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Highly Efficient Chemoenzymatic Synthesis of Methyl (*R*)-*o*-Chloromandelate, a Key Intermediate for Clopidogrel, *via* Asymmetric Reduction with Recombinant *Escherichia coli*

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Abstract: Methyl (*R*)-*o*-chloromandelate [(*R*)-**1**], which is an intermediate for a platelet aggregation inhibitor named clopidogrel, was obtained in >99% *ee* by the asymmetric reduction of methyl *o*-chlorobenzoylformate (**2**) with recombinant *Escherichia coli* overproducing a versatile carbonyl reductase. A remarkable temperature effect on productivity was observed in the whole-cell reduction of **2**, and the optimum productivity as high as 178 g/L was attained at 20 °C on a 2-g scale (1.0 M). The optimized reaction could be scaled up easily to transform 20 g of **2** in 100 mL of buffer. Three synthetic methods for **2** are compared.

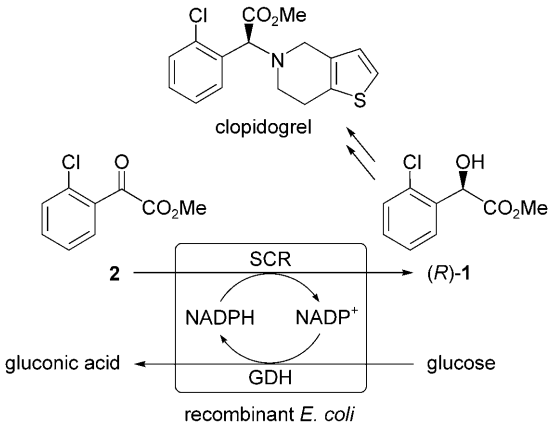
Keywords: asymmetric catalysis; biotransformations; chemoenzymatic synthesis; enzyme catalysis

Introduction

Chemoenzymatic synthesis is becoming more and more important from the viewpoint of green chemistry. An interdisciplinary field encompassing synthetic organic chemistry and biology will play a key role in developing highly efficient and environmentally benign synthetic routes. A synergic use of biotransformation and organic synthesis, taking advantage of the merits of each methodology, can realize a powerful and secure production of a useful compound.[1,2]

Clopidogrel is a platelet aggregation inhibitor widely administered to atherosclerotic patients with the risk of a heart attack or stroke that are caused by the formation of a clot in the blood. Worldwide sales of Plavix (clopidogrel bisulfate) amounted to $6.4 billion per year, which ranks second.[3] The key enantiomer, methyl (*R*)-*o*-chloromandelate [(*R*)-**1**], can be prepared in several ways. (i) The corresponding (*R*)-carboxylic acid[4] can be obtained by the fractional crystallization of the racemic mixture or by the asymmetric reduction of the corresponding α-keto acid.[5] (ii) The direct asymmetric reduction of the corresponding ketone, methyl *o*-chlorobenzoylformate (**2**), is also promising. Although the Ru-catalyzed asymmetric hydrogenation of **2** and the corresponding ethyl ester has been done, (*R*)-**1** and the corresponding ethyl ester have been obtained, respectively, in 50% *ee*[6] and 76% *ee*[7] at most. (iii) The asymmetric hydrocyanation of *o*-chlorobenzaldehyde using *R*-selective hydroxynitrile lyases has also been used to access clopidogrel.[8,9] Among them, the direct asymmetric reduction of **2** is much safer, more straightforward, and more advantageous because the asymmetric center is generated in a step closer to the final product.

Biocatalysts that can transform ketones into optically active alcohols are powerful synthetic tools.[1,2,10] We have been studying a versatile carbonyl reductase called SCR capable of showing catalytic activity for various ketones, such as α-chloro ketones, α-acetoxy ketones, α-keto esters, β-keto esters, γ-keto esters, and β-diketones, and 13 out of 20 alcohols obtained had enantiomeric purities of >98% *ee*.[11] The gene encoding SCR has been cloned and expressed in *E. coli*, and the asymmetric reduction of various ketones with the recombinant *E. coli* cells has afforded 20 synthetically useful alcohols, 11 of which had enantiomeric purities of >98% *ee*.[12] The gene encoding SCR is *Gre2* (*YOL151w*) from *Saccharomyces cerevisiae* (baker's yeast), and it has also been used by other researchers for the purpose of biotransformations.[13] Recently, we have succeeded in the highly efficient asymmetric reduction of **2** using the recombinant *E. coli* as a versatile biocatalyst (Scheme 1).[14] No biotransformations of **2** to **1** had been reported before. Here we report the environmentally benign and practical chemoenzymatic synthesis of the key intermediate for clopidogrel, (*R*)-**1**, in detail.



