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Facile Synthesis of Enantiopure 4-Substituted 2-Hydroxy-4-butyrolactones using a Robust *Fusarium* Lactonase

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Abstract: A facile chemo-enzymatic process has been developed for producing stereoisomers of 4-substituted 2-hydroxy-4-butyrolactones with good to excellent enantioselectivity. This process involves an easy separation of the diastereoisomers by column chromatography and efficient enzymatic resolution by whole cells of *Escherichia coli* JM109 expressing *Fusarium proliferatum* lactonase gene. This biocatalyst shows strong tolerance towards different substrate structures and at least three out four possible isomers could be obtained in excellent enantiomeric purity. Different substrate concentrations (10 mM–200 mM) were examined, giving a substrate to catalyst ratio of up to 26:1. This general and efficient enzymatic process provides access to stereoisomers of 4-substituted 2-hydroxy-4-butyrolactones readily and cost-effectively. The stereochemical assignments were conducted systematically based on NMR, X-ray diffraction and circular dichroism, leading to further understanding of the enzyme's stereoselectivity.

Keywords: chemo-enzymatic process; circular dichroism; *Fusarium proliferatum* lactonase; stereoselectivity; 4-substituted 2-hydroxy-4-butyrolactones

Introduction

4-Substituted 2-hydroxy-4-butyrolactones comprise a vast family of compounds with great importance. As chiral building blocks, they have been widely used in the pharmaceutical industry for the production of ACE inhibitors[1a] such as Enalapril, Cilazapril, Benazepril etc. They are also used in the synthesis of natural products with antitumor or cytotoxic activities, including Apoptolidin,[1b] Epothilones,[1c] Salicylihalamides[1d] and Bengazoles.[1e] Some of these lactones have been found to be human hunger modulators[2] or exhibit significant antitumor and cytotoxic activities against cultures of P388 cells.[3]

Due to the great interest in their four stereoisomers, different strategies to make these chiral lactones have been developed. These include: a) chiral pool synthesis, using glucose,[1d] malic acid,[1c] xylose[3] and so on; b) chiral auxiliaries, such as hydrazine SAEP[4] and c) asymmetric catalysis, including enzymatic[5] and non-enzymatic routes.[6] However, strategies a and b share common problems, such as expensive starting materials or auxiliaries and complicated synthetic routes.[4,7] On the other hand, asymmetric catalysis has the advantage that it can produce large quantities of optically pure compounds from simple starting materials using relatively small amount of catalysts.[8]

However, the development of a suitable asymmetric catalytic method is a challenging task. A successful asymmetric catalysis should meet several criteria including ready availability and reusability of the catalysts and the method should have general applicability and afford both enantiomers in high *ee*.

Indeed, none of the existing catalytic methods (strategy c) could be considered ideal or economical. Casy et al.[5] reported the enzymatic reduction of the α-keto group of 2,4-diketo acids and subsequent non-enzymatic *syn*- or *anti*-selective reduction, but the bio-reduction process requires a very low substrate-to-catalyst ratio (from 1/20[9] to 8/1[10]) and prolonged reaction time (from 2 days to 12 days, respectively).

In addition, only one substrate, 4-phenyl-2,4-diketobutyric acid, was studied in this method. Carpentier et al.[6] reported asymmetric hydrogenation of readily available ethyl 2,4-diketo acids using a series of metal/ligand systems. The efficiency and selectivity of the catalysts were highly dependent on the substrate structures and only two [(2*R*,4*R*)- and (2*R*,4*S*)-lactones] of the four stereoisomers of 4-substituted 2-hydroxy-4-butyrolactones could be prepared.

Aiming for a practical catalytic approach to prepare the four stereoisomers of 4-substituted 2-hydroxy-4-butyrolactones, we developed a novel procedure *via* separation of 4-substituted 2-hydroxy-4-butyrolactones into *cis*- and *trans*-isomers and subsequent bioresolution by *Fusarium proliferatum* lactonase.

Fusarium lactonases had been shown to readily catalyze the enantioselective hydrolysis of 2-hydroxy-γ-lactones to optically pure hydroxy acids twenty years ago,[11] since then recombinant *E. coli* cells expressing *Fusarium* lactonase have gradually become an easy-to-make biocatalyst.[12] However, most of the applications were focused on the production of the (*R*)-isomer of 2-hydroxy-γ-lactones, especially D-pantolactone.[13] In our group, we had cloned *Fusarium proliferatum* lactonase gene[14] and expressed it into *E. coli* JM109. In this paper, we report for the first time the successful application of this biocatalyst in the enantioselective synthesis of 4-substituted 2-hydroxy-4-butyrolactones with two chiral centres and four stereoisomers.

