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Tetrahedron: *Asymmetry* 15 (2004) 2749–2756

TETRAHEDRON: ASYMMETRY REPORT NUMBER 72

# 'Low-temperature method' for a dramatic improvement in enantioselectivity in lipase-catalyzed reactions

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Received 1 June 2004; accepted 13 July 2004

**Abstract**—Temperature control in lipase-catalyzed resolutions has been recently focused attention due to its simplicity and reliability for enhancement of the enantioselectivity. Lowering the reaction temperature usually increases the enantioselectivity. Lipase immobilized on porous ceramics was found to greatly accelerate the low-temperature reaction, and made the method practical. Our discovery, properties, and practical uses are summarized, and its applications are reviewed.


## Contents



## 1. Introduction

Among a variety of methods for increasing the enantioselectivity in enzymatic reactions,[1] a temperature control in the lipase-catalyzed resolution of alcohols is now accepted as a simple and theoretically reliable method, since we demonstrated that a lipase exerts its function even at very low temperatures down to −80 °C in an organic solvent.[2a] For example, in the case of resolution of 3-phenyl-2*H*-azirine-2-methanol **1** the enantioselectivity was maximal at −40 °C in ether as reported in 1997 (Fig. 1). Enzymes have long been believed to be temperature labile and have been used at around ambient temperatures; however, our finding opened the way to the temperature control of enantioselectivity in enzymatic reactions like a chemical asymmetric reaction. We proved that the temperature effect is widely applicable to the lipase-catalyzed resolution of alcohols, and the course of enhancement of the enantioselectivity can be explained in terms of physical chemistry. Moreover, an immobilized lipase on porous ceramics is found to be useful for enhancement of the reaction rate even at low temperatures. Here, the discovery,[2a] features,[2b] practical utility,[2c,d,3,4] and current applications are summarized.

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doi:10.1016/j.tetasy.2004.07.058

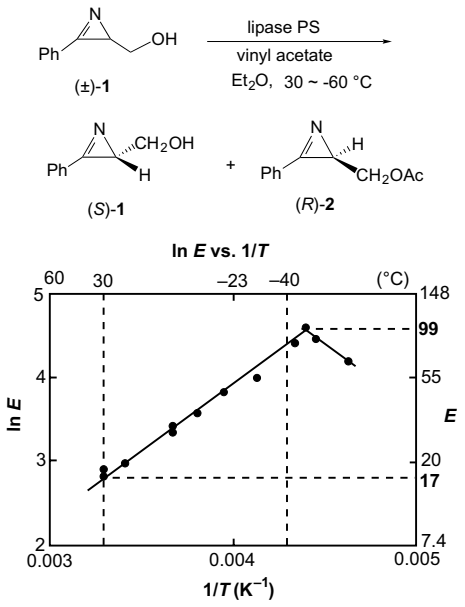



**Figure 1.** 'Low-temperature method' in the lipase-catalyzed resolution of 3-phenyl-2*H*-azirine-2-methanol **1**.

## 2. Discovery and applicability of the 'low-temperature method' in the lipase-catalyzed kinetic resolution

We reported the lipase-catalyzed resolution of azirine-2-methanol **1**,[1a,5] which we expected to have a versatile synthetic utility. As expected for primary alcohols,[6] the enantioselectivity obtained in the transesterification with lipase PS (*Burkholderia cepacia*) in ether was low ($E$[7] = 17 at best) at room temperature despite considerable efforts such as screening of lipases, solvents, additives, and acylating agents.[8–12] As the final choice, we examined the reaction at low temperatures, and found that lowering the temperature to −40 °C (Fig. 2) increased the $E$ value of 17 (at 30 °C) to a practically acceptable level of 99, and the plot of the temperature effect between ln $E$ and $1/T$ showed a linear correlation, obeying Eyring's equation (1).[13] This was the first finding of the enzymatic reaction at such a very low temperature for a synthetic purpose.

$$\ln E = \Delta\Delta S^{\ddagger}/R - \Delta\Delta H^{\ddagger}/(RT) \qquad (1)$$

Interestingly, the plot consists of two lines intersecting at −40 °C, and this temperature is called the 'inversion temperature' ($T_{inv}$).[14] The transition state is kept in the temperature range of the linear correlation. At $T_{inv}$ (−40 °C), the transition-state structure may be changed for some reasons; a temperature-induced structural change of enzyme and/or a solute–solvent cluster change, and so on. The phenomena of $T_{inv}$ are interesting,[15,16] and will be discussed elsewhere.