Scheme 1. Asymmetric synthesis of (*R*)-**1**, a key intermediate for clopidogrel, by the asymmetric reduction of **2** with recombinant *E. coli*.

Results and Discussion

α-Keto ester **2** was prepared according to the literature,[15] and the whole-cell reduction of **2** was done with the *E. coli* strain coproducing SCR and GDH (glucose dehydrogenase) in the same way as reported previously (Scheme 1).[12b] α-Keto ester **2** was added to a mixture of glucose, $NADP^+$, and *E. coli* wet cells in 0.1 M phosphate buffer. The mixture was stirred at a regulated temperature for 24 h, during which 2 N NaOH was added to neutralize the solution acidified upon formation of gluconic acid. The product was extracted with EtOAc and purified by column chromatography or distillation. The results are summarized in Table 1.

When the reaction conditions previously optimized for β-diketone were applied to **2**,[12b] the desired alcohol (*R*)-**1** was obtained in 76% yield with >99% *ee* (entry 1). We immediately tried to improve the efficiency of this biotransformation because of the industrial utility of (*R*)-**1** as a synthetic intermediate for clopidogrel. What we did was to find the best reaction temperature, at which the enzyme denaturation caused by a large amount of substrate/product as well as temperature is suppressed well, and at which the asymmetric reduction of **2** proceeds smoothly, giving the highest productivity of (*R*)-**1**. This working hypothesis turned out to be fine. Table 1 outlines how we optimized the productivity by changing the substrate concentration and the reaction temperature.

When the reaction temperature decreased by 5 °C, the conversion and isolated yield increased (entry 2), which prompted us to double the substrate concentration. Even at the substrate concentration of 0.6 M, the conversion reached 94% (entry 3). Therefore, we further increased the substrate concentration up to 1.0 M, which resulted in 90% conversion (entry 4). Finally, we further lowered the reaction temperature (entries 5 and 6) to find the best temperature giving the highest conversion at the same substrate concentration. Thus, the whole-cell reduction of 1.0 M of **2** at 20 °C gave 99% conversion and 1.78 g of isolated product (*R*)-**1** (entry 5), which corresponds to the productivity of 178 g/L (weight of isolated product per liter of initial reaction volume). When the progress of

Table 1. Asymmetric reduction of **2** with recombinant *E. coli*.[a]

Entry	[**2**]			$NADP^+$ [mg]	Cells [g]	*T* [°C]	*C* [%][b]	Yield [%][c]	*ee* [%][d]
	[g]	[g·L^{-1}]	[M]						
1	0.60	60	0.3	10	2	30	92	76	>99
2	0.60	60	0.3	10	2	25	>99	88	>99
3	1.20	120	0.6	10	2	25	94	88	>99
4	1.98	198	1.0	10	2	25	90	85	>99
5	1.98	198	1.0	10	2	20	99	89	>99
6	1.98	198	1.0	10	2	15	86	82	>99
7	1.98	198	1.0	10	2[e]	20	>99	89	>99
8	2.58	258	1.3	10	2	20	57	53	>99
9	2.58	258	1.3	10	2[e]	20	76	70	>99
10	1.98	198	1.0	5	2	20	69	62	>99
11	1.98	198	1.0	0	2	20	47	43	>99
12	1.98	198	1.0	10	1	20	42	36	>99
13	19.9	199	1.0	100	20	20	98	76	>99

[a] *Conditions:* **2** (quantity and concentration indicated above), wet cells of *E. coli* BL21(DE3) harboring pESCR and pABGD (quantity indicated above), glucose (2 equiv. with respect to **2**), $NADP^+$ (quantity indicated above), 0.1 M phosphate buffer [pH 7.0, 10 mL except for entry 13 (100 mL)], 24 h.
[b] Conversion determined by ^{1}H NMR.
[c] Isolated yield of (*R*)-**1**.
[d] Determined by HPLC [Chiralpak AD-H, hexane/*i*-PrOH (9:1)].
[e] CFE prepared from wet cells of *E. coli* BL21(DE3) harboring pESCR and pABGD (2 g) was used as a catalyst.

