamides or cyanoacids with high enantiomeric excesses could be achieved instead of amideacids, the reaction may be terminated earlier and be more practical. Herein we report in detail the hydrolysis of α-benzyl-α-methylmalononitrile **1a** and the synthesis of a variety of (*S*)-α,α-disubstituted-α-cyanoacetamides via asymmetric hydrolysis of α,α-disubstituted malononitriles catalyzed by the strain

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Rhodococcus sp. CGMCC 0497. The products were achieved with enantiomeric excesses of up to >99%.

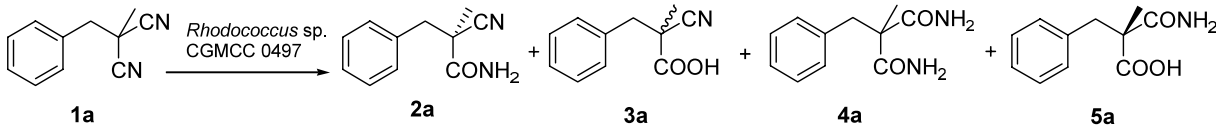
2. Results and discussion

In order to gain reproducible results, the fresh microorganism cultivated under optimal conditions was used. The enantiomeric excesses of cyanoamides **2** were determined directly or after conversion to the corresponding ethyl esters by HPLC with chiral stationary phase. The enantiomeric excesses of cyanoacids **3** and amideacids **5** were determined by means of HPLC with chiral stationary phase after conversion to the corresponding methyl or ethyl esters. The absolute configurations of **3a**[17]and **5a**[13] were obtained by comparing the direction of specific rotations with those in literature, and that of **2a** was assigned by comparing the retention time of the corresponding ethyl ester with the samples derived from **3a** using HPLC with chiral stationary phase. The absolute configurations of other cyanoamide compounds **2** were assumed by the analogy with known enzyme-catalyzed reaction. The cyanoacids **3** were converted to the corresponding cyanoamides and their retention time on chiral HPLC or direction of specific rotations were compared with **2** to determine the absolute configurations.

2.1. Hydrolysis of α-benzyl-α-methylmalononitrile

To investigate the possibility of transforming dinitriles to chiral cyanoamides or cyanoacids by *Rhodococcus* sp. CGMCC 0497, the hydrolysis of α-benzyl-α-methylmalononitrile **1a** at different temperatures and pH was studied in detail (Scheme 1). As shown in Table 1, in all cases, the substrate was hydrolyzed completely, but the products isolated were a complex mixture of hydrolysis intermediates in most cases. The absolute configuration and enantiomeric excess of **3a** seemed less regular, while the absolute configuration of **2a** was consistently *S* and its enantiomeric excess evidently increased with prolonged reaction time (entries 3–6). The cyanoamide (*S*)-**2a** could be obtained with an excellent enantiomeric excess of >99% in a yield of 52% within 72 h (entry 5). Considering the easy removal of acids **3a** by basification, the isolation of (*S*)-**2a** can be achieved conveniently.

Though it is well known that biotransformations of nitriles to the corresponding acids proceed by two distinct routes: by nitrilase or by a combination of nitrile hydratase and amidase,[1] the detailed information on the pathway of the hydrolysis of **1a** to the four products **2a**, **3a**, **4a** and **5a** remains still largely unexplored. Our early work has demonstrated that product **5a** was mostly derived from **4a** by (*R*)-selective amidase, rather than from **3a** by nitrile hydratase.[16] To shed more light on the multi-step biotransformation procedure, (*rac*)-2-cyano-2-methyl-3-phenylpropanamide **2a** was synthesized and subjected to the reaction catalyzed by *Rhodococcus* sp. CGMCC 0497. The results were illustrated in Scheme 2. It is clear that cyanoacid **3a** came from **2a** by an (*R*)-selective amidase. Calculation according to the yields and enantiomeric excesses of **2a** and **3a**, as well as the rules of kinetic resolution, demonstrated that more (*S*)-**2a** converted to **4a** and **5a** than (*R*)-**2a** did. Thus, it can be inferred that diamide **4a** came from **2a** by an (*S*)-selective nitrile hydratase and the total effect of the two steps, **2a** to **3a** and **2a** to **4a**, was (*R*)-selective, because the recovered **2a** was of (*S*)-configuration. That is consistent with the results obtained in Table 1, entries 3–5. The enantiomeric excess of (*S*)-**2a** increased as the reaction continued, for (*R*)-**2a** was preferred in the following steps and the (*S*)-**2a** was retained. Prolonging the reaction time to 90 h, the enantiomeric excess of

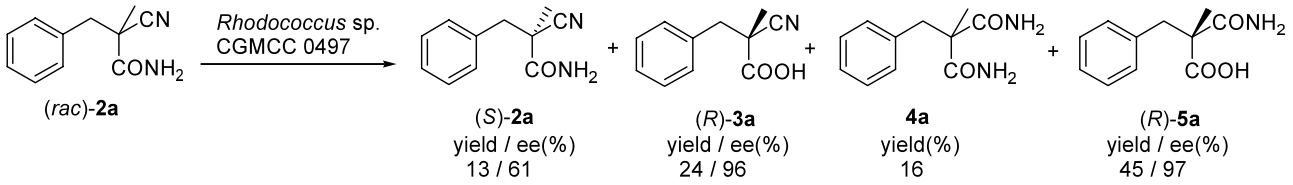


Scheme 1.

Table 1. Hydrolysis of α-benzyl-α-methyl malononitrile **1a** by *Rhodococcus* sp. CGMCC

Entry	pH	T (°C)	Time (h)	(*S*)-**2a**		**3a**		**4a**
				Yield (%)	Ee (%)	Yield (%)	Ee (%)	Yield (%)
1	7.0	10	24	66	45	9	16 *S*	17
2[a]	7.0	20	24	21	46	12	78 *R*	52
3	7.0	30	24	70	48	19	72 *R*	8
4	7.0	30	66	56	93	33	52 *R*	8
5	7.0	30	72	52	>99	35	49 *R*	7
6[b]	7.0	30	90	22	>99	35	6 *R*	–
7	8.0	30	72	49	87	46	40 *R*	–
8	8.9	30	72	45	81	50	30 *R*	–

[a] **5a** obtained in 13% yield.
[b] (*R*)-**5a** obtained in 41% yield and 88% ee.



Scheme 2.

(*S*)-**2a** remains >99%, while the yield decreased (Table 1, entry 6). In this situation, (*R*)-**2a** has been exhausted and (*S*)-**2a** was consequently converted to **4a** and **4a** to **5a**, or to **3a** to make the enantiomeric excess of (*S*)-**3a** decrease dramatically. We can further deduced from the >50% yield and >99% enantiomeric excess of (*S*)-**2a** (Table 1, entry 5) that the process of **1a** to **2a** was catalyzed by an (*S*)-selective nitrile hydratase. The enantiomeric excess of **2a** was thus enhanced by the processes that produce it (**1a** to **2a**) and consume it (**2a** to **3a** and **4a**), which exhibit the selectivity of the opposite direction.

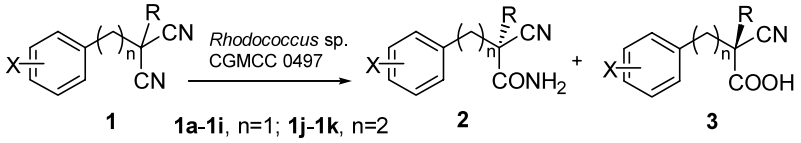
2.2. Hydrolysis of various α,α-disubstituted malononitriles

Based on the above results, a variety of α,α-disubstituted malononitriles **1** were subjected to the enzymatic transformation. The reactions were monitored by TLC to produce chiral cyanoamides **2** with both good yield and ee (Scheme 3).