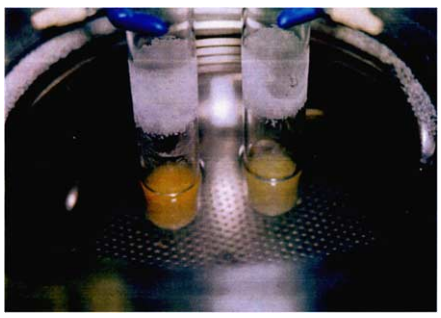


**Figure 2.** Lipase-catalyzed reactions at −40 °C in a cooling apparatus.

General applicability of the low-temperature method has been then demonstrated by its application to some primary and secondary alcohols (Fig. 3).[2b] For example, solketal, 2,2-dimethyl-1,3-dioxolane-4-methanol,[17] had been known to show low enantioselectivity in the lipase-catalyzed resolution (lipase AK, *Pseudomonas fluorescens*; $E$ = 16 at 23 °C, 27 at 0 °C);[17a] however, the $E$ value was successfully raised up to 55 by lowering the temperature to −40 °C (Table 1). Further lowering the temperature rather decreased the $E$ value. Interestingly, the lipase once cooled below −40 °C could be reused by allowing it to warm higher than −40 °C. The results mean that the temperature-induced conformational change, if it occurs, is reversible.

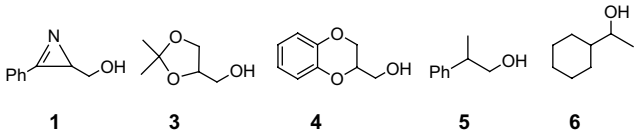



**Figure 3.** Substrates for the lipase-catalyzed resolutions at low temperatures.

Other alcohols exhibited similar temperature effect as shown in Figure 4. These results suggest that the temperature effect is the generally applicable phenomena regardless of primary or secondary alcohols and an origin of lipase.

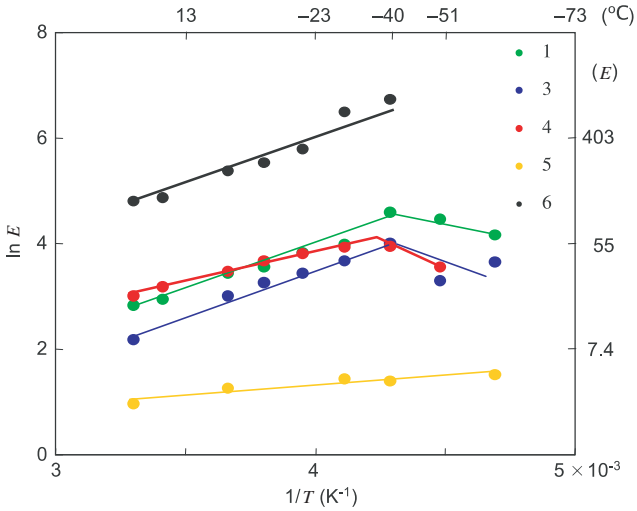
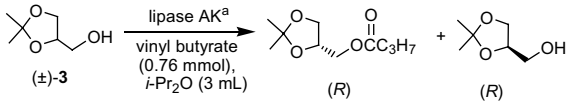



**Figure 4.** Plots of ln $E$ versus $1/T$ for the lipase-catalyzed resolutions.

The thermodynamic parameters ($\Delta\Delta H^{\ddagger}$ and $\Delta\,\Delta S^{\ddagger}$) and the racemic temperatures $T_r$ ($\Delta\Delta H^{\ddagger}/\Delta\Delta S^{\ddagger}$)[13] estimated for these alcohols are shown in Table 2. The results indicate that the enantioselectivity in all of these reactions is