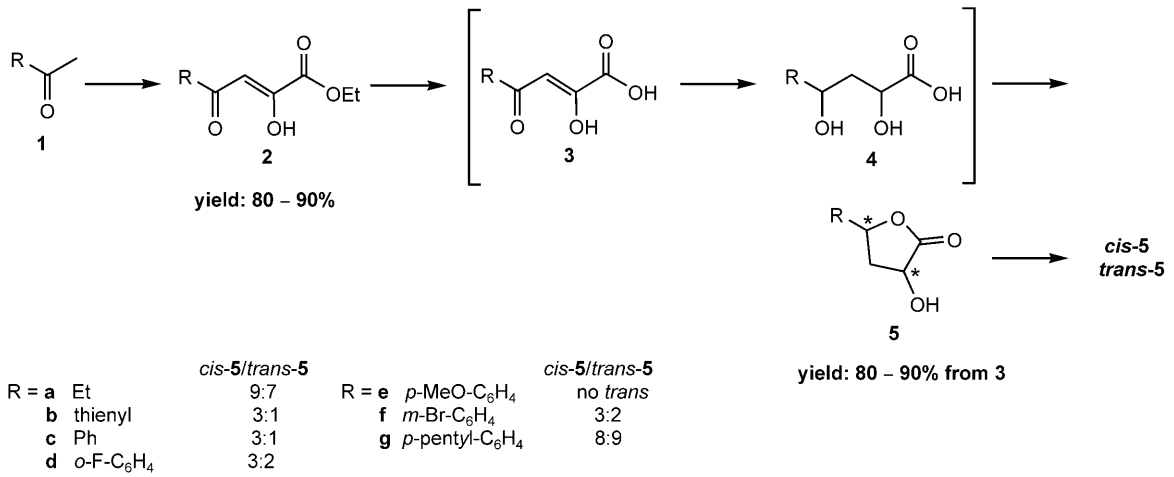
Results and Discussion

Synthesis of the Racemic Substrates

The present study began with the preparation of *cis*- and *trans*-4-substituted 2-hydroxy-4-butyrolactone isomers (Scheme 1) as substrates of reFPL. Crude compound **2,** prepared according to a previous literature method,[5] with 80%–90% purity, was subjected to NaOH hydrolysis without further purification. After acidification of the reaction mixture, the precipitate of keto acid **3** was collected in nearly quantitative yield as colourless crystals. It was reduced with $NaBH_4$ in methanol, and subsequent *in situ* cyclization afforded a mixture of *cis*- and *trans*-**5** in 80–90% yield, which was separated by column chromatography on silica gel. We also examined the effects of temperature on the diastereoselectivity in $NaBH_4$ reduction. Reduction of 2,4-dioxo-4-(*o*-fluorophenyl)-butyrate (**3d**) was carried out at different temperatures, and the ratio of *syn*-**4d** to *anti*-**4d** was monitored using ^{19}F NMR. Moderate diastereoselectivity of 1.25:1–1:1.5 (*anti*-**4d**/*syn*-**4d**) was observed when the temperature was varied between −40 °C and 60 °C.[15] We did not further optimize this ratio, but Casy[5] reported that it was possible to obtain a ratio from 6:1 to 1:50 (*cis*-**5c**/*trans*-**5c**), which would help to improve our theoretical yield from 50% to 100%. In this process, only one column chromatography was needed to separate the *cis*- and *trans*-isomers, which makes the synthesis of racemic lactones more cost effective.