To our disappointment, the reactions were generally unsuccessful with the conversion of substrates incomplete and the enantiomeric excesses of products low to medium. During the reaction time of 75–120 h, compounds **2** and **3** were obtained in most cases, while the formations of **4** and **5** were not observed as that in the hydrolysis of **1a**.

Substrates **1b** to **1h** are most similar to **1a** in structure with a methyl and a benzyl group at the α position of the malononitrile, but the benzyl is substituted by groups such as methyl-, methoxy-, chloro-, bromo-, etc. These substitutions especially relatively large *p*-methyl, *p*-methoxy and *o*-chloro groups, seem to exert distinct influences on the reaction. As show in Table 1, **1a** could be converted to amides and acids completely within 24 h, while **1b**–**h** could not be converted even after 75 h (Table 2, entries 1–9); hydrolysis of **1a** could afford (*S*)-**2a** successfully in 52% yield and >99% ee, while hydrolysis of **1b**–**h** only afforded **2b**–**h** in yields of 20–42% and ee of 2–81%.

It seemed that the substitutions increased steric hindrance, which made the conversion of dinitriles **1** to cyanoamides **2** much slower. The slowness of the formation of compound **2** led to the hydrolysis of (*S*)-**2**, which had been expected to remain, as well as (*R*)-**2** to cyanoacid **3**. At the same time, hydration of the remaining dinitrile **1** to afford **2** was continuing with low to medium enantioselectivity, which led to the low ee value of product **2**. On the contrary, α-benzyl-α-methyl malononitrile **1a** was converted completely to



Scheme 3.

Table 2. Hydrolysis of various α,α-disubstituted malononitriles **1** by *Rhodococcus* sp. CGMCC at 30°C

Entry	Substrate **1**	X	R	Time (h)	Recovered **1** (%)	(*S*)-**2**		**3**	
						Yield (%)	Ee (%)	Yield (%)	Ee (%)
1	**1b**	*p*-Me	CH_3	75	24	30	64	30	14 *S*
3	**1c**	*p*-F	CH_3	75	10	42	78	40	16 *R*
4	**1d**	*p*-Cl	CH_3	75	11	39	42	39	ND *S*
5	**1e**	*p*-Br	CH_3	75	14	39	78	17	ND[a]
6	**1f**	*p*-MeO	CH_3	75	33	20	2	33	30 *S*
8	**1g**	*m*-Cl	CH_3	75	16	35	81	41	ND *R*
9	**1h**	*o*-Cl	CH_3	75	34	26	44	29	ND[a]
11	**1i**	H	CH_2CH_3	90	–	92	65	–	–
12	**1j**	H	CH_2CH_3	72	44	37	64	–	–
13	**1k**	H	Allyl	120	46	47	60[a]	–	–

[a] Absolute configuration undetermined.

cyanoamide **2a** first by *Rhodococcus* sp. CGMCC (Table 1, entry 3), and **2a** to **3a** or **4a** in succession. Though the first step was less selective, the enantiomeric excess of **2a** was enhanced by the next steps.

The products cyanoacids **3** were not our concern, but their unexpected absolute configuration caught our attention. Unlike any other situations in our enzymatic hydrolysis of nitriles using *Rhodococcus* sp. CGMCC, which gave products with concordant absolute configuration, the hydrolysis of dinitriles gave cyanoacids **3** with absolute configuration of both *R* and *S*. We speculated that this phenomenon may not be caused by the difference of selectivity of the amidase towards different substrates, for there are both (*R*)- and (*S*)-cyanoacids for the same substrate (Table 1), but caused by the complexity of the multi-step reaction. The amidase is most probably (*R*)-selective consistently. The transformation of (*S*)-**2** following (*R*)-**2** because of the slow conversion of **1** to **2** may result in the formation of (*S*)-cyanoacids **3** as well as the low ee of (*S*)-cyanoamides **2**.

Compounds **1i**–**k** are substrates bearing a benzyl or phenylethyl group and a substitution of ethyl or allyl, larger than methyl group of the substrates **1a**–**h**, on the α position of the malononitriles. It seems that amidase in *Rhodococcus* sp. CGMCC 0497 is very sensitive to the steric hindrance, for no acid was formed in the three reactions. As observed in our early study, it is difficult to hydrolyze this kind of amides with two substituents both larger than methyl by the amidase in *Rhodococcus* sp. CGMCC 0497.[18] Though the hydrolysis of **1i** to **2i** proceeded smoothly and completely with medium enantioselectivity, the ineffectiveness of amidase towards **2i** made the enantiomeric excess of **2i** remain as it formed. Prolonged reaction time took no effect. **1j** and **1k** were recovered nearly half of the feeding with **2j** and **2k** as the sole product.

To improve the yields and enantiomeric excesses of (*S*)-cyanoamides **2**, we carried out reactions at 20°C instead of 30°C. We have observed that lower reaction temperatures favor the conversion of the substrates in the hydrolysis β-hydroxy nitriles by *Rhodococcus* sp. CGMCC 0497.[11] It was expected that the reactions at 20°C may result in the faster transformation of dinitriles **1** to cyanoamides **2**, then more (*S*)-**2** could be retained in the sequent amidase-catalyzed reaction.

Substrates **1b**–**h** were subjected to the hydrolysis reaction at 20°C. The reactions were monitored by TLC. It was observed that in the process of the hydrolysis of **1c**, **1d** and **1f**, there was very little **2** all along and the reaction mixture consisted mainly of cyanoacids **3**, amideacids **5** and unreacted dinitriles **1**. It seemed that on this reaction condition, the conversion of **2** to the diamide **4** accelerated as well as the conversion of **1** to **2**, which made the accumulation of product **2** hard to achieve and the amideacids **5** formed instead.

While under the same condition, the hydrolysis of **1b**, **1f** and **1h**, the relatively bulky substrates, gave products (*S*)-**2** with good to excellent enantiomeric excesses (Table 3). The enantiomeric excess of **2b** increased from 64% (Table 2, entry 1) to 88% (Table 3, entry 1); that of **2f** increased from 2% (Table 2, entry 6) to 96% (Table 3, entry 2); that of **2h** increased from 44% (Table 2, entry 9) to >99% (Table 3, entry 3); and the yields of these three cyanoamides increased as well. In these cases, decreased temperatures enhanced the conversion of dinitriles **1** to cyanoamides **2** more than **2** to diamides **4**, thus (*S*)-**2** were successfully accumulated.

Interestingly, the absolute configurations of cyanoacids **3b** and **3f** are *R*, opposite to that got at 30°C. The absolute configuration of **3** seems in close relation to the enantiomeric excess of **2**. The accelerated conversion of **1** to **2**, the enantioselective hydrolysis of (*R*)-**2** and the retaining of (*S*)-**2** resulted in both the formation of (*R*)-**3** and the excellent enantiomeric excess of **2**.

