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New synthesis of harzialactone A via kinetic resolution using recombinant *Fusarium proliferatum* lactonase

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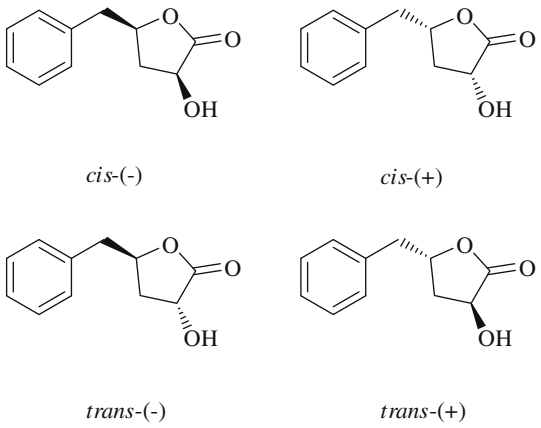
ABSTRACT

A new synthesis of harzialactone A together with its stereoisomers was achieved starting from phenyl acetone. The key step of this new route is the kinetic bioresolution of racemic *cis*- and *trans*-isomers by recombinant *Escherichia coli* JM109 cells expressing *Fusarium proliferatum* lactonase gene (reFPL). Harzialactone A was isolated in excellent ee (>99%), while moderate to good enantioselectivities (80% to >99% ee) were obtained for its isomers.

1. Introduction

Harzialactone A (Scheme 1) is an antitumor marine metabolite isolated from *Trichoderma harianium* OUPS-N115 strain and has been reported to exhibit cytotoxic activity against the P388 lymphocytic leukaemia test system in cell cultures.[1] Much effort has been directed towards the asymmetric synthesis of this natural product. Although Ito et al. established its absolute configuration by the Mosher ester method, Mereyala et al. revised its configuration and developed synthetic routes from D-glucose (seven steps, 15% overall yield).[2] They also prepared (−)-harzialactone A from D-xylose (eight steps, 24% yield).[3] In 2007, Sudalai et al. reported another route employing proline-catalyzed sequential α-aminoxylation–HWE olefination of 3-phenylpropanal (four steps, 26.1% overall yield).[4] Jian et al. also synthesized (−)-harzialactone A in 3 steps from malic acid (yield of 40%).[5]

Previously, we have developed a method to prepare 4-substituted 2-hydroxy-4-butyrolactone using robust recombinant *Fusarium proliferatum* lactonase and assign the relative and absolute structure.[6] Herein, we applied this method to synthesize the natural product harzialactone A and its three stereoisomers in a facile and 'green' way for the first time using recombinant *F. proliferatum* lactonase (reFPL) with defined relative and absolute structure.



Scheme 1. Structures of four isomers of harzialactone A.

2. Results and discussion

Condensation (Scheme 2) of phenyl acetone and ethyl oxalate by freshly prepared NaOEt (1.1 equiv) in ethyl ether solution was carried out at −20 °C for 2 h, and then left to warm to room temperature overnight. Routine work-up of this reaction mixture afforded compound **2**. After saponification and acidification, compound **3** precipitated out as white needle crystals from water and was submitted directly to subsequent $NaBH_4$ reduction. After acidification and lactonization, compound **5** was obtained (dr *cis*:*trans*/3:2) and separated by column chromatography on silica gel. Previous reports have demonstrated the asymmetric *syn*- and *anti*-reduction of 1,3-diketo may be well manipulated by changing the reducing agents and reaction temperature.[7]

With *cis*-**5** and *trans*-**5** in hand, we submitted them to enzymatic resolution (Scheme 2). Both racemic *cis*-**5** and *trans*-**5** were readily recognized by recombinant *Escherichia coli* JM109 cells harboring plasmid pET28a carrying *F. proliferatum* lactonase (reFPL) gene.[8] No side reactions were observed. *E. coli* JM109 harboring