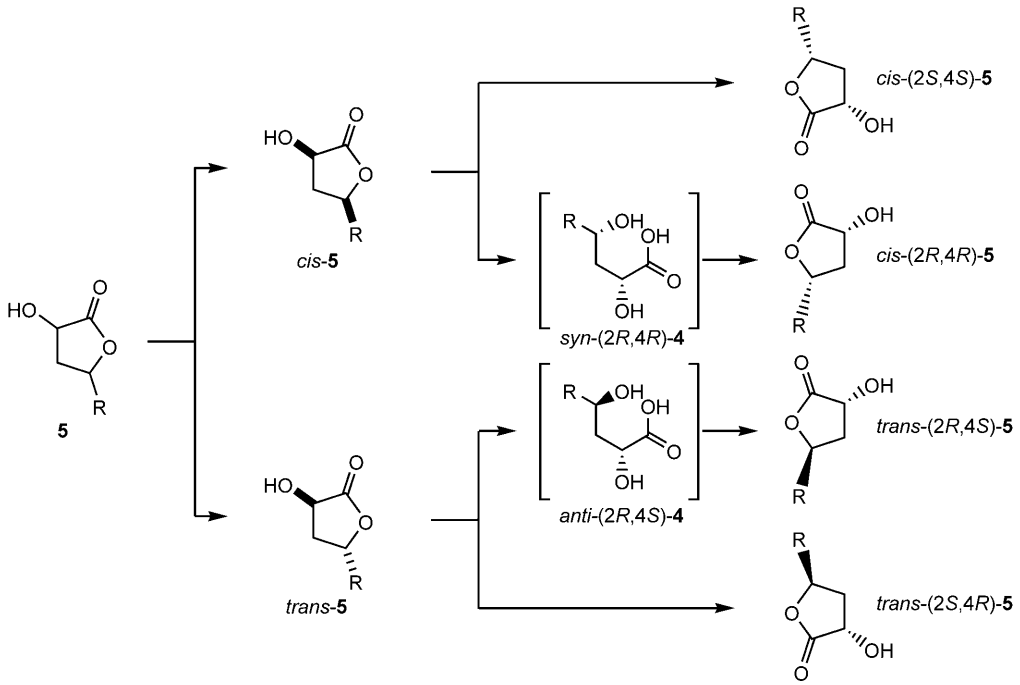
Enzymatic Resolution

After *cis*-**5** and *trans*-**5** had been separated, we submitted them to enzymatic resolution (Scheme 2). Both racemic *cis*-**5** and *trans*-**5** were readily recognized (except **5g**, possibly because the *p*-pentylphenyl group is too bulky and could not be accepted by the active site of the enzyme) by recombinant *E. coli* JM109 cells harboring plasmid pET28a carrying *Fusarium proliferatum* lactonase (reFPL) gene. No side reaction was observed. *E. coli* JM109 harboring plasmid



	cis-**5**/*trans*-**5**		*cis*-**5**/*trans*-**5**
R = **a** Et	9:7	R = **e** *p*-MeO-C_6H_4	no *trans*
b thienyl	3:1	**f** *m*-Br-C_6H_4	3:2
c Ph	3:1	**g** *p*-pentyl-C_6H_4	8:9
d *o*-F-C_6H_4	3:2		

Scheme 1. Synthesis of 4-substituted 2-hydroxy-4-butyrolactones.



Scheme 2. *Fusarium* lactonase catalyzed chemo-enzymatic synthesis of four isomers of 4-substituted 2-hydroxy-4-butyrolactones (R = aromatic or heteroaromatic rings).

pET28a alone showed no hydrolytic activity, indicating that the asymmetric hydrolysis was totally due to the activity of reFPL.

In a typical resolution process, we chose *cis*-**5c** as the model substrate. To prepare (2*S*)-**5c**, a 10 mM solution of racemic *cis*-**5c** in 10 mL potassium phosphate buffer (KPB, 50 mM, pH 6.4) was treated with an appropriate amount of the biocatalyst (wet cells of *E. coli* JM109). MeCN (200 μL) was chosen as co-solvent to enhance the solubility of **5c** in water. The biocatalytic hydrolysis took one hour, and by the end of the resolution, (2*S*)-isomer of *cis*-**5c** was isolated by simple extraction in near theoretical yield and >99% *ee* (Table 1).[15,16]

When (2*R*)-**5c** was the target compound, undesired (2*S*)-**5c** needs to be extracted completely, and the residue was acidified with 2 N HCl to pH 2.0 and heated for one hour at 60 °C.[15] The cyclized (2*R*)-**5c** was then isolated (>99% *ee*) by extraction.

Relaxed Substrate Specificity of reFPL

A series of lactones were then tested in the resolution process described above. Various substrate structures could be accepted by this robust catalyst, indicating a great generality of this method (Table 1). Excellent enantioselectivity (>99% *ee*) and isolated yield (50.8%–57.8%) were obtained for all of the (2*S*)-**5** substrates in the process of enzymatic resolution (in the case of **5e**, the isolated isomer turned out to be 2*R* instead of 2*S*). For (2*R*)-**5**, good to excellent enantioselectivity (84.3% to >99.0% *ee*) could also be obtained using the same biocatalyst. Most of the *cis*-**5** isomers, except **5a**, could be crystallized, which facilitates the isolation and purification of these compounds. Therefore at least three of the four possible stereoisomers could be prepared in optically pure form by using one single robust biocatalyst. For *trans*-(2*R*,4*S*)-**5**, good (76.5% *ee*) to excellent (95.3% *ee*) enantioselectivities were achieved.