2.3. Synthesis of enantiopure amino acids

The products of the enantioselective hydrolysis of α,α-disubstituted malononitriles, (*S*)-α,α-disubstituted cyanoacetamides **2**, could be conveniently converted to either (*R*)- or (*S*)-α-alkylated amino acids using routine rearrangement reactions and hydrolysis reactions. For example, enantiopure (*S*)-2-cyano-2-methyl-3-phenylpropanamide **2a** was transferred to (*R*)-**9** or (*S*)-**9** in a yield of 89 or 93% (Scheme 4). This non-proteinogenic amino acid is an efficient β-turn and helix former, much stronger than its non-methylated parent compound phenylalanine.[14a]

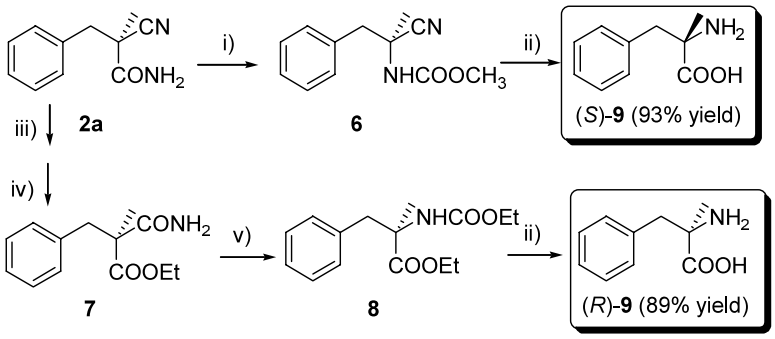
3. Conclusion

We have shown a new application of nitrile-converting enzymes to the synthesis of optically active α,α-disubstituted-α-cyanoacetamides from α,α-disubstituted-malononitriles with whole cells of *Rhodococcus* sp.

Table 3. Hydrolysis of α,α-disubstituted malononitrile **1** by *Rhodococcus* sp. CGMCC 0497 at 20°C

Entry	Substrate **1**	X	R	Time (h)	Recovered **1** (%)	(*S*)-**2**		**3**	
						Yield (%)	Ee (%)	Yield (%)	Ee (%)
1	**1b**	*p*-Me	CH_3	85	7	49	88	27	51 *R*
2	**1f**	*p*-MeO	CH_3	90	11	51	96	26	21 *R*
3	**1h**	*o*-Cl	CH_3	95	6	53	>99	27	ND[a]

[a] Absolute configuration undetermined.



Scheme 4. *Reagents and conditions*: (i) DMF, $Hg(OAc)_2$, NBS, MeOH, rt; (ii) 20% HCl, reflux; (iii) EtOH, 98% H_2SO_4, reflux; (iv) EtOH, 30% H_2O_2, 10N NaOH, rt; (v) DMF, $Hg(OAc)_2$, NBS, EtOH, rt.

CGMCC 0497. The products were obtained with enantiomeric excesses of up to >99%, and yields of up to 53%. It was found that reaction temperature had a great effect on the yields and enantiomeric excesses of the products. As far as we know, homochiral α,α-disubstituted-α-cyanoacetamides have never been synthesized directly by chemical or enzymatic methods in spite that they are very useful homochiral intermediates. In this paper, the enzymatic method, combined with the convenient conversion of the products to amino acids, provides a new way to the synthesis of enantiopure α,α-disubstituted amino acids.

4. Experimental

4.1. Materials and methods

The commercially available reagents were used without further purification. Melting points were determined on a Mettler FP62 and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker AMX-300 (300 MHz) spectrometer at room temperature with TMS as internal standard. Chemical shifts in ppm were positive for upfield shifts. IR spectra were recorded neat or in KBr and measured in cm^{-1}, using a Shimadzu IR-440 IR spectrophotometer. EI-MS spectra were recorded on an HP 5989A. High-resolution mass spectra were obtained on a Finnigan MAT8430. Microanalyses were carried out on an Italian Carlo-Erba 1106. Polarimetry was carried out using an optical activity Perkin–Elmer 241ML polarimeter and the measurements were made at the sodium D-line with a 10 cm pathlength cell. Concentrations (c) are given in g/100 ml. Enantiomeric excesses: Chiral HPLC was conducted with a PE NELSON NCI900 using Chiralpak AS, AD or Chiralcel OJ, OD column at a flow rate of 0.7 ml/min with 2-propanol/hexane as the mobile phase.

4.2. General procedure for dinitriles synthesis

Method A (for **1a**–**i**): To a suspension of NaH (60%) 400 mg (10 mmol) in 40 ml THF and 4 ml DMF, was added dropwise a solution of malononitrile (1.32 g, 20 mmol) in 5 ml THF at room temperature. After 2 h, benzyl bromide or chloride (10 mmol) in THF (10 ml) was added within 2 h. Stirring was continued at room temperature for 3–10 h. The reaction was quenched by aqueous NH_4Cl solution, extracted with ethyl acetate, dried on $MgSO_4$ and purified by flash chromatography. The product was dissolved in acetone (50 ml). Methyl iodide, or ethyl bromide, or allyl bromide (20 mmol) and solid K_2CO_3 (25 mmol) were added. The mixture was stirred overnight at room temperature. After filtration and concentration, the residue was purified by flash chromatography to yield α,α-disubstituted malononitriles.

Method B (for **1j** and **1k**): To a solution of malononitrile (1.32 g, 20 mmol) and phenylethyl bromide (10 mmol) in THF (40 ml) cooled by cryohydrate, was added solid *tert*-BuOK (10 mmol). The mixture was stirred overnight at room temperature. The following procedure was as described in Method A.

4.2.1. 2-Benzyl-2-methylmalononitrile, 1a. Mp 94.3–95.3°C, Lit.[19] 94.5–95.5°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.35–7.45 (m, 5H, Ar-H), 3.22 (s, 2H, CH_2), 1.81 (s, 3H, CH_3); IR (KBr): 2251 (CN); MS m/z (%): 170 (M^+, 1), 91 (100).

4.2.2. 2-(4′-Methylbenzyl)-2-methylmalononitrile, 1b. Mp 88.6–89.6°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.25, 7.20 (AB, 4H, J=8.1 Hz, ArH), 3.17 (s, 2H, CH_2), 2.37 (s, 3H, CH_3), 1.79 (s, 3H, CH_3); IR (KBr): 2253 (CN); MS m/z (%): 184 (M^+, 5), 105 (100). Anal. calcd for $C_{12}H_{12}N_2$: C, 78.23; H, 6.57; N, 15.20. Found: C, 78.36; H, 6.50; N, 15.33.

4.2.3. 2-(4′-Florobenzyl)-2-methylmalononitrile, 1c. Mp 126.1–127.1°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.38–7.33 (m, 2H, ArH), 7.14–7.08 (m, 2H, ArH), 3.19 (s, 2H, CH_2), 1.82 (s, 3H, CH_3); IR (KBr): 2253 (CN); MS m/z (%): 188 (M^+, 2), 173 (1), 109 (100). Anal. calcd for $C_{11}H_{19}FN_2$: C, 70.20; H, 4.82; F, 10.09; N, 14.88. Found: C, 70.22; H, 4.98; F, 10.05; N, 14.99.

4.2.4. 2-(4′-Chrolobenzyl)-2-methylmalononitrile, 1d. Mp 88.9–89.9°C; ^{1}H NMR (300 MHz, $CHCl_3$): δ 7.40, 7.31 (AB, 4H, J=8.4 Hz, ArH), 3.18 (s, 2H, CH_2), 1.82 (s, 3H, CH_3); IR (KBr): 2254 (CN); MS m/z (%): 206 (M^++2, 1), 204 (M^+, 4), 127 (33), 125 (100). Anal. calcd for $C_{11}H_9ClN_2$: C, 64.56; H, 4.43; Cl, 17.32; N, 13.69. Found: C, 64.43; H, 4.60; Cl, 17.39; N, 13.68.