**Table 1.** Temperature effect in the lipase-catalyzed kinetic resolution of solketal




| Entry | Temp (°C) | Lipase (mg) | Time (h) | Ester (% ee) | Alcohol (% ee) | Conv.[b] | *E* |
|---|---|---|---|---|---|---|---|
| 1 | 30 | 20 | 3 | 63 | 69 | 0.52 | 9 |
| 2 | 0 | 20 | 6 | 88 | 25 | 0.22 | 20 |
| 3 | −10 | 60 | 8 | 84 | 77 | 0.48 | 26 |
| 4 | −20 | 60 | 11 | 92 | 32 | 0.26 | 31 |
| 5 | −30 | 100 | 16 | 93 | 39 | 0.30 | 39 |
| 6 | −40 | 200[c] | 24 | 93 | 63 | 0.41 | 55 |
| 7 | −50 | 200[c] | 48 | 74 | 97 | 0.57 | 27 |
| 8 | −60 | 200[c] | 48 | 93 | 51 | 0.35 | 44 |

[a] MW = ca. 33,000. Lipase (ca. 1% w/w) is adsorbed on Celite.
[b] Calculated from ee(s).
[c] TTN is ca. 3000 at 50% conversion.

**Table 2.** Thermodynamic parameters for the lipase-catalyzed resolutions (*i*-$Pr_2O$, vinyl acetate)

| Compd | Lipase | $\Delta\Delta H^{\ddagger}$ (kcal mol$^{-1}$) | $\Delta\Delta S^{\ddagger}$ (cal deg$^{-1}$ mol$^{-1}$) | $T_r$ (°C) |
|---|---|---|---|---|
| **1** | PS[b] | −3.01 ± 0.13 | −4.31 ± 0.50 | 425 |
| **3** | AK[a] | −3.53 ± 0.20 | −7.18 ± 0.79 | 219 |
| **4** | AK | −2.24 ± 0.12 | −1.35 ± 0.40 | 1386 |
| **5** | PS | −1.13 ± 0.05 | −1.77 ± 0.96 | 365 |
| **5** | AK | −0.74 ± 0.05 | −1.51 ± 0.19 | 217 |
| **6** | PS | −3.73 ± 0.25 | −3.02 ± 0.92 | 962 |

[a] With vinyl butyrate.
[b] In $Et_2O$.

governed by the activation–enthalpy differences ($\Delta\Delta H^{\ddagger}$), which originate from the difference in the steric and electronic interactions operating between the faster- and slower-reacting enantiomers in the transition state. The higher enantioselectivity for secondary alcohol **6** comes from the larger negative $\Delta\Delta H^{\ddagger}$ and the low selectivity for **5** depends on the smaller ones. The negative values of $\Delta\Delta H^{\ddagger}$ are compensated by the negative values of $\Delta\Delta S^{\ddagger}$ in all cases.

The low-temperature method was effectively applied to the resolution of (±)-2-hydroxy-2-(pentafluorophenyl)acetonitrile **7** (Fig. 5),[2c] which was used for the syntheses of a variety of ethane diols, amino alcohols containing $C_6F_5$ groups as novel chiral ligands.[18] After screening lipases, LIP (*P. aeruginosa* lipase immobilized on Hyflo Super-Cel, Toyobo, Co., Ltd, Japan) was found to be the best choice ($E = 113$, 1.5 h, 44% conv. at 30 °C). The *E* value was increased by lowering the temperature to 0 °C to reach >427 °C (4.0 h, 44% conv.), which is a satisfactory and practical level.