This enzyme showed higher hydrolytic activity towards **5a** than other lactones with more bulky aromatic or heteroaromatic ring substituents, possibly due to the smaller size of the ethyl group in **5a** which may fit better in the enzyme's active site. For **5b**–**5f**, the enzyme showed higher hydrolytic activity towards *trans*-**5** than the corresponding *cis*-**5** with the same aromatic ring substituent but different conformations, indicating the conformations of substrates also play an important role in the process of enzymatic hydrolysis. The enzymatic activity was also affected by the substituents on the aromatic ring. Substrate **5e** with an electron-donating group on the phenyl ring inhibited the activity of reFPL.

We also tested the biocatalyst's hydrolytic activity towards other substrates (Figure 1). As reported previously, lactones **7a** and **7b** could be readily recognized by this enzyme. When the hydroxy group was alkylated (**8**), the enzyme could no longer hydrolyze this compound. Compounds **6a**, **6b** and **9** are not suitable substrates for this enzyme either. It is well

Table 1. Biocatalyzed asymmetric hydrolysis of 4-substituted 2-hydroxy-4-butyrolactones.

Entry	Biocatalyst (IU)[a]	Configuration	*ee* [%]	Conversion [%]
1	3	*cis*-(2*R*,4*S*)-**5a**	96.5[c]	34.5[f]
2		*cis*-(2*S*,4*R*)-**5a**	>99[c]	51.3 (0.3 h)[g]
3	10[b]	*cis*-(2*R*,4*R*)-**5b**	84.3[d]	25.4[f]
4		*cis*-(2*S*,4*S*)-**5b**	>99[d]	57.3 (2 h)[g]
5		*trans*-(2*S*,4*R*)-**5b**	>99	52.1 (1.5 h)[g]
6	10	*cis*-(2*R*,4*R*)-**5c**	>99[d]	30.0[f]
7		*cis*-(2*S*,4*S*)-**5c**	>99[d]	51.4 (1.5 h)[g]
8	10	*trans*-(2*R*,4*S*)-**5c**	95.3[e]	36.9[f]
9		*trans*-(2*S*,4*R*)-**5c**	>99[e]	53.4 (1.0 h)[g]
10	10	*cis*-(2*R*,4*R*)-**5d**	>99[e]	37.8[f]
11		*cis*-(2*S*,4*S*)-**5d**	>99[e]	50.8 (1.5 h)[g]
12	10	*trans*-(2*R*,4*S*)-**5d**	86.5[d]	32.1[f]
13		*trans*-(2*S*,4*R*)-**5d**	>99[d]	53.6 (1.2 h)[g]
14	15	*cis*-(2*S*,4*S*)-**5e**	80.3[d]	34.2[f]
15		*cis*-(2*R*,4*R*)-**5e**	>99[d]	53.4 (3 h)[g]
16	10	*cis*-(2*R*,4*R*)-**5f**	98.3[e]	34.6 (1 h)[g]
17		*cis*-(2*S*,4*S*)-**5f**	>99[e]	52.1 (1.5 h)[g]
18	10	*trans*-(2*R*,4*S*)-**5f**	76.5[e]	40.2[f]
19		*trans*-(2*S*,4*R*)-**5f**	>99[d]	55.4 (1.4 h)[g]

[a] *Reaction conditions:* 30 °C; 10 mL KPB buffer (pH 6.4, 50 mM) containing 2% (v/v) MeOH was mixed with 10^{-4} mol substrate (10 mM); *E. coli* wet cells were added in various amounts; details for enzyme assay are summarized in the Supporting Information.
[b] Due to the unstable nature of *trans*-**5c**, we did not prepare *trans*-(2*S*,4*R*)-**5c**, and the absolute configuration was postulated according to the enantioselectivity of reFPL toward *cis*-**5c**.
[c] Analyzed by chiral GC β-cyclodextrin column.
[d] Analyzed by chiral HPLC with an AD-H column.
[e] Analyzed by chiral HPLC with an OJ-H column.
[f] Conversion was calculated from formula: $c = ee_s/(ee_s + ee_p)$.[16]
[g] Isolated yield is given.

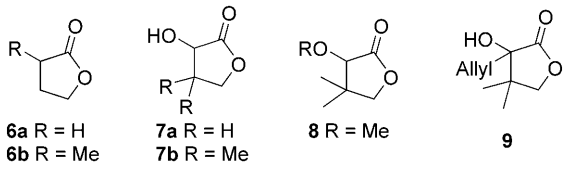


Figure 1. Compounds tested in the enzymatic hydrolysis.

known that the 2-hydroxy group is essential in this enzymatic hydrolysis. Our results on 2-methoxylactone suggest that the hydrogen in the hydroxy group may also play an important role in this process.