4.2.5. 2-(4′-Bromobenzyl)-2-methylmalononitrile, 1e. Mp 86.3–87.3°C; ^{1}H NMR (300 MHz, $CHCl_3$): δ 7.55, 7.25 (AB, 4H, $J=8.4$ Hz, ArH), 3.17 (s, 2H, CH_2), 1.82 (s, 3H, CH_3); IR (KBr): 2247 (CN); MS m/z (%): 250 (M^++2, 5), 248 (M^+, 5), 171 (94), 169 (100). Anal. calcd for $C_{11}H_9BrN_2$: C, 53.04; H, 3.64; Br, 32.08; N, 11.25. Found: C, 53.05; H, 3.48; Br, 32.20; N, 11.06.

4.2.6. 2-(4′-Methoxybenzyl)-2-methylmalononitrile, 1f. Mp 68–69°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.28, 6.93 (AB, 4H, $J=9.0$ Hz, ArH), 3.82 (s, 3H, OMe), 3.16 (s, 2H, CH_2), 1.79 (s, 3H, CH_3); IR (KBr): 2253 (CN); MS m/z (%): 200 (M^+, 4), 121 (100). Anal. calcd for $C_{12}H_{12}N_2O$: C, 71.98; H, 6.04; N, 13.99. Found: C, 71.87; H, 6.05; N, 13.80.

4.2.7. 2-(3′-Chrolobenzyl)-2-methylmalononitrile, 1g. Mp 81.3–82.3°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.39–7.46 (m, 4H, ArH), 3.19 (s, 2H, CH_2), 1.84 (s, 3H, CH_3); IR (KBr): 2250 (CN); MS m/z (%): 206 (M^++2, 4), 204 (M^+, 12), 127 (34), 125 (100). Anal. calcd for $C_{11}H_9ClN_2$: C, 64.56; H, 4.43; Cl, 17.32; N, 13.69. Found: C, 64.39; H, 4.24; Cl, 17.15; N, 13.72.

4.2.8. 2-(2′-Chrolobenzyl)-2-methylmalononitrile, 1h. Mp 61.9–62.9°C; ^{1}H NMR (300 MHz, $CHCl_3$): δ 7.55–7.51 (m, 1H, ArH), 7.51–7.46 (m, 1H, ArH), 7.36–7.26 (m, 2H, ArH), 3.48 (s, 2H, CH_2), 1.85 (s, 3H, CH_3); IR (KBr): 2253 (CN); MS m/z (%): 206 (M^++2, 2), 204 (M^+, 6), 127 (34), 125 (100). Anal. calcd for $C_{11}H_9ClN_2$: C, 64.56; H, 4.43; Cl, 17.32; N, 13.69. Found: C, 64.79; H, 4.65; Cl, 17.29; N, 13.69.

4.2.9. 2-Benzyl-2-ethylmalononitrile, 1i. White solid, mp 59.4–60.4°C, Lit.[20] mp. 60°C; ^{1}H NMR (300 MHz, $CHCl_3$): δ 7.42–7.36 (m, 5H, ArH), 3.21 (s, 2H, CH_2), 2.02 (q, 2H, $J=7.2$ Hz, CH_2), 1.32 (t, 3H, $J=7.5$ Hz, CH_3); IR (KBr): 2247 (CN); MS m/z (%): 184 (M^+, 2), 155 (M^+–Et, 2), 91 (100).

4.2.10. 2-Phenylethyl-2-ethylmalononitrile, 1j. Oil, ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.38–7.33 (m, 2H, ArH), 7.30–7.23 (m, 3H, ArH), 3.04–2.98 (m, 2H, CH_2), 2.24–2.19 (m, 2H, CH_2), 2.06 (q, 2H, $J=7.5$ Hz, CH_2), 1.32 (t, 3H, $J=7.5$ Hz, CH_3); IR (film): 2248 (CN); MS m/z (%): 198 (M^+, 21), 105 (46), 91 (100); HRMS calcd for $(C_{13}H_{14}N_2)^+$: 198.11570. Found: 198.11300.

4.2.11. 2-Phenylethyl-2-allylmalononitrile, 1k. Oil, ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.37–7.31 (m, 2H, ArH), 7.29–7.21 (m, 3H, ArH), 5.98–5.84 (m, 1H, CH=C), 5.46–5.37 (m, 2H, CH_2=C), 3.02–2.96 (m, 2H, CH_2), 2.74 (d, 2H, $J=7.5$ Hz, CH_2), 2.23–2.17 (m, 2H, CH_2); IR (film): 2249 (CN); MS m/z (%): 211 (M^++1, 4), 210 (M^+, 27), 91 (100); HRMS calcd for $(C_{14}H_{14}N_2)^+$: 210.11570. Found: 210.11255.

4.3. Microorganism and cultivation

The strain *Rhodococcus* sp. CGMCC 0497 is available in CGMCC (China General Microbiological Culture Collection Center). *Rhodococcus* sp. CGMCC 0497 was subcultured at 30°C for 24 h in a 100 ml shaking flask containing 20 ml of a medium consisting of 0.5 g of polypepton, 0.5 g of beef extract and 1 g of glucose per 100 ml of tap water, pH 7.0. Then the subculture was inoculated into a 5 l shaking flask containing 1 l of the rich medium consisting of 1 g of glucose, 0.5 g of beef extract, 0.25 g of methacrylamide, 100 mg of $K_2HPO_4{\cdot}3H_2O$, 75 mg of KH_2PO_4, 10 mg of NaCl, 0.1 ml of mineral medium per 100 ml of tap water with methacrylamide added 24 h later. The pH of each medium was adjusted to around 7.0–7.2 by addition of 2N NaOH or 3N HCl. After incubation at 30°C with reciprocal shaking for 48 h, the organism was harvested by centrifugation using an HIMAC centrifuge CR20B2 (Hitachi, Japan) with a RPR9-2 rotor (6800*g*, 30 min 10°C). Cells were washed with 100 mM potassium phosphate buffer (pH 7.0) and centrifugated.

4.4. General procedure with whole cells and determination of enantiomeric excess

A suspension of 10 g washed wet cells and 80 ml 0.1 mM potassium phosphate buffer (pH 7.0) was incubated at 30 or 20°C for 30 min with continuous magnetic stirring before the addition of the substrate, a solution of 100 mg of α,α-disubstituted malononitrile dissolved in 100 μl of acetone. The reaction was quenched by centrifugation. The resulting supernatant was made alkaline with 2N NaOH to pH 12 and extracted with diethyl ether. The organic solutions, after drying ($MgSO_4$) and concentration, gave the amide **2** and **4**, and unreacted dinitrile **1**. Separation was effected by column chromatography. The aqueous solution was then acidified using 3N HCl to pH 2 and extracted with diethyl ether. After concentration, the residue was purified by flash chromatography on silica gel (elute: petroleum ether/EtOAc/AcOH 150:100:1) to afford the acids **3** and **5**.

To a solution of α,α-disubstituted cyanoacetic acid **3** (0.1 mmol) in DMF (0.1 ml) was added bromoethane or iodomethane (2 mmol) and anhydrous K_2CO_3 (2 mmol). The reaction was carried out at room temperature for one day to achieve the esters and the esters were subjected to chiral HPLC.

Amides **2** were subjected to chiral HPLC directly, or dissolved in ethanol gently refluxed at the presence of catalytic concentrated sulfuric acid for 3 h to yield the corresponding esters.