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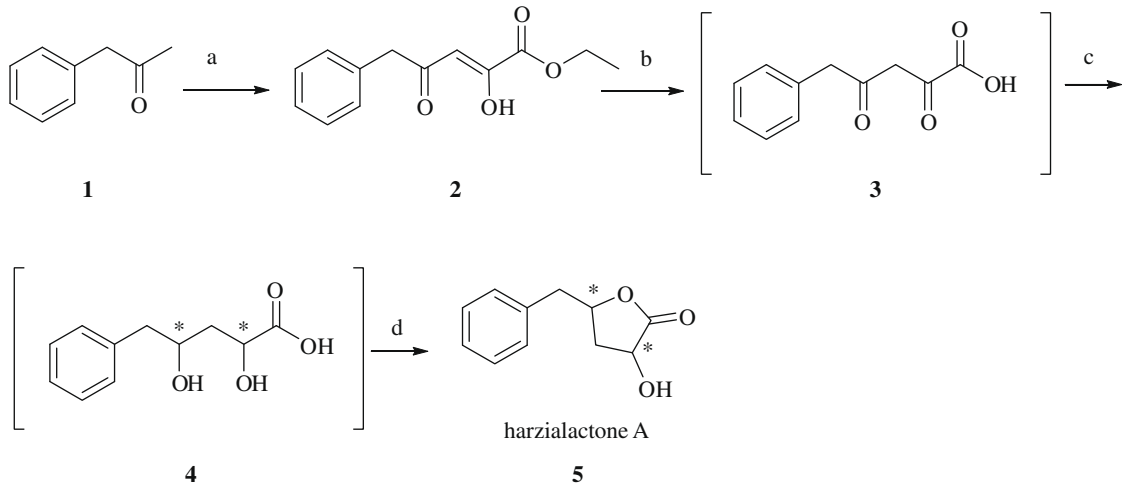
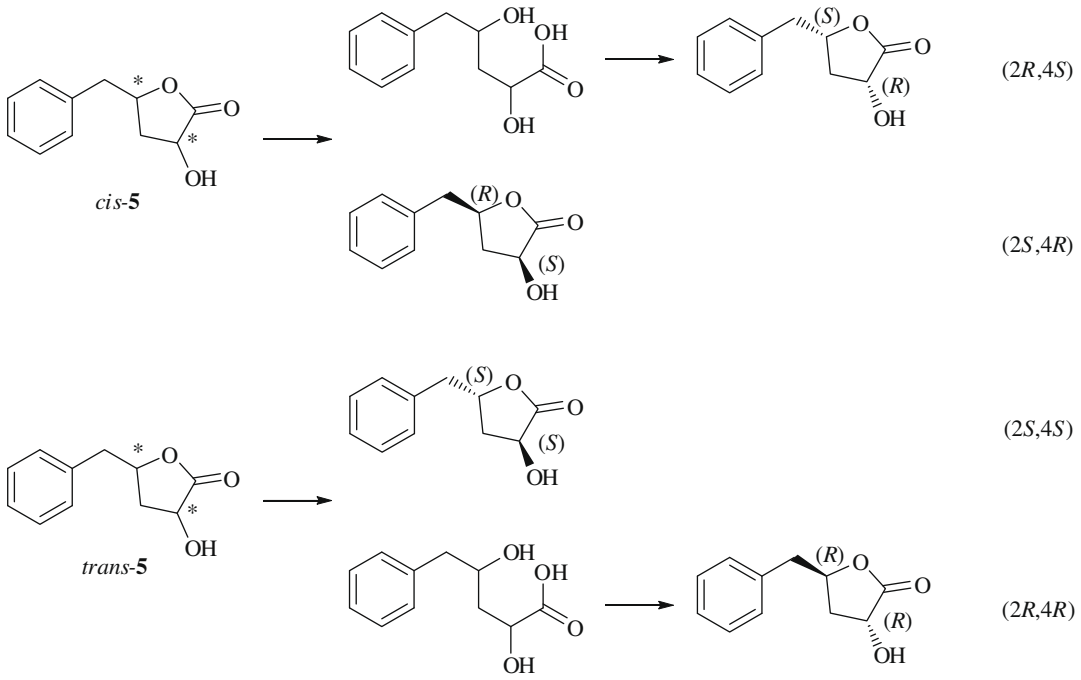


Scheme 2. The synthetic route to harzialactone A [(±)-**5**]. Reagents and conditions: (a) 1.1 equiv NaOEt/1.1 equiv ethyl oxalate/Et_2O/−20 °C/overnight; (b) (i) LiOH/THF–H_2O (1:1), (ii) Concentrated HCl; (c) 4 equiv $NaBH_4$/CH_3OH; (d) concentrated HCl/60 °C.