Increased Substrate Load

A high ratio of substrate-to-catalyst (nearly 26 g of substrate/1 g of biocatalyst in dry cell weight) could also be achieved in this strategy, suggesting high efficiency of this biocatalyst. When *cis*-**5c** was again chosen as the model substrate, the substrate concentration could be increased to 100–200 mM and the reaction is complete within 1–3 h with conversion of *ca.* 65% [*ee* of *cis*-(2*S*)-**5c** >99.0%] using a catalytic amount of 0.13 g of wet cells (about 0.02 g of dry cells) in a 10 mL reaction system (Figure 2). When the substrate concentration was increased to 300 mM, the percentage of MeCN had to be increased to 40% (v/v) leading to complete loss of the enzymatic activity of the whole cells. Moreover, this biocatalyst could

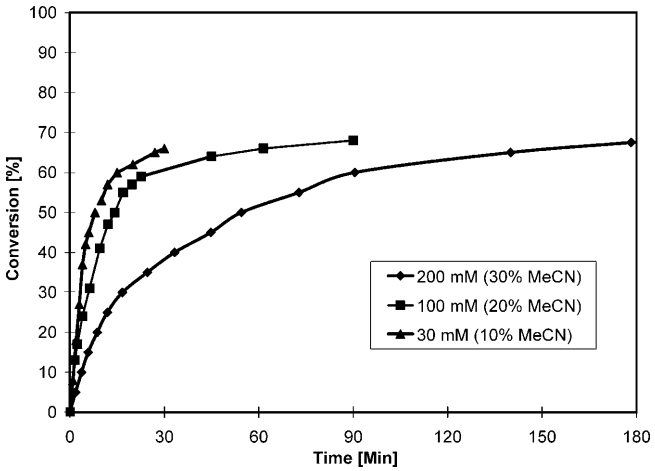


Figure 2. ReFPL-catalyzed asymmetric hydrolysis of *cis*-**5c** at different substrate concentrations.

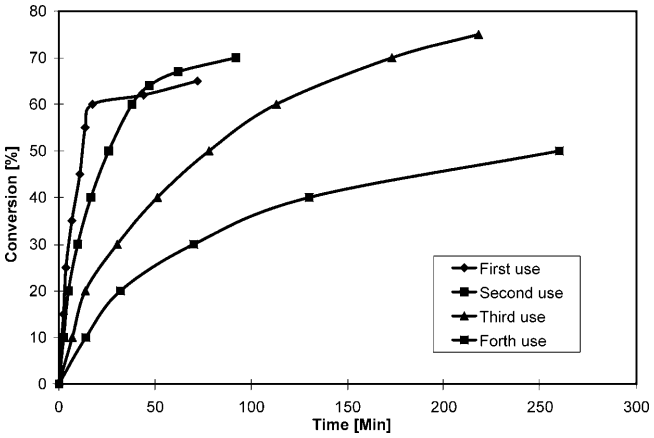


Figure 3. Reuse of whole cells of recombinant *E. coli* JM109 expressing *Fusarium proliferatum* lactonase gene.

be reused for at least three times at a substrate concentration of 100 mM (20% MeCN, v/v) with acceptable conversion rate (Figure 3) in less than 8 h, which could improve the ratio of substrate to catalyst from 8.8 g substrate/1 g catalyst to 26 g substrate/1 g catalyst. About 10% of the initial enzyme activity remained[15] in the fourth use and it was necessary to prolong the reaction time to more than 8 h. We are currently working on the immobilization of the whole cells that may improve the enzyme's stability against high concentration of MeCN to further improve the ratio of substrate-to-biocatalyst and reduce the cost of this process.

Stereochemical Assignment of Chiral 4-Substituted 2-Hydroxy-4-buyrolactones

We used ^{1}H NMR and NOE experiments to assign the relative configuration (Figure 4).[6,17] In *cis* lactones H-2 exhibits a dd peak with coupling constants of ~8.1 Hz ($J_{2,3'}$) and ~11.1 Hz ($J_{2,3}$) while H-4 also shows a dd peak with coupling constants of ~5.1 Hz ($J_{4,3'}$) and ~11.1 Hz ($J_{4,3}$).[18,19] The ^{1}H NMR spectra of *trans* lactones show the proton signal at the 2-position as a dd peak with the same coupling constants of ~7.8 Hz ($J_{2,3}$ and $J_{2,3'}$) while the proton signal at the 4-position is a dd peak with the coupling constants of ~4.5 Hz and ~7.5 Hz. NOE experiments also validate these findings.