4.4.1. Enzymatic hydrolysis of 2-benzyl-2-methylmalononitrile, 1a. (*S*)-2-Cyano-2-methyl-3-phenylpropanamide **2a**. Mp 96.5–97.5°C; $[\alpha]_D^{17}=+46.85$ (*c* 0.98, $CHCl_3$), >99% ee; enantiomeric excess was determined by HPLC on a Chiralcel OD column with hexane/2-propanol mixtures 8:2 and the retention time for the (*R*)- and (*S*)-enantiomers was 13.8 and 16.8 min, respectively; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.40–7.20 (m, 5H, Ar-H), 6.04 (s, br, 1H, NH), 5.52 (s, br, 1H, NH), 3.26 (d, 1H, $J=13.5$ Hz, CH), 2.96 (d, 1H, $J=13.5$ Hz, CH), 1.66 (s, 3H, CH_3); IR (KBr): ν 3378, 3320 (NH), 2244 (CN), 1666 (C=O); MS m/z (%): 189 (M^++1, 3), 188 (M^+, 21), 173 (M^+–CH_3, 4), 144 (M^+–

$CONH_2$, 19), 91 (100). Anal. calcd for $C_{11}H_{12}N_2O$: C, 70.19; H, 6.43; N, 14.88. Found: C, 69.98; H, 6.51; N, 14.81. (*R*)- or (*S*)-2-Cyano-2-methyl-3-phenylpropanoic acid **3a**. Mp 94.6–95.6°C, Lit.[17] 84°C; $[\alpha]_D^{25}=-15.1$ (*c* 0.53, $CHCl_3$), 52% ee, *R*, {Lit.[17] $[\alpha]_D=27.2$ (*c* 2, $CHCl_3$), *S*}; ^1H NMR (300 MHz, $CDCl_3$): δ 9.83 (br s, 1H, OH), 7.37–7.28 (m, 5H, Ar-H), 3.27 (d, 1H, *J*= 13.5 Hz, CH), 3.07 (d, 1H, *J*=13.8 Hz), 1.65 (s, 3H, CH_3); IR (KBr): *v* 3074 (br OH), 2263 (CN), 1747 (C=O); MS *m*/*z* (%): 189 (M^+, 2), 174 (M^+-CH_3, 3), 144 (M^+-COOH, 3), 91 (100). 2-Benzyl-2-methylmalonamide **4a**. White solid, mp 195.4–196.4°C, Lit.[21] 202–203°C; ^1H NMR (300 MHz, DMSO): δ 7.22–7.12 (m, 9H, ArH, $2NH_2$), 3.06 (s, 2H, CH_2), 1.10 (s, 3H, CH_3); IR (KBr): *v* 3389, 3205 (NH), 1692, 1663 (C=O); MS *m*/*z* (%): 206 (M^+, 7), 189 (10), 162 (M^+-CONH_2, 100), 160 (41). (*R*)-2-Benzyl-2-methylmalonamic acid **5a**. White solid, mp 117.9–118.9°C, Lit.[13] 120–121°C; $[\alpha]_D^{15}=-15.0$ (*c* 1.07, MeOH), 94% ee; {Lit.[13] $[\alpha]_D=-4.4$ (*c* 0.5, MeOH), *R*}; ^1H NMR (300 MHz, acetone-d_6): δ 7.29–7.19 (m, 5H, ArH), 3.61 (br s, 3H, NH_2, OH), 3.25 (d, 1H, *J*=13.8 Hz), 3.20 (d, 1H, *J*=13.5 Hz), 1.37 (s, 3H, CH_3); IR (KBr): *v* 3423, 3204 (NH), 3034 (br OH), 1745, 1659 (C=O); MS *m*/*z* (%): 207 (M^+, 2), 91 (100).

4.4.2. Enzymatic hydrolysis of 2-(4′-methylbenzyl)-2-methylmalononitrile, 1b. (*S*)-2-Cyano-2-methyl-3-(4′-methylphenyl)propanamide **2b**. Mp 114.1–115.1°C; $[\alpha]_D^{26}=+12.0$ (*c* 0.25, $CHCl_3$), 64% ee; $[\alpha]_D^{25}=+16.7$ (*c* 0.76, $CHCl_3$), 88% ee; enantiomeric excess was determined by HPLC on a Chiralpak AD column with hexane/2-propanol mixtures 9:1 and the retention time for *R* and *S* enantiomer was 17.2 and 11.2 min, respectively; ^1H NMR (300 MHz, $CDCl_3$): δ 7.19, 7.14 (AB, 4H, *J*=8.4 Hz, ArH), 6.04 (br s, 1H, NH), 5.59 (br s, 1H, NH), 3.22 (d, 1H, *J*=13.5 Hz, CH), 2.92 (d, 1H, *J*=13.5 Hz, CH), 2.34 (s, 3H, CH_3), 1.64 (s, 3H, CH_3); IR (KBr): 3443, 3420, 3189 (br NH), 2237 (CN), 1695 (C=O); MS *m*/*z* (%): 202 (M^+, 6), 105 (100). Anal. calcd for $C_{12}H_{14}N_2O$: C, 71.26; H, 6.98; N, 13.85. Found: C, 71.32; H, 7.04; N, 13.78. (*R*) or (*S*)-2-Cyano-2-methyl-3-(4′-methylphenyl)propanoic acid **3b**. $[\alpha]_D^{24}=+3.5$ (*c* 1.05, $CHCl_3$), 14% ee, *S*; ^1H NMR (300 MHz, $CDCl_3$): δ 7.19, 7.14 (AB, 4H, *J*=8.4 Hz, ArH), 6.45 (br s, 1H, OH), 3.23 (d, 1H, *J*=13.5 Hz, CH), 3.01 (d, 1H, *J*=13.5 Hz, CH), 2.33 (s, 3H, CH_3), 1.65 (s, 3H, CH_3); IR (film): 2850–3250 (br OH), 2251 (CN), 1739 (C=O); MS *m*/*z* (%): 203 (M^+, 4), 105 (100); HRMS calcd for $(C_{12}H_{13}NO_2)^+$: 203.09463. Found: 203.09419.

4.4.3. Enzymatic hydrolysis of 2-(4′-florobenzyl)-2-methylmalononitrile, 1c. (*S*)-2-Cyano-2-methyl-3-(4′-florophenyl)propanamide **2c**. Mp 138.4–139.4°C; $[\alpha]_D^{22}=+26.4$ (*c* 0.582, $CHCl_3$). 78% ee; enantiomeric excess was determined by HPLC on a Chiralpak AD column with hexane/2-propanol mixtures 9:1 and the retention time for (*R*)- and (*S*)-enantiomer was 17.3 and 15.0 min, respectively; ^1H NMR (300 MHz, $CDCl_3$): δ 7.31–7.26 (m, 2H, ArH), 7.06–7.01 (m, 2H, ArH), 6.05 (br s, 1H, NH), 5.55 (br s, 1H, NH), 3.25 (d, 1H, *J*=13.5 Hz, CH), 2.92 (d, 1H, *J*=13.5 Hz, CH), 1.65 (s, 3H, CH_3); IR (KBr): 3472, 3316, 2249 (CN), 1664 (C=O); MS *m*/*z* (%): 206 (M^+, 5), 162 (M^+-CONH_2, 8), 109 (100). Anal. calcd for $C_{11}H_{11}FN_2O$: C, 64.07; H, 5.38; F, 9.21; N, 13.58. Found: C, 64.05; H, 5.39; F, 9.05; N, 13.62. (*R*)-2-Cyano-2-methyl-3-(4′-florophenyl)propanoic acid **3c**. $[\alpha]_D^{21}=-3.8$ (*c* 0.177, $CHCl_3$), 16% ee; ^1H NMR (300 MHz, $CDCl_3$): δ 8.58 (br s, 1H, OH), 7.31–7.27 (m, 2H, ArH), 7.05–6.99 (m, 2H, ArH), 3.25 (d, 1H, *J*=13.8 Hz, CH), 3.02 (d, 1H, *J*=13.5 Hz, CH), 1.64 (s, 3H, CH_3); IR (film): 2950–3200 (br OH), 2254 (CN), 1733 (C=O); MS *m*/*z* (%): 207 (M^+, 2), 162 (6), 161 (7), 109 (100); HRMS calcd for $(C_{11}H_{10}FNO_2)^+$: 207.06960. Found: 207.07429.