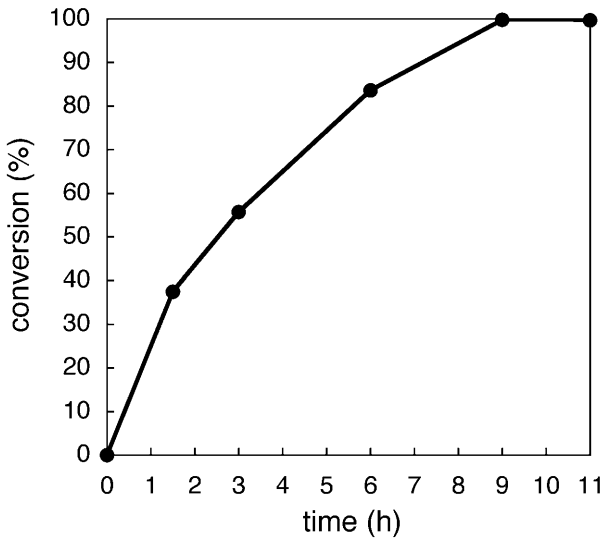


Figure 1. Time course of the bioreduction of **2**. For the reaction conditions, see entry 5 in Table 1. The reaction was monitored by ^{1}H NMR.

the reaction was monitored by NMR, the reaction was almost complete (>99% conversion) in 9 h as shown in Figure 1.

In the whole-cell reduction described above, the cells are not intact but permeabilized partially by exposure to a large amount of substrate and by magnetic stirring. Nevertheless, the cell membrane can hinder the mass transfer. Indeed, the cell-free extract (CFE) prepared from 2 g of the recombinant *E. coli* gave >99% conversion under otherwise the same reaction conditions (entry 7). Further comparison between the whole-cell reduction and the CFE reduction at a higher concentration (1.3 M) revealed that the latter was superior to the former (entries 8 and 9). The cell membrane therefore seems to retard the enzymatic reactions to some degree despite the facts that the local concentrations of the enzymes (SCR and GDH) in the cells are higher and that the enzymes in the cells are exposed to a lower amount of substrate/product. Despite these results, we selected the whole-cell reduction for further investigation because it is much more convenient than the CFE reduction.

Despite our efforts, all the attempts to further improve the efficiency of the production of (*R*)-**1** failed. For example, the decrease in the amount of $NADP^+$ or *E. coli* cells resulted in a decrease in conversion (entries 10, 11, and 12). We therefore concluded that the reaction conditions indicated in entry 5 are the best. We then scaled up the biotransformation by tenfold under the optimized reaction conditions (entry 13), and the bioreduction of 20 g of **2** in 100 mL of buffer at 20 °C for 24 h followed by distillation gave 15 g of optically pure (*R*)-**1** successfully (76% yield).

Although other researchers had gained the highest productivity at 20 °C in the whole-cell asymmetric reduction of ethyl 4-chloroacetoacetate,[16] the remarkable temperature effect on productivity as described above was beyond our expectation. We speculate that lowering the temperature led to a decrease in the mobility of SCR by strengthening the intramolecular hydrogen bonds, which might suppress the protein denaturation caused by heat and/or a large amount of substrate/product to retain the catalytic activity of SCR. Apart from the mechanistic origin, the present whole-cell reduction is quite promising from a practical viewpoint; only several examples of microbial reduction systems capable of giving productivity higher than 100 g/L have been reported.[1,2,10b,g,k,l,17–20]