As described above, the temperature effect is found to be a general phenomenon, and useful for enhancing the enantioselectivity; however, an inevitable problem is the decrease in the reaction rate. For example, although in a lipase AK-catalyzed resolution of solketal, the *E* value (9 at 30 °C, Table 1, entry 1) is increased up to

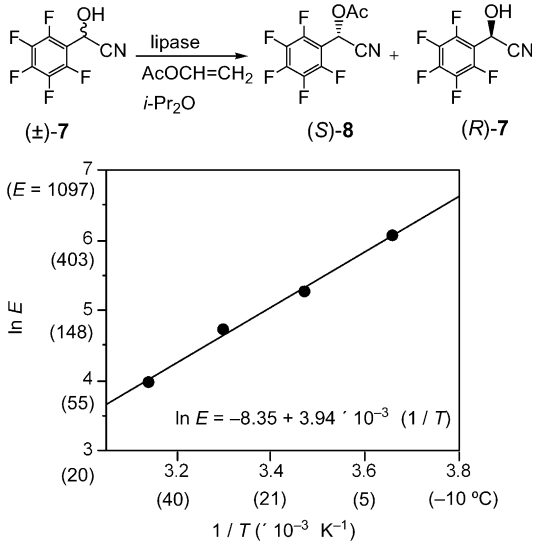



**Figure 5.** The correlation between ln *E* and 1/*T* in the lipase LIP-catalyzed transesterifications of (±)-**7**.

55 by lowering the temperature to −40 °C, 10 times amount of lipase and 8-fold of the reaction time are required as compared with those at 30 °C.[2a] Thus, the rate acceleration is an important subject especially to make the low-temperature reaction practical.

## 3. Immobilization of lipase on porous ceramic support (Toyonite) for acceleration[2d]

We attained an acceleration by using a lipase immobilized on porous ceramic support (Toyonite).[19] The immobilized lipase PS is commercially available as lipase PS-C II, which has (methacryloyloxy)propylsilanetrioxyl bridges on the ceramic surface, and lipases are immobilized on the bridges. Immobilization of an enzyme is known to affect the enzyme conformation, rigidity, and aggregation state to alter the enantioselectivity and reactivity.[20] As shown in Table 3, the use of the

Toyonite-immobilized lipase AK accelerates the reaction from 11,000 (TTN/h, total turnover number per hour) for Celite-immobilized one to 53,000 at 30 °C. Observed maximal acceleration is 80 times in the case of entry 4 as compared with that of entry 3. The great acceleration ability is significant for practical use[21–23] especially at low temperatures. In an organic solvent, lipase molecules usually form aggregation structures, even on Celite, which reduce the activity, while on Toyonite lipases may be highly dispersed by immobilization with organic bridges on the porous ceramic to exert the inherent high activity.

**Table 3.** Toyonite-immobilized lipase-catalyzed resolution of solketal (±)-**3** (vinyl butylate)

| Entry | Lipase | Organic bridge | *E* | | TTN/h | |
|---|---|---|---|---|---|---|
| | | | 30 °C | −40 °C | 30 °C | −40 °C |
| 1 | AK | Celite | 9.0 | 55 | 11,000 | 110 |
| 2 | AK | Toyonite | 3.2 | 21 | 53,000 | 1400 |
| 3 | PS | Celite | 6.8 | 15 | 6400 | 22 |
| 4 | PS | Toyonite | 6.8 | 15 | 110,000 | 1700 |

## 4. Structural optimization of organic bridges on Toyonite[2d]

Structures of organic bridges attached on the support are also crucial for the conformational engineering of enzymes. The ability of organic bridges in the low temperature reaction was then examined by taking the substrate of 2-hydroxymethyl-1,4-benzodioxane **4** as shown in Table 4 and Figure 6.[2d] The low immobilization ability of aliphatic bridge **9i** suggests that the alkoxycarbonyl moiety is essential probably for the binding of lipase through hydrogen bonding. Terminal C–C double bond in **9a–c** is not necessary, because saturated bridges **9d–f** exhibited rather better results. Figure 6 shows that choice of organic bridges is crucially important both to accelerate the reaction and to enhance the enantioselectivity. Surprisingly, a small structural change in the bridges remarkably affects the temperature effect probably by a difference in the manner of hydrogen bonding between the bridge and lipase molecules. As