4.4.4. Enzymatic hydrolysis of 2-(4′-chrolobenzyl)-2-methylmalononitrile, 1d. (*S*)-2-Cyano-2-methyl-3-(4′-chlorophenyl)propanamide **2d**. Mp 118.8–119.8°C; $[\alpha]_D^{26}=+15.7$ (*c* 0.57, $CHCl_3$), 42% ee; enantiomeric excess was determined by HPLC on a Chiralpak AD column with hexane/2-propanol mixtures 9:1 and the retention time for (*R*)- and (*S*)-enantiomer was 17.8 and 15.5 min, respectively; ^1H NMR (300 MHz, $CDCl_3$): δ 7.47, 7.18 (AB, 4H, *J*=8.1 Hz, ArH), 6.06 (br s, 1H, NH), 5.57 (br s, 1H, NH), 3.23 (d, 1H, *J*=13.5 Hz, CH), 2.90 (d, 1H, *J*=13.2 Hz, CH), 1.66 (s, 3H, CH_3); IR (KBr): 3456, 3311, 3196, 2249 (CN), 1664 (C=O); MS *m*/*z* (%): 224 (M^++2, 3), 222 (M^+, 7), 127 (33), 125 (100). Anal. calcd for $C_{11}H_{12}ClNO_3$: C, 59.33; H, 4.98; Cl, 15.92; N, 12.58. Found: C, 59.46; H, 4.98; Cl, 15.90; N, 12.64. (*S*)-2-Cyano-2-methyl-3-(4′-chlorophenyl)propanoic acid **3d**. $[\alpha]_D^{21}=+8.0$ (*c* 1.46, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 7.6 (br s, 1H, OH), 7.32, 7.25 (AB, 4H, *J*=8.4 Hz, ArH), 3.25 (d, 1H, *J*=14.1 Hz, CH), 3.02 (d, 1H, *J*=13.8 Hz, CH), 1.65 (s, 3H, CH_3); IR (film): 2800–3200 (br OH), 2254 (CN), 1731 (C=O); MS *m*/*z* (%): 225 (M^++2, 3), 223 (M^+, 9), 127 (33), 125 (100); HRMS calcd for $(C_{11}H_{10}ClNO_2)^+$: 223.04001. Found: 223.04123.

4.4.5. Enzymatic hydrolysis of 2-(4′-bromobenzyl)-2-methylmalononitrile, 1e. (*S*)-2-Cyano-2-methyl-3-(4′-bromophenyl)propanamide **2e**. Mp 119.5–120.5°C; $[\alpha]_D^{26}=+23.0$ (*c* 0.33, $CHCl_3$), 78% ee; enantiomeric excess was determined by HPLC on a Chiralpak AD column with hexane/2-propanol mixtures 9:1 and the retention time for (*R*)- and (*S*)-enantiomer was 17.3 and 14.8 min, respectively; ^1H NMR (300 MHz, $CDCl_3$): δ 7.32, 7.24 (AB, 4H, *J*=8.4 Hz, ArH), 6.06 (br s, 1H, NH), 5.57 (br s, 1H, NH), 3.24 (d, 1H, *J*=13.5 Hz, CH), 2.92 (d, 1H, *J*=13.2 Hz, CH), 1.66 (s, 3H, CH_3); IR (KBr): 3389, 3312, 3205, 2248 (CN), 1669 (C=O); MS *m*/*z* (%): 268 (M^++2, 9), 266 (M^+, 8), 224 (4), 222 (4), 171 (91), 169 (100). Anal. calcd for $C_{11}H_{11}BrN_2O$: C, 49.46; H, 4.15; Br, 29.91; N, 10.49. Found: C, 49.59; H, 4.16; Br, 30.05; N, 10.55. 2-Cyano-2-methyl-3-(4′-bromophenyl)propanoic acid **3e**. $[\alpha]_D^{18}=+8.75$ (*c* 1.37, $CHCl_3$); ^1H NMR (300 MHz, $CDCl_3$): δ 7.48, 7.24 (AB, 4H, *J*=8.7 Hz, ArH), 6.99 (br s, 1H, OH), 3.23 (d, 1H, *J*=13.8 Hz, CH), 3.01 (d, 1H, *J*=13.5 Hz, CH), 1.65 (s, 3H, CH_3); IR (film): 2800–3200 (br OH), 2254 (CN), 1731 (C=O); MS *m*/*z* (%): 269 (M^++2, 9), 267 (M^+, 9), 171 (96), 169 (100), 115 (17), 90 (38); HRMS calcd for $(C_{11}H_{10}BrNO_2)^+$: 266.98949. Found: 266.98855.

4.4.6. Enzymatic hydrolysis of 2-(4′-methoxybenzyl)-2-methylmalononitrile, 1f. (*S*)-2-Cyano-2-methyl-3-(4′-methoxyphenyl)propanamide **2f**. Mp 95.9–96.9°C; $[\alpha]_D^{28}=+41.0$ (*c* 1.60, $CHCl_3$), 96% ee; enantiomeric excess was determined by HPLC on a Chiralpak AS column with hexane/2-propanol mixtures 8:2 and the retention time for (*R*)- and (*S*)-enantiomer was 19.3 and 16.1 min, respectively; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.22, 6.86 (AB, 4H, $J=8.4$ Hz, ArH), 6.04 (br s, 1H, NH), 5.61 (br s, 1H, NH), 3.80 (s, 3H, OMe), 3.19 (d, 1H, $J=13.5$ Hz, CH), 2.90 (d, 1H, $J=13.5$ Hz, CH), 1.63 (s, 3H, CH_3); IR (KBr): 3427, 3307, 3201 (br NH), 2247 (CN), 1670 (C=O); MS *m*/*z* (%): 218 (M^+, 6), 174 (M^+-CONH_2, 1), 121 (100). Anal. calcd for $C_{12}H_{14}N_2O_2$: C, 66.04; H, 6.47; N, 12.84. Found: C, 66.00; H, 6.23; N, 12.97. (*R*) or (*S*)-2-Cyano-2-methyl-3-(4′-methoxyphenyl)propanoic acid **3f**. $[\alpha]_D^{19}=+7.5$ (*c* 1.0, $CHCl_3$), 30% ee, *S*; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.22, 6.87 (AB, 4H, $J=8.4$ Hz, ArH), 6.58 (br s, 1H, OH), 3.80 (s, 3H, OMe), 3.22 (d, 1H, $J=13.5$ Hz, CH), 3.00 (d, 1H, $J=13.8$ Hz, CH), 1.62 (s, 3H, CH_3); IR (film): 2850–3250 (br OH), 2253 (CN), 1747 (C=O); MS *m*/*z* (%): 219 (M^+, 2), 121 (100); HRMS calcd for $(C_{11}H_{13}NO)^+$: [(M^+-CH_3)] 175.09971. Found: 175.09795.