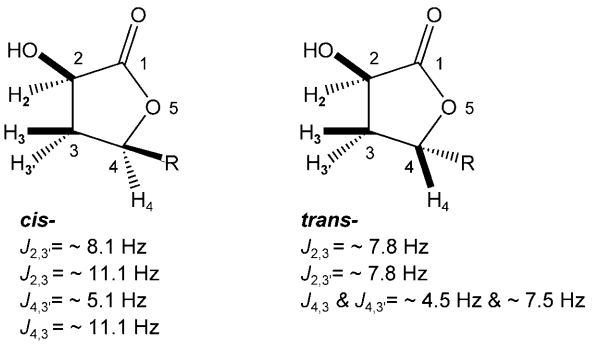


Figure 4. Coupling constants of H-2 and H-4 in 4-substituted 2-hydroxy-4-butyrolactones.

The absolute structure (AS) assignment of chiral 4-substituted 2-hydroxyl-4-butyrolactones was a challenge. Ito et al. assigned[3] the AS of natural product (+)-harzialactone A (4-benzyl 2-hydroxy-4-butyrolactone) using the Mosher ester method but the results were challenged by Mereyala et al.[3] Kiegiel et al. established the configuration of chiral 4-methyl 2-hydroxy-4-butyrolactone unambiguously by transforming this compound into its derivative which could crystallize to afford the single crystals suitable for X-ray analysis.[20] However, this method is limited to crystalline compounds (or its derivatives) only. Although the limitation to crystalline compounds could be overcome by synthesis from chiral building blocks, it could be time-consuming to do so.

Circular dichroism (CD), which has been widely used in the determination of absolute configurations of natural products and small molecule drugs,[21] is our choice for the assignment of the absolute configuration of 2-hydroxy-4-butyrolactones in a facile and direct way. We examined the CD spectra of nine compounds[16] and divided them into two types. Type a includes *trans*-(2*S*,4*R*)-**5c**, **5d**, **5f** with positive C.E. curves in the n→π* transition region at *ca.* 223.5–233 nm indicating *S* configuration of the hydroxy group.[22] Type b consists of *cis*-**5b**, **5c**, **5d**, **5e** and **5f**, among which *cis*-(2*S*,4*S*)-**5b**, **5c**, **5d** and **5f** show a positive sign in the n→π* transition region (*ca.* 230–250 nm) to confirm the *S* configuration of the hydroxy group while *cis*-(2*R*,4*R*)-**5e** and *cis*-(2*R*,4*R*)-**5f** give a negative sign in the n→π* transition region indicating the *R* configuration of the hydroxy group. From these CD results, we could deduce that γ-lactones with a (2*S*)-hydroxy group would give a positive Cotton effect curve in the n→π* region, obeying the Okuda's rule.[23] The substituent attached to the γ-carbon (C-4) has little, if any, contribution in the sign of the C.E. curve.

X-ray diffraction analyses of crystalline *cis*-(2*S*,4*S*)-**5b** and *cis*-(2*S*,4*S*)-**5f**[24] gave unambiguous stereochemical assignments including relative and absolute configurations and the "equatorial" orientation of both hydroxy and aromatic (heteroaromatic) groups (Figure 5).

Conclusions

An example of the successful asymmetric catalysis was described in this contribution. The readily available biocatalyst affords high *ee* towards both enantio-

