# Enzymatic resolution and evaluation of enantiomers of *cis*-5′-hydroxythalidomide†

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**The straightforward synthesis of both enantiomers of *cis*-5′-hydroxythalidomide, a major metabolite of thalidomide, has been accomplished by enzymatic kinetic resolution of a racemic substrate catalyzed by *Pseudomonas stutzeri* lipase TL. *cis*-5′-Hydroxythalidomide shows resistance to racemization (and epimerization) at physiological pH. A tube formation assay to assess the ability to inhibit angiogenesis revealed that *cis*-5′-hydroxythalidomides are inactive.**

Despite the notorious medical disaster caused by thalidomide (**1**), huge numbers of papers have been published about this drug since its formulation in 1956, because of its potential for treating a number of intractable diseases, such as leprosy, human immunodeficiency virus replication in acquired immune deficiency syndrome, and cancer.[1] The pharmacological aspects of thalidomide and its derivatives have been frequently investigated; however, studies focusing on their enantioselective biological activities are limited[2] due to the ease of racemization,[3] as well as the lack of availability of the enantiomers.[4] Therefore, the answer to the question of whether thalidomide is enantiospecifically teratogenic is still a matter of concern.[5] Incidentally, the teratogenic properties of thalidomide appear to require prior biotransformation,[6] and its anticancer activities have been attributed to the formation of metabolites.[7] Therefore, much attention has recently been focused on the synthesis and biological activities of thalidomide metabolites (Fig. 1).[6–8]

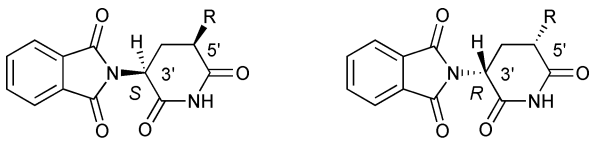


**Fig. 1** Structures of (*S*)- and (*R*)-thalidomides (**1**) and their metabolites, (3′*S*,5′*R*)- and (3′*R*,5′*S*)-5′-hydroxythalidomides (**2**).

*cis*-5′-Hydroxythalidomide (**2**), one of the major metabolites of thalidomide, is found to show enhanced inhibition of TNF-α production.[8l] Although the first synthesis of racemic **2** was reported 10 years ago,[9a] there has not been any method for the asymmetric synthesis of **2**.[9b] During the course of our recent investigations, Hashimoto *et al.* reported the synthesis of (3′*S*,5′*R*)-**2** from L-hydroxyproline.[8l] The synthetic study of optically active **2** was also examined by Luzzio *et al.*[9c] Previously, we reported the synthesis of fluorinated thalidomide[10a] and a three step synthesis of thalidomide enantiomers.[10b] Here, we report an efficient enzymatic asymmetric synthesis of both enantiomers of the thalidomide metabolite **2**. Racemization studies showed that **1** was found to racemize; however, **2** shows resistance to racemization (and epimerization) at physiological pH. Preliminary biological evaluation of the enantiomers of **1** and **2** in an angiogenic assay[11] is also briefly described.

Several approaches to the asymmetric synthesis of **2** could be considered. Enzymatic kinetic resolution[12] of the racemic substrate should be the most practical and direct route for the preparation of enantiomeric **2**, because both enantiomers of **2** are needed for biological evaluation. In the first set of experiments, the efficiency of different commercially available lipases in the catalysis of the acylation of the hydroxy group in **2** was investigated using an excess of vinyl acetate as the acyl donor in 1,4-dioxane at 37 °C (Table 1, and Table S1 in the supplementary information†). After screening of the enzymes, lipase TL from *Pseudomonas stutzeri* was found to be effective for this enzymatic kinetic resolution. Optimization of the reaction conditions under different acyl donors, solvents and temperatures, identified lipase TL from *Pseudomonas stutzeri*, with isopropenyl acetate in acetone at 37 °C as the best combination for the enzymatic kinetic resolution of **2**. Enantiomerically pure (3′*S*,5′*R*)-**2** (>99.9% ee, 45% yield) and (3′*R*,5′*S*)-**3** (81% ee, 55% yield) were obtained with an *E* value of 70. (3′*R*,5′*S*)-**3** was easily converted to enantiomerically pure (3′*R*,5′*S*)-**2** (>99.9% ee) under refluxing in MeOH in the presence of TsOH followed by single recrystallization. Optically pure enantiomers of **2** showed characteristic CD spectra reciprocal to one another: CD spectra of the pair of enantiomers of **2** shown in Fig. 2 indicate quite good agreement with those in the literature (Fig. 2).[6g] The absolute configurations of (3′*S*,5′*R*)- and (3′*R*,5′*S*)-**2** were also identified by optical rotation.[8l] Although the asymmetric synthesis of (3′*S*,5′*R*)-**2** was recently reported,[8l] the lipase-mediated direct acylation of the racemic substrate offers a simple and efficient route to access both enantiomers of **2**.