Scheme 3. Separation of *cis*- and *trans*-4-benzyl 2-hydroxy-4-butyrolactones by column chromatography combined with bioresolution catalyzed by *Fusarium* lactonase. Bioresolution conditions: 30 °C; 10 ml KPB buffer (pH 6.4, 50 mM); 2% (v/v) MeCN; 10 mM substrate; 4 IU wet cells.

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

In a typical resolution (Scheme 3), racemic *trans*-harzialactone A (0.1 mmol) in 10 ml (10 mM) of buffer (KPB, 50 mM, pH 6.4) was treated with 30 μl of the biocatalyst (4 IU, substrate-to-catalyst ratio of 20/1),[9] and MeCN was used as the cosolvent (2%, v/v). On completion of the resolution (~10 min), the mixture was centrifuged at 10,000 *g* to remove the biocatalyst. The undesired (−)-(2*S*,4*S*) harzialactone A was removed by extensive extraction of the supernatant with ethyl acetate, and the aqueous layer was acidified to pH 2.0 with dilute HCl and heated for 1 h at 60 °C. The cyclized lactone was then extracted with ethyl acetate, and the combined extract was concentrated under vacuum to afford (+)-(2*R*,4*R*)-harzialactone A as colorless crystals.

(−)-(2*S*,4*S*)-Harzialactone A isolated in this process was contaminated with unreacted (+)-isomer. The enzymatic reaction was extended to 30 min to hydrolyze completely the undesired

Table 1
Results of resolution of harzialactone A catalyzed by recombinant *Fusarium proliferatum* (reFPL)

Entry	Absolute configuration	ee (%)	Isolated yield (%)	$[\alpha]_D^{20}$
1	(2*R*,4*S*)-**5**	80.2[a]	43.8	—
2	(2*S*,4*R*)-**5**	97.5[a]	44.8	−9.0
3	(2*S*,4*S*)-**5**	>99[b]	47.2	−32.5
4	(2*R*,4*R*)-**5**	>99[b]	45.9	+33.5

[a] Analyzed by a chiral HPLC AD-H column.
[b] Analyzed by a chiral HPLC OJ-H column.

(2*R*,4*R*)-**5**. Then (−)-(2*S*,4*S*)-harzialactone A was isolated from the reaction mixture as colorless crystals (>99% ee) by simple extraction.

The asymmetric hydrolysis of racemic *cis*-harzialactone using reFPL was also accomplished using similar procedure. Both (2*S*,4*R*)-**5** and (2*R*,4*S*)-**5** were obtained in moderate to high ee values and the results are summarized in Table 1.

We have previously reported the hydrolytic properties of reFPL towards various 4-aromatic 2-hydroxy-4-butyrolactone.[6] Herein, when the phenyl group was replaced with a benzyl group an increase in reFPL activity was observed. When (+)-(2*R*,4*R*)-harzialactone A was the desired product, the bioresolution process was completed within less than 10 min in a substrate-to-catalyst ratio of 20/1, which was far more efficient (about 15-fold) than that of 4-phenyl 2-hydroxy-4-butyrolactone reported previously. This feature of reFPL with the combination of convenient access to racemic substrates could compensate for the drawbacks in the theoretical yield and contribute to the robustness of this process.

The relative configurations of *cis*- and *trans*-lactones were confirmed by ^{1}H NMR. The assignment of the absolute structure was a challenge.[1–5] Ito et al. first assigned the configuration of native harzialactone A to be (2*S*,4*S*) (+33.3) using a Mosher ester method in 1998, after they isolated this compound. However, this conclusion was contested by Mereyala et al. in 1999, who described the concise synthesis of harzialactone A from D-glucose. They assigned the native harzialactone A to be (2*R*,4*R*), and this assignment is further supported by later results. Our specific rotations (Table 1) were consistent with Mereyala et al.'s reports.

We had studied the enzymatic hydrolysis of 2-hydroxy-4-butyrolactones and found that *Fusarium* lactonase specifically recognizes (2*R*)-hydroxyl group and leaves (2*S*)-lactones intact.[10] As for 4-substituted 2-hydroxy-4-butyrolactone, we also have developed a CD method[6] to assign the absolute structure and this method was confirmed by X-ray diffraction. Herein, we applied this method to assign the absolute structure of harzialactone A. The CD spectra of (2*S*,4*R*)-**5** and (2*S*,4*S*)-**5** (Figure 1) both showed positive C.E. curves in the $n\rightarrow\pi^*$ transition region at ca. 225 nm indicating an (*S*)-configuration of the hydroxyl group. These results are also consistent with Mereyala et al.'s finding.