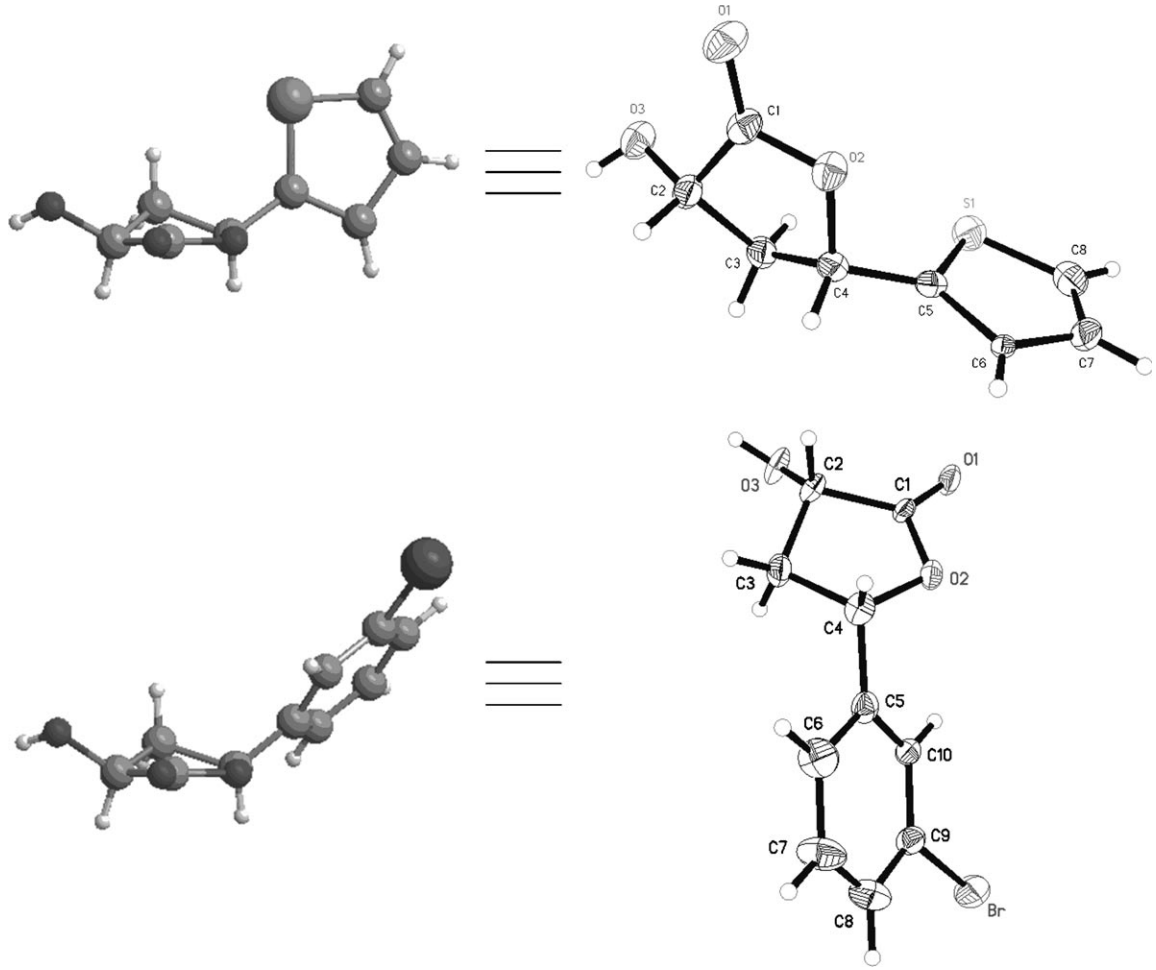


Figure 5. Crystal structure of *cis*-(2*S*,4*S*)-**5b** (*top*) and *cis*-(2*S*,4*S*)-**5f** (*bottom*).

mers with reusability and great generality. The biocatalytic resolution is highly efficient, and the optically pure product was isolated by simple extraction. In addition to the "elegance" of the biocatalysis, the substrate was prepared in a facile way from readily available starting materials. All of these could compensate for the drawbacks in theoretical yield and contribute to the robustness of this process.

In summary, we have developed a versatile process towards chiral 4-substituted 2-hydroxy-4-butyrolactones with a single biocatalyst. We also developed a CD method to establish the stereochemistry of these compounds. This process combines the ready availability of the starting materials and a highly efficient biocatalyst and should prove to be useful in the synthesis of natural products and pharmaceutical compounds.

Experimental Section

Synthesis of Intermediate 2

To a slurry of newly prepared NaOEt (0.1 mol) and Et_2O (50 mL), a mixture of diethyloxalic acid (0.1 mol) and compound **1** (0.1 mol) in dry Et_2O (15 mL) solution was added carefully in small portions (in the case of **1b** and **1e**, the temperature was controlled at −20 °C) and kept overnight. The mixture was then poured into dilute HCl and extracted with ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum. Purification of the product (compound **2**) was done by column chromatography on silica gel (petroleum ether: ethyl acetate = 5:1).

Synthesis of Intermediate 3

To a 250-mL round-bottom flask was added crude compound **2** (42 mmol) in 50 mL THF, followed by LiOH (50.4 mmol) in 50 mL water in one portion. Saponification was terminated when compound **2** was consumed completely by TLC analysis (petroleum ether:ethyl acetate = 1:1).

The mixture was concentrated under vacuum to remove the THF and then was extracted with ethyl acetate (50 mL) three times. The aqueous layer was acidified with 6 N HCl and a large amount of compound **3** precipitated. This precipitate was filtered and washed with water (100 mL) three times and dried under vacuum. It can be used without further purification.