We next investigated the stabilities of **1** and **2** toward racemization (epimerization) and hydrolysis (decay).[3b,13] Optically pure **1** and **2** were incubated at 37 °C and varying pH values, and monitored by HPLC using reported methods by Hashimoto *et al.* for racemization,[3b] and by Lafont *et al.*[13a] and Schumacher *et al.*[13b]

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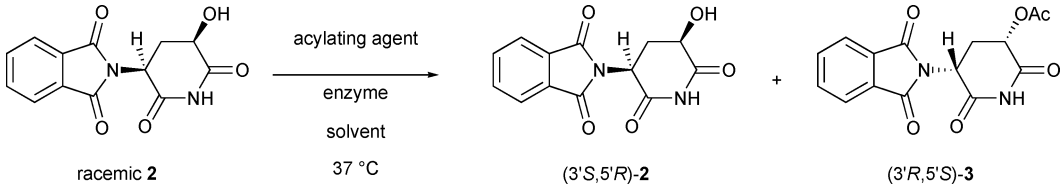
**Table 1** Enzymatic kinetic resolution of racemic **2**[a]




| Run | Enzyme | Solvent | Acylating agent | Time/h | (3′*S*,5′*R*)-**2** ee (%)[b] | (3′*R*,5′*S*)-**3** ee (%)[b] | Conv. **3** (%) | *E*[c] |
|---|---|---|---|---|---|---|---|---|
| 1 | Lipase AS | 1,4-Dioxane | Vinyl acetate | 24 | 3 | 14 | 4 | 5 |
| 2 | Lipase QLM | 1,4-Dioxane | Vinyl acetate | 24 | 11 | 30 | 27 | 2 |
| 3 | Lipase PS | 1,4-Dioxane | Vinyl acetate | 24 | <1 | 33 | <3 | <2 |
| 4 | CALB | 1,4-Dioxane | Vinyl acetate | 72 | 0 | 0 | 0 | — |
| 5 | Lipase TL | 1,4-Dioxane | Vinyl acetate | 24 | 54 | 70 | 44 | 10 |
| 6 | Lipase TL | 1,4-Dioxane | Allyl acetate | 12 | 7 | 19 | 27 | 2 |
| 7 | Lipase TL | 1,4-Dioxane | *t*-Butyl acetate | 12 | 29 | 79 | 27 | 11 |
| 8 | Lipase TL | 1,4-Dioxane | i-Propenyl acetate | 12 | 39 | 81 | 33 | 14 |
| 9 | Lipase TL | $CH_2Cl_2$ | Vinyl acetate | 12 | 22 | 67 | 25 | 6 |
| 10 | Lipase TL | $^{i}Pr_2O$ | Vinyl acetate | 12 | 22 | 54 | 29 | 4 |
| 11 | Lipase TL | Toluene | Vinyl acetate | 12 | 24 | 79 | 23 | 11 |
| 12 | Lipase TL | MeCN | Vinyl acetate | 12 | 78 | 73 | 52 | 15 |
| 13 | Lipase TL | Acetone | Vinyl acetate | 12 | 96 | 68 | 59 | 20 |
| 14 | Lipase TL | Acetone | i-Propenyl acetate | 12 | 50 | 88 | 36 | 26 |
| 15 | Lipase TL | Acetone | i-Propenyl acetate | 18 | 85 | 88 | 49 | 42 |
| 16 | Lipase TL | Acetone | i-Propenyl acetate | 67 | >99.9 | 81 | 55 | >70 |