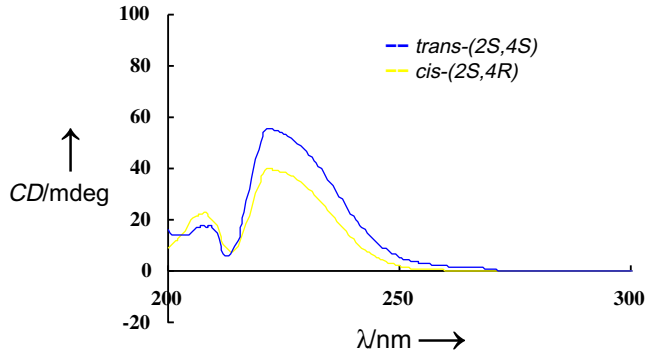


Figure 1. CD spectra of harzialactone A (10^{-4} mM in MeOH).

3. Conclusion

In conclusion, we have developed a chemo-enzymatic route to harzialactone A for the first time with a defined configuration, which is considered to be more eco-friendly.

4. Experimental

4.1. Synthesis of intermediate 2

To a slurry of newly prepared NaOEt (0.1 mol) and Et_2O solution (50 ml), a mixture of diethyl oxalic acid (0.1 mol) and phenyl acetone (0.1 mol) in dry Et_2O (25 ml) solution was added carefully in small portions at −20 °C within 2 h and then kept overnight at room temperature. The mixture was then poured into diluted HCl and extracted with ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo. Purification of the product **2** was carried out by column chromatography on silica gel (petroleum ether/ethyl acetate = 4:1).

4.2. Synthesis of intermediate 3

To a 250-ml round-bottom flask was added crude compound **2** (40 mmol) in 50 ml THF, followed by LiOH (60 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 in vacuo to remove THF and the residue was extracted with ethyl acetate (50 ml) three times. The aqueous layer was acidified with 6 N HCl and large amount of compound **3** precipitated. This precipitate was filtered and washed with water (100 ml) three times and dried in vacuo. It can be used without further purification.

4.3. Synthesis of harzialactone

To a stirred 250-ml round-bottom flask, crude compound **3** (40 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. Next, 50 ml water was added to decompose residual $NaBH_4$ and the mixture of **4** was concentrated in vacuo to remove the solvent. Then the mixture was acidified to pH 1.0–2.0 and heated at 60 °C to afford racemic harzialactone **5**. Compound **5** was then 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). NMR data of *cis*-harzialactone A: ^{1}H NMR (300 MHz, CDCl3) δ 7.21–7.32 (m, 5H), 4.49–4.60 (m, 2H), 3.46 (br, 1H), 2.91–3.17 (m, 2H), 2.56–2.65 (m, 1H), 1.91–1.99 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 177.6, 135.5, 129.4, 128.7, 127.2, 77.4, 68.6, 41.3, 36.6 ppm. NMR data of *trans*-harzialactone A: ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.19–7.33 (m, 5H), 4.87–4.95 (m, 1H), 4.07–4.13 (dd, J = 8.1 Hz, 8.1 Hz, 1H), 2.96–2.98 (m, 2H), 2.39–2.23 (m, 2H) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ 177.7, 135.3, 129.6, 128.8, 127.3, 78.4, 67.1, 41.1, 34.5 ppm.

4.4. Expression of recombinant *F. proliferatum* (reFPL) lactonase

FPL gene sequence was ligated to pET28a. The ligation mixture was introduced by the 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, at which point 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 were then used for further studies.

4.5. Enzyme assay

The standard assay mixture comprised of 2.5% (w/v) racemic pantolactone and 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 the 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.

4.6. Resolution of substrates by recombinant *E. coli* cells

Appropriate amounts of wet cells were 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 10 IU/100 μl. General procedures were as following: To a 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 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 when the desired product reached the appropriate enantiomeric purity. Compound (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 M HCl) and heating (60 °C, 1 h) after which the product was extracted with ethyl acetate.

Acknowledgements

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9. New appropriate amount of wet cells were 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. Then this solution was used as the biocatalyst.
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