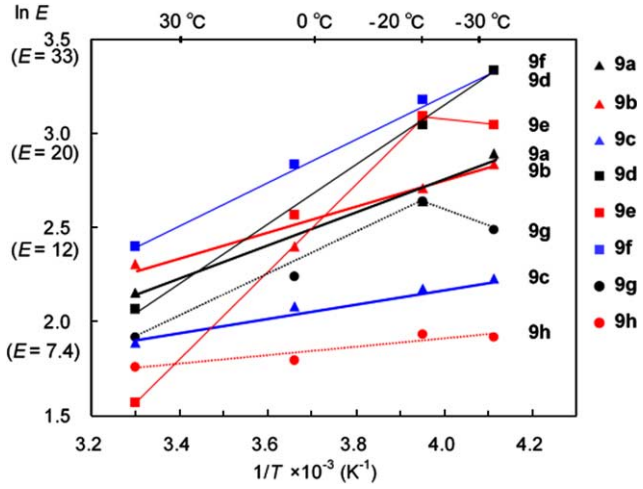
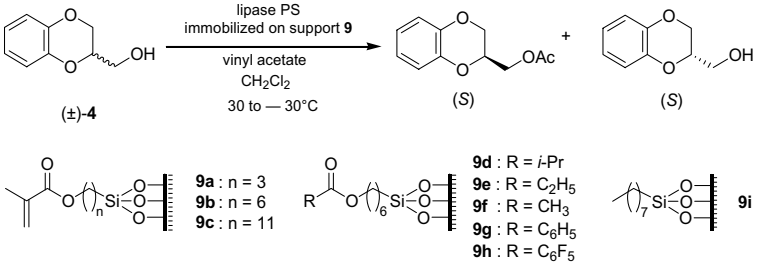



**Figure 6.** Temperature effect on the Toyonite-immobilized lipase-catalyzed resolution of (±)-**4** by varying the organic bridges.

**Table 4.** Effect of organic bridges on the Toyonite-immobilized lipase-catalyzed resolution of (±)-**4**




| Entry | Organic bridge | *E* | | TTN/h | | Bridge wt% | Protein wt% |
|---|---|---|---|---|---|---|---|
| | | 30 °C | −30 °C | 30 °C | −30 °C | | |
| 1 | None[a] | 18 | 33 | 3100 | 26 | — | — |
| 2 | None[b] | 19 | 31 | 5300 | 52 | — | 0.75 |
| 3 | **9a** | 8.6 | 18 | 7300 | 130 | 5.3 | 1.3 |
| 4 | **9b** | 10 | 17 | 14,000 | 280 | 5.7 | 1.3 |
| 5 | **9c** | 6.6 | 9.3 | 14,000 | 230 | 6.5 | 1.6 |
| 6 | **9d** | 7.9 | 28 | 8700 | 280 | 5.5 | 1.6 |
| 7 | **9e** | 4.8 | 21 | 27,000 | 840 | 5.3 | 1.1 |
| 8 | **9f** | 11 | 28 | 12,000 | 100 | 5.3 | 1.3 |
| 9 | **9g** | 6.8 | 12 | 57,000 | 940 | 6.9 | 1.1 |
| 10 | **9h** | 5.9 | 6.8 | 7200 | 61 | 7.7 | 1.6 |
| 11 | **9i** | — | — | — | — | 6.4 | 0.23 |

[a] Commercially available lipase PS immobilized on Celite.
[b] Toyonite without organic bridges.

far as examined here, **9d** was the best choice from the points of reaction efficiency and of availability.