[a] All reactions were performed on a 7.3 μmol scale with 4 mg of enzyme in 0.2 mL of solvent with 0.1 mL of acylating agent. [b] Enantiomeric excesses were determined by HPLC using a CHIRALCEL OD-RH with ethanol as elute. [c] *E* value was calculated from $E = \ln[(1 - c)(1 - ee_s)]/\ln[(1 - c)(1 + ee_s)]$, where $c = ee_s/(ee_s + ee_p)$.

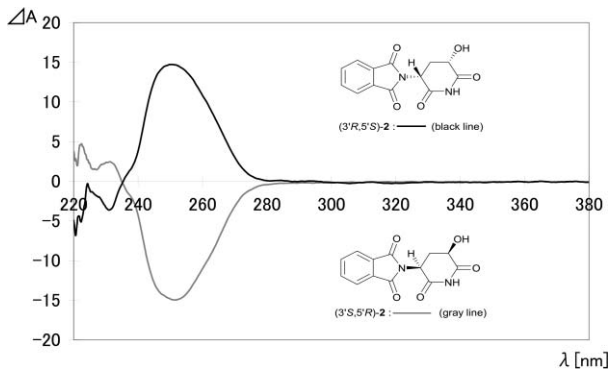



**Fig. 2** CD spectra of (3′*S*,5′*R*)-**2** and (3′*R*,5′*S*)-**2**.

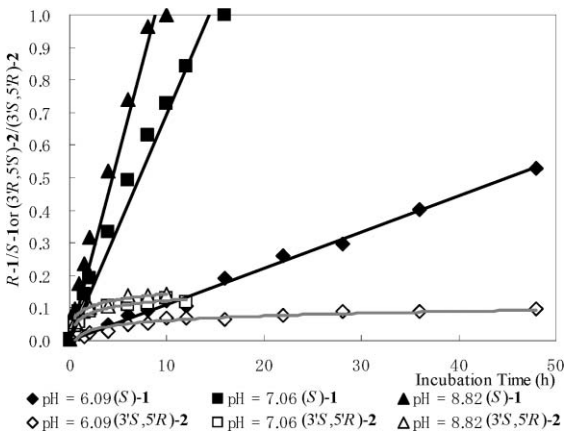



**Fig. 3** Racemization study of (*S*)-**1** and racemization–epimerization study of (3′*S*,5′*R*)-**2**.

for hydrolysis. CHIRALCEL OD-RH with ethanol was used for the separation. Three buffer systems, pH 6.09 (100 mM sodium phosphate monobasic, 100 mM sodium phosphate dibasic), pH 7.06 (100 mM sodium phosphate monobasic, 100 mM sodium phosphate dibasic) and pH 8.82 (40 mM Tris base, 40 mM hydrochloric acid) were employed. The results are shown in Fig. 3 and 4. In the racemization study, **1** was racemized using all buffer solutions, the rate of racemization was rather quick in neutral and alkaline buffer solutions, which was consistent with earlier studies.[4] On the other hand, under these conditions less than 12% racemization of **2** was observed (Fig. 3) and no detectable epimerization from *cis*-**2** to its *trans*-isomer occurred. The half-life of the racemization of **1** was estimated by a plot of the experimental data: $t_{0.5}$ is where $R/S = 0.5$. While the racemization half-lives of **1** were found to be 45 h at 6.09 pH, 7.2 h at 7.06 pH, and 4.4 h at 8.82 pH in buffer solution at 37 °C, those of **2** were $2.7 \times 10^{10}$ h at 6.09 pH, $4.3 \times 10^{7}$ h at 7.06, and $1.4 \times 10^{7}$ h at 8.82. The configurational stability of *cis*-**2** can be explained as shown in Fig. 5. The 3′-5′-diequatorial conformation in the glutarimide moiety of *cis*-**2** was revealed by X-ray crystallography.[6f,14] $^1$H NMR data also supported the preferable diequatorial conformation of *cis*-**2** (3′H: dd, $J$ = 5.2, 13.2 Hz; 5′H: dd, $J$ = 5.2, 12.6 Hz). These observations allowed us to propose a mechanism for the epimerization–racemization process of **2** *via* the axial–equatorial conformation. Even though the compound *cis*-**2**, as well as **1**, is configurationally labile at C-3′, its epimer (*trans*-isomer of **2**) has its hydroxy group in the more unfavourable axial position *via* an inversion at C-3′ followed by conformational flipping. Thus the apparent stability of **2** is mainly caused by the chemical equilibrium (thermodynamic control), resulting in the low racemization rate. This epimerization–racemization process is also supported by