4.4.7. Enzymatic hydrolysis of 2-(3′-chrolobenzyl)-2-methylmalononitrile, 1g. (*S*)-2-Cyano-2-methyl-3-(3′-chlorophenyl)propanamide **2g**. Mp 137.0–138.0°C; $[\alpha]_D^{25}=+33.7$ (*c* 0.895, $CHCl_3$), 81% ee; enantiomeric excess was determined by HPLC on a Chiralpak AD column with hexane/2-propanol mixtures 9:1 and the retention time for (*R*)- and (*S*)-enantiomer was 18.3 and 13.6 min, respectively; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.29–7.19 (m, 4H, ArH), 6.15 (br s, 1H, NH), 5.95 (br s, 1H, NH), 3.24 (d, 1H, $J=13.5$ Hz, CH), 2.91 (d, 1H, $J=13.5$ Hz, CH), 1.65 (s, 3H, CH_3); IR (KBr): 3461, 3325, 3190, 2245 (CN), 1668 (C=O), 1626, 1406, 1243, 792; MS *m*/*z* (%): 224 (M^++2, 9), 222 (M^+, 23), 180 (13), 178 (36), 127 (33), 125 (100). Anal. calcd for $C_{11}H_{11}ClN_2O$: C, 59.33; H, 4.98; Cl, 15.92; N, 12.58. Found: C, 59.39; H, 5.01; Cl, 15.82; N, 12.67. (*R*)-2-Cyano-2-methyl-3-(3′-chlorophenyl)propanoic acid **3g**. Mp 101.0–102.0°C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.32–7.21 (m, 4H, ArH), 6.22 (br s, OH), 3.25 (d, 1H, $J=13.8$ Hz, CH), 3.03 (d, 1H, $J=13.5$ Hz, CH), 1.66 (s, 3H, CH_3); IR (film): 3477 (br OH), 2253 (CN), 1741 (C=O); MS *m*/*z* (%): 225 (M^++2, 2), 223 (M^+, 7), 127 (32.8), 125 (100); HRMS calcd for $(C_{11}H_{10}ClNO_2)^+$: 223.04001. Found: 223.03706.

4.4.8. Enzymatic hydrolysis of 2-(2′-chrolobenzyl)-2-methylmalononitrile, 1h. (*S*)-2-Cyano-2-methyl-3-(2′-chlorophenyl)propanamide **2h**. Mp 138.9–139.9°C; $[\alpha]_D^{25}=+25.4$ (*c* 1.14, $CHCl_3$), 44% ee; $[\alpha]_D^{25}=+64.9$ (*c* 1.54, $CHCl_3$), >99% ee; enantiomeric excess was determined by HPLC on a Chiralpak AD column with hexane/2-propanol mixtures 9:1 and the retention time for *R* and *S* enantiomer was 18.3 and 15.1 min, respectively; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.44–7.37 (m, 2H, ArH), 7.28–7.24 (m, 2H, ArH), 6.22 (br s, 1H, NH), 5.78 (br s, 1H, NH), 3.41 (d, 1H, $J=14.1$ Hz, CH), 3.33 (d, 1H, $J=14.1$ Hz, CH), 1.68 (s, 3H, CH_3); IR (KBr): 3408, 3199, 3140 (NH), 2244 (CN), 1702 (C=O); MS *m*/*z* (%): 225 (M^++3, 1), 223 (M^++1, 2), 187 (M^+-Cl, 77), 127 (34), 125 (100). Anal. calcd for $C_{11}H_{12}ClNO_3$: C, 59.33; H, 4.98; Cl, 15.92; N, 12.58. Found: C, 59.28; H, 4.96; Cl, 16.18; N, 12.60. 2-Cyano-2-methyl-3-(2′-chlorophenyl)propanoic acid **3h**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.48–7.40 (m, 2H, ArH), 7.30–7.26 (m, 2H, ArH), 6.05 (br s, 1H, OH), 3.45 (d, 1H, $J=14.1$ Hz, CH), 3.37 (d, 1H, $J=14.4$ Hz, CH), 1.69 (s, 3H, CH_3); IR (film): *v* 3483 (br OH), 2253 (CN), 1731 (C=O); MS *m*/*z* (%): 225 (M^++2, 3), 223 (M^+, 10), 127 (37), 125 (100); HRMS calcd for $(C_{11}H_{10}ClNO_2)^+$: 223.04001. Found: 223.03989.

4.4.9. Enzymatic hydrolysis of 2-benzyl-2-ethylmalononitrile, 1i. (*S*)-2-Cyano-2-ethyl-3-phenylpropanamide **2i**. Mp 115.2–116.2°C; $[\alpha]_D^{19}=+38.9$ (*c* 0.941, $CHCl_3$), 65% ee; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.36–7.28 (m, 5H, ArH), 6.05 (br s, 1H, NH), 5.79 (br s, 1H, NH), 3.23 (d, 1H, $J=13.5$ Hz, CH), 2.96 (d, 1H, $J=13.5$ Hz), 2.13 (m, 1H, CH), 1.83 (m, 1H, CH), 1.11 (t, 3H, $J=7.2$ Hz, CH_3); IR (KBr): 3390, 3185 (br NH), 2245 (CN), 1695 (C=O); MS *m*/*z* (%): 202 (M^+, 15), 187 (1), 91 (100). Anal. calcd for $C_{12}H_{14}N_2O$: C, 71.26; H, 6.98; N, 13.85. Found: C, 71.22; H, 6.95; N, 13.87.

4.4.10. Enzymatic hydrolysis of 2-phenylethyl-2-ethylmalononitrile, 1j. (*S*)-2-Cyano-2-ethyl-4-phenylbutamide **2j**. 64% ee, $[\alpha]_D^9=+11.1$ (*c* 1.076, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.32–7.29 (m, 2H, ArH), 7.23–7.18 (m, 3H, ArH), 6.42 (s, br, 1H, NH), 6.14 (s, br, 1H, NH), 2.84 (td, 1H, $J_1=12.9$ Hz, $J_2=4.8$ Hz, CH), 2.70 (td, 1H, $J_1=12.9$ Hz, $J_2=5.0$ Hz, CH), 2.25 (td, 1H, $J_1=12.9$ Hz, $J_2=5.2$ Hz, CH), 2.06–1.98 (m, 2H), 1.81 (dt, $J_1=21.3$ Hz, $J_2=7.2$ Hz, CH), 1.11 (t, 3H, $J=7.2$ Hz, CH_3); IR (KBr): 3383, 3182 (NH), 2245 (CN), 1694 (C=O); MS *m*/*z* (%): 217 (M^++1, 4), 200 (2), 112 (100), 105 (25); HRMS calcd for $(C_{13}H_{16}N_2O)^+$: 216.12626. Found: 216.12489.

4.4.11. Enzymatic hydrolysis of 2-phenylethyl-2-allylmalononitrile, 1k. (*S*)-2-Cyano-2-allyl-4-phenylbutamide **2k**. Mp 99.8–108.8°C; $[\alpha]_D^{17}=+5.5$ (*c* 0.86, $CHCl_3$), 60% ee; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.32–7.14 (m, 5H, ArH), 6.34 (s, br, 1H, NH), 5.95 (s, br, 1H, NH), 5.91–5.76 (m, 1H, =CH), 5.30 (d, 1H, $J=6.3$ Hz, =CH), 5.25 (s, 1H, =CH), 2.89–2.79 (m, 1H), 2.75–2.63 (m, 2H), 2.53–2.45 (m, 1H), 2.32–2.17 (m, 1H), 2.05–1.93 (m, 1H); IR (KBr): 3388, 3178 (NH), 2246 (CN), 1695 (C=O); MS *m*/*z* (%): 229 (M^++1, 1), 124 (100), 91 (49); HRMS calcd for $(C_{14}H_{16}N_2O)^+$: 228.12626. Found: 228.12724.