## 5. Practical resolution of azirine 1 by the 'low-temperature method' combined with Toyonite-immobilized lipase and optimized acylating agent[2e]

In the first attempt of the low-temperature method for azirine-2-methanol **1** using the Celite-immobilized lipase, the *E* value was increased from 17 (30 °C) up to 99 (−40 °C); however, the TTN/h was decreased from 4700 to 210. On the other hand, use of Toyonite D-M-immobilized lipase[24] in the reaction remarkably raised the TTN/h to 4200 at −40 °C. In contrast, it lowered the *E* value to 33. Therefore, adjustment of the two conflicting features, the reaction rate and the enantioselectivity, is essential for the practical use of the low-temperature method. To achieve this, acylating agents[25] were screened, and found to be highly effective on the *E* values. The results for the reactions carried out at −40 °C are shown in Figure 7.[2e] Elongation of the acyl chain to vinyl butanoate dramatically increased the *E* value up to 96, a comparable value in the Celite-immobilized lipase, together with a sufficient TTN/h of 3600. Further improvement was attained by using Toyonite

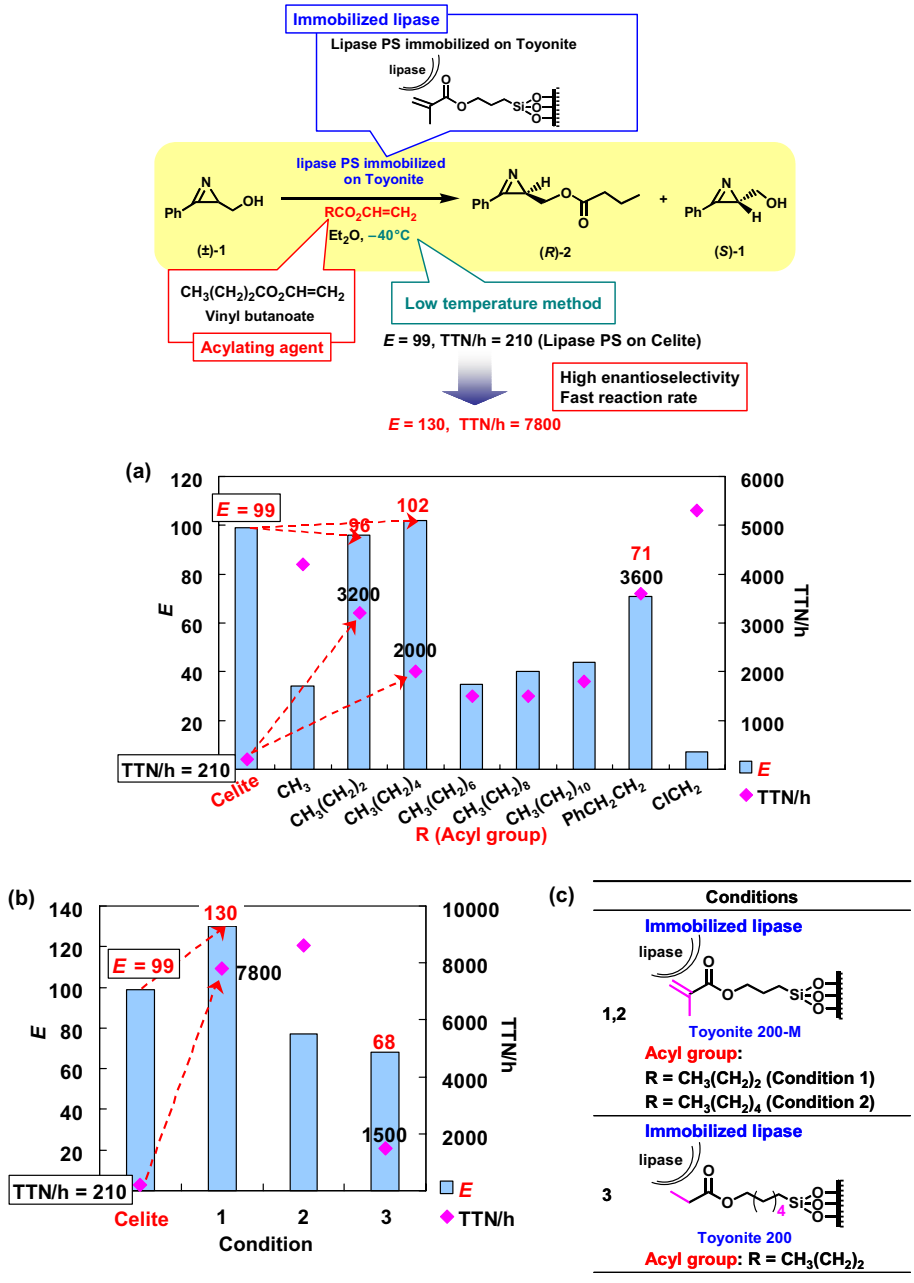