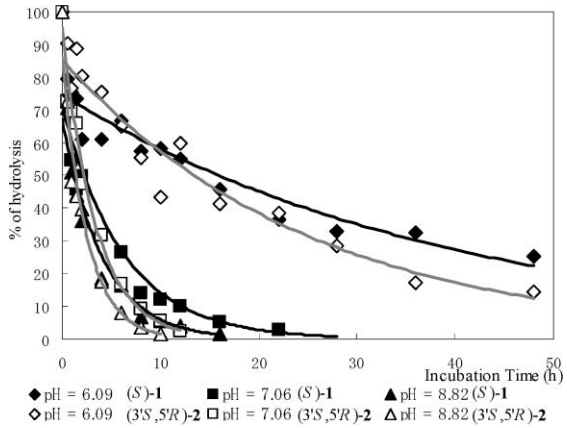



**Fig. 4** Hydrolysis (decay) studies of (*S*)-**1** and (3′*S*,5′*R*)-**2**.

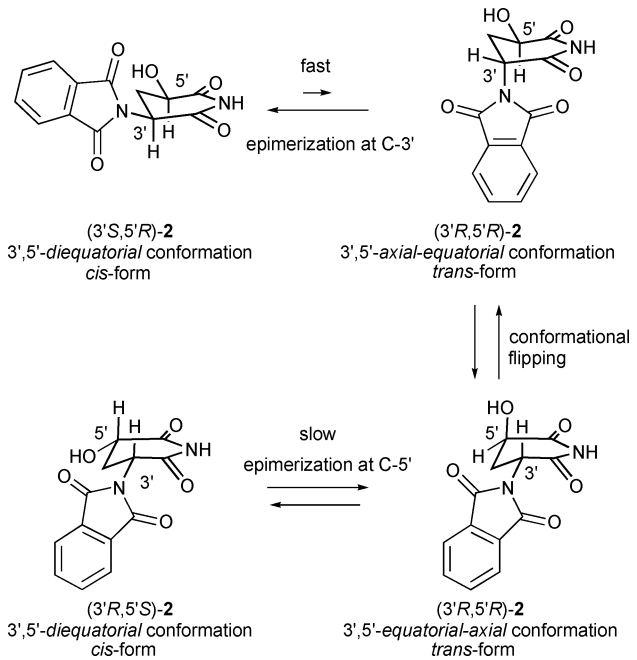



**Fig. 5** Postulated scheme for the epimerization–racemization process of **2**.

the reports by Blaschke *et al.*[6f,g] Although the possibility of direct epimerization at C-5′ *via* an enolate is not ruled out, the deprotonation at C-5′ would not be easy due to the presence of a free hydroxy group. The effects of the pH dependent hydrolysis (decay) of **1** and **2** are shown in Fig. 4. The data for **1** and **2** were essentially similar to each other in the range of pH 6–9 (Fig. 4).