4.4.12. Enzymatic hydrolysis of (*rac*)-2-cyano-2-methyl-3-phenylpropanamide, 2a. A suspension of 7 g of washed wet cells and 56 ml of 0.1 mM potassium phosphate buffer (pH 7.0) was incubated at 30°C for 30 min with continuously magnetic stirring, then a solution of 70 mg (*rac*)-2-cyano-2-methyl-3-phenylpropanamide **2a** dissolved in 70 μl of acetone was added. The reaction was continued for 24 h and quenched by centrifugation. The post treatment was carried out as described in General procedure. The

resulting product was purified by flash chromatography to afford 9 mg (*S*)-2-cyano-2-methyl-3-phenylpropanamide **2a** (13% yield, 61% ee), 17 mg (*R*)-2-cyano-2-methyl-3-phenylpropanoic acid **3a** (24% yield, 96% ee), 12 mg 2-benzyl-2-methylmalonamide **4a** (16% yield) and 35 mg (*R*)-2-benzyl-2-methylmalonamic acid **5a** (45% yield, 97% ee). The physical data of the products are the same as those described in Section 4.4.1.

4.5. Synthesis of amino acids

4.5.1. (*S*)-2-Methoxycarbonylamino-2-methyl-3-phenylpropanonitrile, 6. The product **2a** (0.16 mmol) was dissolved in dry DMF (1.5 ml). $Hg(OAc)_2$ (61 mg, 0.19 mmol), dry methanol (5 mmol) and NBS (37 mg, 0.21 mmol) were added at room temperature. After 16 h, the resulting mixture was poured into water and extracted with ether, washed and dried on Na_2SO_4. The solvent was removed under reduced pressure and the resulting product was purified by flash chromatography to afford (*S*)-**6** (96% yield). Lit.[17] mp 81°C; $[\alpha]_D^{22}=-46.6$ (*c* 0.798, $CHCl_3$), {Lit.[17] $[\alpha]_D=-46.1$ (*c* 2, $CHCl_3$), *S*}; enantiomeric excess was determined by HPLC on a Chiralcel OJ column with hexane/2-propanol mixtures 9:1 and the retention time for (*R*)- and (*S*)-enantiomer was 36.6 and 40.5 min, respectively; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.40–7.34 (m, 3H, ArH), 7.30–7.27 (m, 2H, ArH), 4.94 (br s, 1H, NH), 3.74 (s, 3H, OMe), 3.29 (d, 1H, $J=13.8$ Hz, CH), 3.19 (d, 1H, $J=13.5$ Hz, CH), 1.66 (s, 3H, CH_3); IR (KBr): *v* 3325 (NH), 2240 (CN), 1699 (C=O); MS *m*/*z* (%): 219 (M^++1, 8), 218 (M^+, 15), 192 (M^+–CN, 99), 91 (100).

4.5.2. (*S*)-2-Benzyl-2-methylmalonamic acid ethyl ester, 7. (*S*)-**2a** (28 mg, 1.5 mmol) was dissolved in 2 ml anhydrous ethanol. Three drops of concentrated sulfate acid was added and the mixture was refluxed gently. The resulting mixture was poured into cool $NaHCO_3$ aqueous solution and extracted with ethyl acetate, washed and dried on $MgSO_4$. The solvent was removed under reduced pressure and the resulting product was purified by flash chromatography (98%). The product was dissolved in 1 ml ethanol. 30% H_2O_2 (2 ml) and 10 M NaOH (0.05 ml) were added at 0°C. After 3 h at room temperature, the resulting mixture was extracted with ethyl acetate, washed and dried on $MgSO_4$. The solvent was removed under reduced pressure and the resulting product was purified by flash chromatography to give 34 mg (*S*)-**7** (98%). Mp 90.9–91.9°C; $[\alpha]_D^{16}=+3.3$ (*c* 2.6, $CHCl_3$); enantiomeric excess was determined by HPLC on a Chiralcel AS column with hexane/2-propanol mixtures 85: 15 and the retention time for *R* and *S* enantiomer was 21.0 and 16.4 min, respectively; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.30–7.23 (m, 3H, Ar-H), 7.17–7.14 (m, 2H, Ar-H), 6.98 (s, br, 1H, NH), 5.79 (s, br, 1H, NH), 4.19 (q, 2H, $J=7.2$ Hz), 3.37 (d, 1H, $J=13.5$ Hz), 3.12 (d, 1H, $J=13.5$ Hz, CH), 1.45 (s, 3H, CH_3), 1.27 (t, 3H, $J=7.2$ Hz, CH_3); IR (KBr): *v* 3395, 3327 (NH), 1722 (C=O), 1694, 1667 (C=O); MS *m*/*z* (%): 235 (M^+, 5), 218 (8), 190 (16), 162 (22), 91 (100). Anal. calcd for $C_{13}H_{17}NO_3$: C, 66.36; H, 7.28; N, 5.95. Found: C, 66.35; H, 7.07; N, 5.88.

4.5.3. (*R*)-2-Ethoxycarbonylamino-2-methyl-3-phenylpropanoic acid ethyl ester, 8. The product **7** was dissolved in dry DMF (1.5 ml). $Hg(OAc)_2$ (61 mg, 0.19 mmol), dry ethanol (220 mg, 5 mmol) and NBS (37 mg, 0.21 mmol) were added at room temperature. After 16 h, the resulting mixture was poured into water and extracted with ether, washed and dried on Na_2SO_4. The solvent was removed under reduced pressure and the resulting product was purified by flash chromatography to afford (*R*)-**8** (98% yield). Oil; enantiomeric excess was determined by HPLC on a Chiralcel OD column with hexane/2-propanol mixtures 8:2 and the retention time for (*R*)- and (*S*)-enantiomer was 10.4 and 17.8 min, respectively; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.30–7.23 (m, 3H, Ar-H), 7.08–7.05 (m, 2H, Ar-H), 5.37 (br s, 1H, NH), 4.27–4.10 (m, 4H, $2OCH_2$), 3.43 (d, 1H, $J=13.8$ Hz, CH), 3.18 (d, 1H, $J=13.2$ Hz, CH), 1.64 (s, 3H, CH_3), 1.33–1.23 (m, 6H, $2CH_3$); IR (film): *v* 3358 (NH), 1721 (C=O), 704; MS *m*/*z* (%): 279 (M^+, 1), 206 (M^+–COOEt, 43), 188 (100); HRMS calcd for $(C_{15}H_{21}NO_4)^+$: 279.14706. Found: 279.15017.

4.5.4. (*S*)- and (*R*)-α-Methylphenylalanine, 9. (*S*)-2-Methoxycarbonylamino-2-methyl-3-phenylpropanonitrile **7** or (*R*)-2-ethoxycarbonylamino-2-methyl-3-phenylpropanoic acid ethyl ester **8** (0.13 mmol) was hydrolyzed by refluxing for 3 h with 20% aqueous hydrochloric acid (3 ml). The solution was evaporated under vacuum. To the residue was added distilled water (3 ml) and evaporated again. The residue was purified by Dowex 50×2-400 ion-exchange resin (Acros), yield 95%. $[\alpha]_D^{17}=-22.0$ (*c* 0.61, H_2O), *S*; $[\alpha]_D^{17}=+21.8$ (*c* 0.73, H_2O), *R*; {Lit.[22] $[\alpha]_D=-22$ (*c* 1, H_2O), *S*}; ^{1}H NMR (300 MHz, D_2O): δ 7.22–7.19 (m, 3H, ArH), 7.10–7.07 (m, 2H, ArH), 3.11 (d, 1H, $J=14.4$ Hz, CH), 2.79 (d, 1H, $J=14.4$ Hz, CH), 1.37 (s, 3H, CH_3); IR (KBr): 2500–3300, 1650 (C=O); MS (ESI): 202 ([M+Na]$^+$), 180 ([M+H]$^+$).

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