The result that (*R*)-**1** was obtained in >99% *ee* (Table 1) was unexpected for the following reason. Although an analogous alcohol, ethyl (*R*)-mandelate [(*R*)-**3**], had been obtained in 92% *ee* before,[12b] the fact that the (*R*)-enantiomer of **3** was obtained could not be explained well by a stereochemical trend we had found.[11b] Figure 2a illustrates the stereochemical trend observed for fifteen alcohols containing a carbonyl group in the substituent.[12b] We have postulated the fixation of the orientation of the ketone by hydrogen bonding followed by the attack of the β-face of the carbonyl group by NADPH (Figure 2b).[11b] The exception is (*R*)-**3** as shown in Figure 2a. Because of the irregular behavior of (*R*)-**3**, we could not predict even whether the enantiomeric purity of **1** would be higher or lower than that of **3**. Although no consistent models are available at present, the molecular mecha-

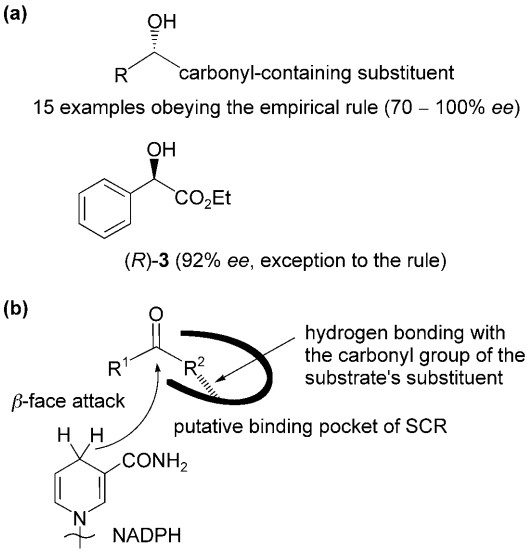
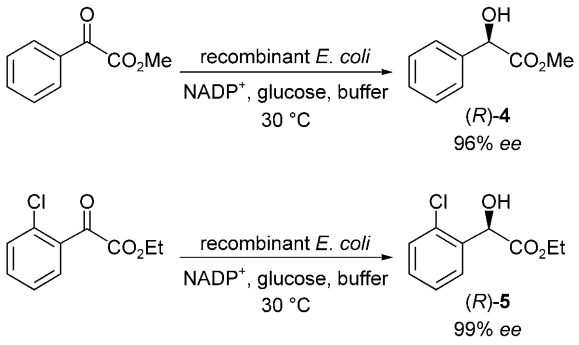


Figure 2. (a) A stereochemical trend observed for fifteen carbonyl-containing alcohols obtained by the bioreduction. The absolute configuration of (*R*)-**3** was opposite to the stereochemical trend. Data taken from ref.[12b] **(b)** A putative binding mode of a ketone bearing a carbonyl-containing substituent.

nism of enantioselectivity could be clarified by determining the crystal structure of SCR in future.

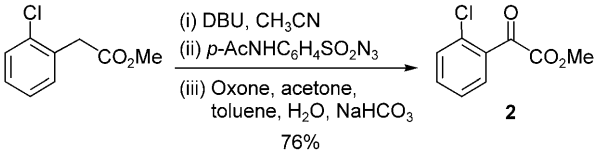
Here we identified the factors responsible for the highly enantioselective production of (*R*)-**1** by examining the enantiomeric purities of **4** and **5** obtained by the whole-cell reduction of the corresponding ketones. As shown in Scheme 2, (*R*)-**4** and (*R*)-**5** were



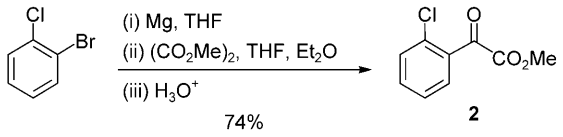
Scheme 2. Asymmetric reduction of α-keto esters.

obtained in 96 and 99% *ee*, respectively. Clearly, the presence of the chlorine atom and the replacement of the ethyl group by the methyl group each contributed to an enhancement of enantioselectivity, and the two modifications led to the production of (*R*)-**1** with >99% *ee*. In the bioreduction of a series of benzoyl formate esters, the difference in the bulkiness of two substituents seems to be much more important for enantioselectivity than the hydrogen bond depicted in Figure 2b, and hence the most unbalanced one, **2**, was converted to the corresponding alcohol with the highest % *ee* value.