Synthesis of 2-Hydroxy-4-butyrolactones 5

To a 250-mL round-bottom flask containing a stirrer, crude compound **3** (47.6 mmol) in 50 mL MeOH was added, followed by $NaBH_4$ (190 mmol) in small portions at room temperature. The mixture was stirred for another 3 h. 50 mL water were added to decompose residual $NaBH_4$ and the mixture of **4** was concentrated under vacuum to remove the solvent. Then the mixture was acidified to pH 1.0–2.0 and heated at 60 °C to afford compound **5. 5** was extracted with ethyl acetate and dried over anhydrous sodium sulfate. The separation of the diastereomers was done by column chromatography on silica gel (petroleum ether:ethyl acetate = 6:1 ~ 3:1).

Expression of Recombinant *Fusarium proliferatum* (reFPL) Lactonase

FPL gene sequence was ligated with Nde I and BamH I digested pET28a. The ligation mixture was introduced by transformation into *E. coli* JM109 and then screening of clones containing the restriction fragment was performed. The resultant plasmid was designated as pET28a-FPL. The recombinant *E. coli* JM109 (DE3) containing pET28a-FPL was cultured aerobically at 37 °C in 50 mL LB medium until OD_{600} reached 0.7, and then the cultures were immediately shifted to 23 °C. IPTG was added at a final concentration of 0.5 mM to induce the *lac* promoter. After further cultivation, the cells were harvested by centrifugation and washed thoroughly with physiological saline, and then were used for further studies.

Enzyme Assay

The standard assay mixture comprised 2.5% (*w/v*) racemic pantolactone and an appropriate amount of the cells with a final volume of 10.0 mL. The reaction was performed at 30 °C, and the pH was automatically controlled at 6.7–6.9 with 0.1 M NaOH. The hydrolysis rate of lactone was calculated based on the rate of NaOH titration. One unit of lactonase (1 IU) was defined as the amount of the enzyme that converted 1.0 μmol of pantolactone into pantoic acid per minute under the above conditions.

Resolution of Substrates by Recombinant *E. coli* Cells

An appropriate amount of wet cells was re-suspended in 10 mL potassium phosphate buffer (KPB, 50 mM, pH 6.4) and the enzyme activity of this aqueous solution was measured as 13.3 IU/100 μL.

General procedures were as following: To 10 mL potassium phosphate buffer (KPB, 50 mM, pH 6.4) was added an appropriate volume of this aqueous solution and 0.1 mmol of *cis*-**5** (or *trans*-**5**) in 200 μL methanol. This mixture in a 100-mL flask was kept at 30 °C and shaken at 180 rpm. The resolution process was monitored by chiral HPLC (AD-H or OJ-H columns) and terminated at the appropriate time. (2*S*)-**5** was extracted with ethyl acetate directly, while the solution containing (2*R*)-**5** was worked up by acidification (pH 2.0, 2 N HCl) and heating (60 °C, 1 hour) after which the product was extracted with ethyl acetate.

Scale-Up Preparation of *cis*-(2*S*,4*S*)-5c

An appropriate amount of wet cells was resuspended in 10 mL potassium phosphate buffer (KPB, 50 mM, pH 6.4). To 500 mL water was added an appropriate volume of this aqueous solution and 17.8 g of *cis*-**5c** in 150 mL MeCN. This mixture in a 1-L beaker was kept in room temperature and stirred. The pH was automatically controlled at 6.3–6.5 with 0.1 M NaOH. The resolution was monitored by HPLC (AD-H column) and terminated when the *ee* value of *cis*-(2*S*,4*S*)-**5c** reached >99%. The biocatalyst was centrifugated and the supernant was extrated with ethyl acetate directly. Concentration of this organic layer afforded 5.5 g of *cis*-(2*S*,4*S*)-**5c** as colourless crystals.

Scale-Up Preparation of *cis*-(2*R*,4*R*)-5c

An appropriate amount of wet cells was resuspended in 10 mL potassium phosphate buffer (KPB, 50 mMм, pH 6.4). To 500 mL water was added an appropriate volume of this aqueous solution and 17.8 g of *cis*-**5c** in 150 mL MeCN. This mixture was kept in room temperature and stirred. The pH was automatically controlled at 6.2–6.4 with 0.1 M $NH_3 \cdot H_2O$. The resolution was terminated when conversion reached around 30%. The biocatalyst was centrifugated and the supernant was concentrated in vacuum to remove MeCN and then extrated with ethyl acetate to remove the *cis*-(2*S*,4*S*)-**5c** completely. While *syn*-(2*R*,4*R*)-**4c** in this aqueous layer was acidified and heated, after which the product was extracted with ethyl acetate to afford 4.9 g of crystals. Recrystallization of these crystals in petroleum ether/ethyl acetate (5:1) gave 4.1 g of colourless crystals in >99% *ee*.

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