**Figure 7.** Optimization of acylating agent and organic bridges in the lipase PS (ceramics-immobilized)-catalyzed reaction of (±)-**1** at −40 °C. (a) Reaction with Toyonite-D-M-immobilized lipase PS and various acylating agents, (b) reaction with Toyonite 200-M (or Toyonite 200) and optimized acylating agent, (c) list of conditions for the reactions in (b).

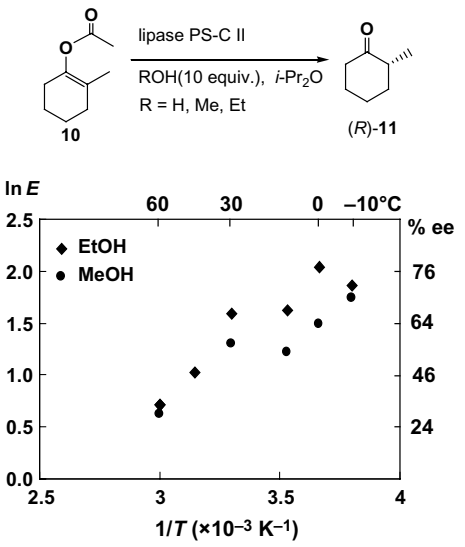



**Figure 8.** Lipase-catalyzed enantioface-selective asymmetric protonation.

200M-immobilized lipase, which gave the highest $E$ value (130) and TTN/h (7800) with vinyl butanoate at −40 °C.

These results indicate that the low-temperature method increases the enantioselectivity, at least above inversion temperature, and the enantioselectivity and reaction rate can be optimized by the use of Toyonite-immobilized lipase and a suitable acylating agent.

## 6. Other applications of temperature control, asymmetric protonation[4] and high-temperature reaction[3]

We recently found that the low-temperature method is effective not only in the kinetic resolution of alcohols but also in the enantioface-selective asymmetric protonation of enol acetate of 2-methylcyclohexanone giving (*R*)-2-methylcyclohexanone.[4] The reaction in $H_2O$ at 30 °C gave 28% ee (98% conv.), which was improved up to 77% ee (82% conv.) by the reaction using lipase