The present whole-cell reduction of α-keto ester **2** was so efficient that the chemical synthesis of **2** became a bottleneck. For the preparation of **2**, we first employed the one-pot oxidation of methyl *o*-chlorophenylacetate according to the literature (Scheme 3).[15] Although it is an excellent method, the productivity of **2** is not so high, and the reagents used are rather expensive. To make this chemoenzymatic synthesis of (*R*)-**1** more successful, we searched for other synthetic methods highly suitable for **2**. We examined various methods and finally found the Grignard reaction shown in Scheme 4. The reaction of *o*-chlorophenylmagnesium bromide prepared from *o*-bromochlorobenzene with dimethyl oxalate gave α-keto ester **2** selectively. It is known that the nucleo-



Scheme 3. Preparation of **2** by the one-pot oxidation.



Scheme 4. Preparation of **2** with the Grignard reagent.

philic attack of a Grignard reagent to dialkyl oxalate forms a 1:1 adduct, which blocks the second attack of the reagent.[21] In this particular case, **2** was isolated in a good yield, although we needed to conduct the reaction at a low temperature to suppress the formation of benzyne generated from *o*-chlorophenylmagnesium bromide. For comparison, we also examined another synthetic method with the organolithium reagent (Scheme 5). Although the desired compound **2** was isolated successfully, the yield was only modest. The former method with the Grignard reagent (Scheme 4) is therefore more economical and suitable for the synthesis of **2**.

Scheme 5. Preparation of **2** with the organolithium reagent.

Conclusions

A highly efficient chemoenzymatic method for methyl (*R*)-*o*-chloromandelate [(*R*)-**1**] has been developed. The asymmetric reduction of methyl *o*-chlorobenzoylformate (**2**) with recombinant *E. coli* overproducing a versatile carbonyl reductase, SCR, gave (*R*)-**1** with >99% *ee*. This is the first example of the direct asymmetric synthesis of (*R*)-**1** with >99% *ee*. A remarkable temperature effect on productivity was observed in the whole-cell reduction of **2**, and the optimum productivity as high as 178 g/L was attained at 20 °C on a 2-g scale (1.0 M). This biotransformation could be scaled up easily, and the bioreduction of 20 g of **2** in 100 mL of buffer at 20 °C for 24 h gave 15 g of optically pure (*R*)-**1** successfully. The bioreduction of **2** is a practical process, because the hydride source is glucose, which is the cheap biomass, and because the catalyst is *E. coli*, which can be multiplied easily and inexpensively. Moreover, the bioreduction is performed in an aqueous solution under air. Two synthetic methods giving α-keto ester **2** in good yields were found. Although more experiments need to be done especially on a much larger scale, the present chemoenzymatic method has good potential for an industrial application because of the pharmaceutical value of the downstream product, clopidogrel.

Experimental Section

General

Methyl *o*-chlorobenzoylformate (**2**) was prepared according to the literature.[15] Methyl benzoylformate was purchased. *E. coli* BL21(DE3) cells harboring pESCR and pABGD were grown as reported previously.[12b] $NADP^+$ was purchased from Oriental Yeast. Silica gel and basic alumina column chromatography was performed using Fuji Silysia BW-127 ZH (100–270 mesh) and Merck aluminum oxide 90 active basic (0.063–0.200 mm), respectively. Thin layer chromatography (TLC) was performed on Merck silica gel 60 F_{254}.

Typical Procedure for the Whole-Cell Asymmetric Reduction of 2 to give Methyl (*R*)-*o*-Chloromandelate [(*R*)-1]