Finally, preliminary enantioselective biological evaluation of **1** and **2** was investigated. Among the diverse biological activities of thalidomide that have been suggested, we were interested in anti-angiogenesis because it is hypothesized that the disruption of blood vessel formation in the fetal limb bud might be responsible for the teratogenicity of thalidomide.[11] Despite the easiness of the racemization of **1** (Fig. 3),[3] the differences in biological activities between thalidomide enantiomers have fragmentally been reported.[2] The tube formation assay,[15] one of the trusted assay systems, was examined using an angiogenesis assay kit according to the manufacturer's instructions. HUVECs co-cultured with fibroblasts were cultivated in the presence or absence of various concentrations of test drugs plus VEGF-A (10 ng ml$^{-1}$). After 11 days, cells were fixed in 70% ethanol. The cells were incubated with diluted primary antibody (mouse anti-human CD31, 1 : 4000) for 1 h at 37 °C, and with the secondary antibody (goat anti-mouse IgG alkaline phosphatase-conjugated antibody, 1 : 500) for 1 h at 37 °C, and visualization was achieved using 5-bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium (BCIP–NBT). Images were obtained from five different fields (5.5 mm$^2$ per field) for each well, and the tube area was quantified using an angiogenesis image analyzer. The results are shown in Fig. 6 and Figures S1–3 in the supplementary information.† We found that racemic **1** and (*S*)-**1** blocked vascular sprout formation in high concentration (tube area <60%), while (*R*)-**1**, racemic **2**, (3′*R*,5′*S*)-**2** and (3′*S*,5′*R*)-**2** failed to inhibit tube formation in this assay (tube area >80%).[16] These results are consistent with the traditional theory that the (*S*)-isomer of thalidomide is responsible for the teratogenicity.[2a]

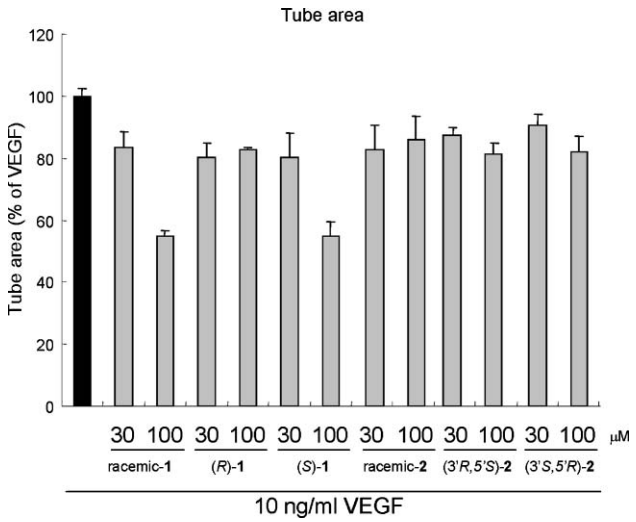



**Fig. 6** Effects of thalidomide on tube area. Comparison of angiogenesis induced by VEGF: mean ± S.E.M (VEGF: $n = 9$, VEGF + compounds: $n = 3$).

In summary, we have achieved the asymmetric synthesis of both enantiomers of the thalidomide metabolite **2** by enzymatic kinetic resolution catalyzed by lipase TL from *Pseudomonas stutzeri*. Incubation experiments revealed that the enantiomerically pure **2** shows resistance to racemization at physiological pH. A preliminary tube formation assay to assess the ability to inhibit angiogenesis suggests that (*S*)-thalidomide is active and its enantiomer (*R*)-**1** and metabolites (3′*R*,5′*S*)-**2** and (3′*S*,5′*R*)-**2** failed to show anti-angiogenesis activity. Although this is a very preliminary experiment and more detailed biological evaluation is clearly required, the results are interesting because 1) metabolism is suggested to play a major role in the teratogenicity of thalidomide, and 2) the anti-angiogenic activity of thalidomide correlates with the teratogenicity[11] but not with the sedative or the mild immunosuppressive properties of thalidomide. Therefore, the teratogenicity of **2** could be dissociable. In addition, **2** possess TNF-α production-inhibitory activity.[8l] These experiments suggest that racemization-free enantomerically pure **2** would be effective for the treatment of diseases dependent on TNF-α production. Asymmetric synthesis and detailed biological studies of **2** and other metabolites of thalidomide are under investigation.

## Acknowledgements

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16 Price *et al.* reported that racemic **2** has moderate anti-angiogenic activity at high concentrations. See reference 8*i*.