To a mixture of glucose (3.60 g, 20.0 mmol), $NADP^+$ (10 mg, 12 μmol), and *E. coli* BL21(DE3) cells harboring pESCR and pABGD (2.0 g) in 0.1 M phosphate buffer (pH 7.0, 10 mL) was added methyl *o*-chlorobenzoylformate (**2**) (1.98 g, 10.0 mmol). The mixture was stirred in a water bath at 20 °C for 24 h, during which 2 N NaOH was added to neutralize the solution acidified by the progress of the reaction. Solid NaCl (5.5 g) was added, and the product was extracted with EtOAc (3×25 mL), where centrifugation (3,200 rpm, 10 min) was conducted to promote the phase separation. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. Purification by silica gel column chromatography (hexane/EtOAc, 10:1) gave methyl (*R*)-*o*-chloromandelate [(*R*)-**1**] as a colorless oil; yield: 1.78 g (89%). $[\alpha]_D^{19}$: −178.3 (*c* 1.3, $CHCl_3$), >99% *ee*, (*R*) {lit.[22] $[\alpha]_D^{22}$: −146.3 (*c* 1.05, $CHCl_3$), 80.8% *ee*, (*R*)}; HPLC: Chiralpak AD-H (Daicel Chemical Industries), hexane/*i*-PrOH (9:1), flow rate 0.5 mL min^{-1}, detection 254 nm, (*S*) 20.3 min, (*R*) 22.7 min; ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 3.56 (d, *J* = 5.4 Hz, 1H), 3.78 (s, 3H), 5.57 (d, *J* = 5.4 Hz, 1H), 7.28–7.29 (m, 2H), 7.39–7.40 (m, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ = 53.2, 70.3, 127.2, 128.8, 129.8, 130.0, 133.5, 135.9, 173.7; IR (film): ν = 3454, 3003, 2955, 1744, 1441, 1223, 1090, 756 cm^{-1}.

Methyl (*R*)-Mandelate [(*R*)-4]

The whole-cell reduction of methyl benzoylformate was performed at 30 °C on a 3.0-mmol scale as described above. Isolated yield 72%. $[\alpha]_D^{23}$: −130.8 (*c* 1.0, MeOH), 96% *ee*, (*R*) {lit.[23] $[\alpha]_D^{20}$: −124.8 (*c* 0.8, MeOH), 86% *ee*, (*R*)}; GC: CP-cyclodextrin-β-2,3,6-M-19 (Chrompack, ø 0.25 mm×25 m), injector 300 °C, column 100 °C, detector 250 °C, (*R*) 77.8 min, (*S*) 83.6 min; ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 3.42 (d, *J* = 5.4 Hz, 1H), 3.77 (s, 3H), 5.18 (d, *J* = 5.4 Hz, 1H), 7.34–7.43 (m, 5H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ = 53.1, 72.9, 126.6, 128.5, 128.6, 138.2, 174.1; IR (KBr): ν = 3443, 3032, 2955, 1740, 1435, 1209, 1096, 739 cm^{-1}.

Ethyl (*R*)-*o*-Chloromandelate [(*R*)-5]

A solution of methyl *o*-chlorobenzoylformate (**2**) (3.01 g, 15.2 mmol) and H_2SO_4 (0.6 mL) in EtOH (380 mL) was heated at reflux for 4 d. After removal of EtOH by rotary evaporation, brine was added, and the product was extracted with EtOAc. The organic layer was dried over $MgSO_4$ and concentrated. The product was purified by basic alumina column chromatography (hexane/EtOAc, 10:1–0:1) to afford ethyl *o*-chlorobenzoylformate[7] as a pale yellow oil; yield: 1.42 g (44%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 1.41 (t, *J* = 7.5 Hz, 3H), 4.43 (q, *J* = 7.5 Hz, 2H), 7.40–7.46 (m, 2H), 7.53 (ddd, *J* = 8.1, 7.8, 1.8 Hz, 1H), 7.77 (dd, *J* = 7.8, 1.8 Hz, 1H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ = 13.9, 62.8, 127.2, 130.5, 131.6, 133.3, 133.9, 134.3, 163.1, 186.6; IR (film): ν = 3071, 2988, 1732, 1699, 1589, 1198, 1065, 1018, 760 cm^{-1}.

The whole-cell reduction of ethyl *o*-chlorobenzoylformate was performed at 30 °C on a 3.0-mmol scale as described above. Isolated yield: 93%. $[\alpha]_D^{22}$: −149.0 (*c* 1.0, $CHCl_3$), 99% *ee*, (*R*) {lit.[24] $[\alpha]_D^{25}$: −45.0 (*c* 4.0, $CHCl_3$), 62% *ee*, (*R*)}; HPLC: Chiralcel OD-H, hexane/*i*-PrOH (20:1), flow rate 1.0 mL min^{-1}, detection 254 nm, (*S*) 12.3 min, (*R*) 14.6 min; ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 1.22 (t, *J* = 6.9 Hz, 3H), 3.56 (d, *J* = 5.1 Hz, 1H), 4.18–4.30 (m, 2H), 5.55 (d, *J* = 5.1 Hz, 1H), 7.27–7.30 (m, 2H), 7.38–7.41 (m, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ = 14.0, 62.5, 70.4, 127.1, 128.8, 129.7, 129.9, 133.6, 136.1, 173.2; IR (film): ν = 3468, 3067, 2984, 1732, 1217, 1090, 756 cm^{-1}.

Preparation of CFE for the Asymmetric Reduction of 2

E. coli BL21(DE3) cells harboring pESCR and pABGD (2.0 g) in 0.1 M phosphate buffer (pH 7.0, 5 mL) were lysed by sonication for 1 min in an ice bath (×10). Centrifugation (20,000 rpm, 4 °C, 30 min) gave a cell-free extract (CFE) as the supernatant. The precipitate was suspended in the phosphate buffer (5 mL), and centrifugation (20,000 rpm, 4 °C, 10 min) gave a CFE. The CFE solutions were combined and used for the asymmetric reduction of **2**. The CFE reduction of **2** was conducted as described for the whole-cell reduction of **2**.

Preparation of Methyl *o*-Chlorobenzoylformate (2) using the Grignard Reagent

A solution of *o*-bromochlorobenzene (1.24 g, 6.48 mmol) in dry THF (8 mL) was added dropwise to a mixture of Mg (0.163 g, 6.71 mmol) and I_2 (1 piece) in dry THF (2 mL) at 10–15 °C over 0.5 h under N_2. To the Grignard reagent thus prepared was added a solution of dimethyl oxalate (0.503 g, 4.26 mmol) in dry THF (10 mL) and dry Et_2O (10 mL) at −70 °C *via* syringe. After the mixture had been stirred at −70 °C for 1 h, the reaction was quenched with 10% HCl. The product was extracted with EtOAc, and the organic layer was dried over $MgSO_4$ and concentrated. Purification by silica gel column chromatography (hexane/EtOAc, 10:1) gave methyl *o*-chlorobenzoylformate (**2**) as a pale yellow oil; yield: 626 mg (74%). ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 3.96 (s, 3H), 7.42 (ddd, *J* = 7.8, 7.2, 1.2 Hz, 1H), 7.45 (dd, *J* = 7.8, 1.2 Hz, 1H), 7.54 (ddd, *J* = 7.8, 7.2, 1.8 Hz, 1H), 7.77 (dd, *J* = 7.8, 1.8 Hz, 1H); ^{13}C NMR ($CDCl_3$, 150 MHz): δ = 53.3, 127.3, 130.5, 131.6, 133.2, 133.9, 134.4, 163.4, 186.2; IR (film): ν = 3013, 2955, 1740, 1701, 1589, 1435, 1254, 1207, 1065, 1007, 760 cm^{-1}.

Preparation of Methyl *o*-Chlorobenzoylformate (2) using the Organolithium Reagent

To a solution of *o*-bromochlorobenzene (1.24 g, 6.48 mmol) in dry THF (10 mL) was added dropwise *n*-BuLi (1.65 M in hexane, 4.6 mL, 7.59 mmol) at −80 °C over 10 min under N_2. The solution was stirred at −80 °C for 20 min. To the aryllithium compound thus prepared was added a solution of dimethyl oxalate (0.982 g, 8.32 mmol) in dry THF (5 mL) at −80 °C via syringe. After the mixture had been stirred at −80 °C for 2 h, the reaction was quenched with saturated aqueous NH_4Cl. The product was extracted with EtOAc, and the organic layer was dried over $MgSO_4$ and concentrated. Purification by silica gel column chromatography (hexane/EtOAc, 10:1) gave **2** as a pale yellow oil; yield: 420 mg (33%).

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