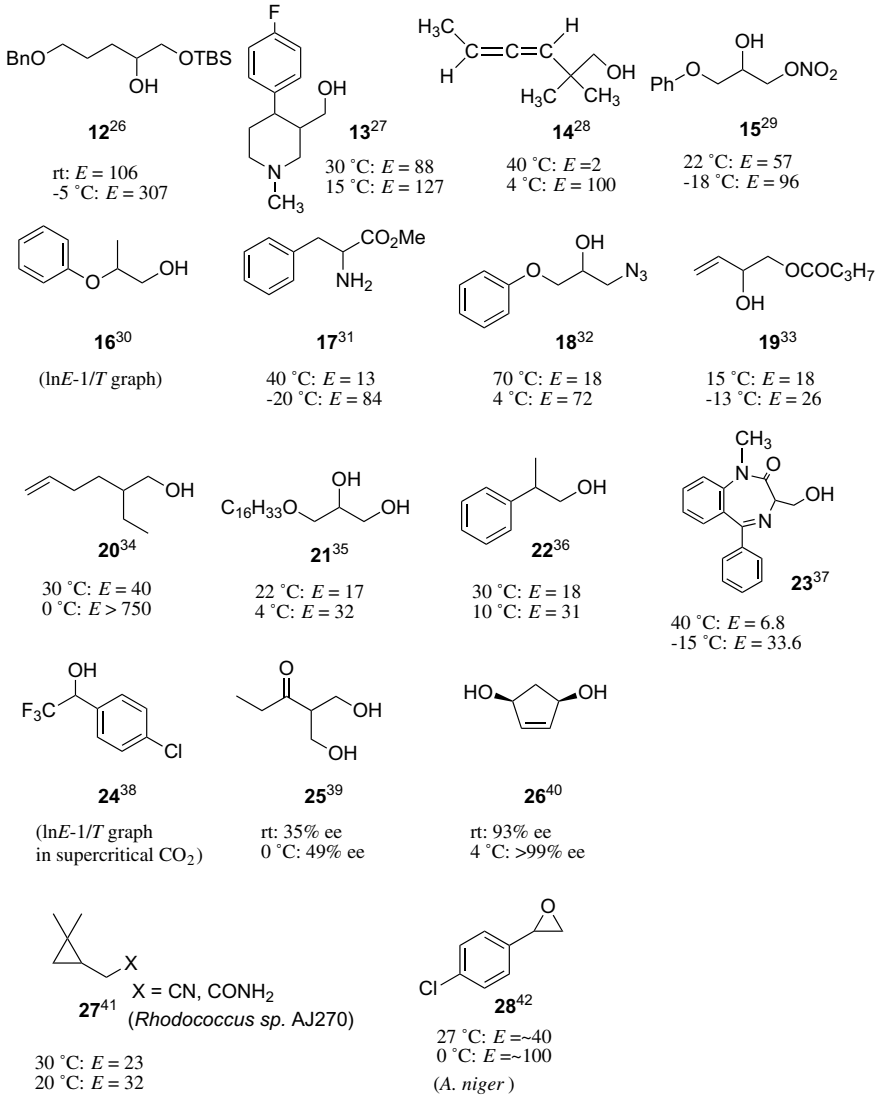
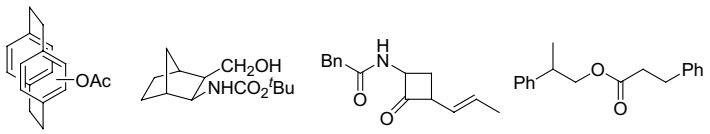



**Figure 9.** Examples of the 'low-temperature method'.




**29**[43] 60 °C: $E = 26$, 22 °C: $E = 2$

**30**[44] rt: $E = 127$, 0 °C: $E = 13$

**31**[45] 55 °C: $E = 130$, 28 °C: $E = 24$

**32**[46] 30 °C: $E = 107$, 4 °C: $E = 5.8$

**Figure 10.**

PS-C II in *i*-$Pr_2O$ and ethanol at 0 °C. Acceleration of the reaction with lipase PS-C II made this reaction possible, because this reaction required a long reaction time. The temperature effect is shown in Figure 8. Each plot of ln $E$ versus $1/T$ for methanol and ethanol shows that lowering the temperature increases the enantioselectivity, however, without obeying an exact linear correlation. Curiously, increase of the $E$ value was not observed between 30 and 10 °C in both cases. This may suggest a transition of the mechanisms, although the details are unclear at present. The protons may be supplied from $H_2O$, methanol, or ethanol, whose bulkiness is important.

On the other hand, lipase PS-C II was found to be useful for a high-temperature reaction in the resolution of a bulky substrate, 1,1-diphenyl-2-propanol.[3] The reaction at 40–120 °C gave an enantiopure product, and the highest conversion (39%) was obtained at 80–90 °C. The results mean that a single enzyme shows the catalytic function at a very wide range of temperatures from −60 to 120 °C. Lipase PS-C II is a robust biocatalyst useful for organic synthesis.

## 7. Low-temperature methods in the literature

Since our first report,[2a] many examples of the low-temperature method have been published. The typical examples for the synthetic purpose are summarized in Figure 9. The $E$ values of these are increased by lowering the temperature. Compound **24** is used in media of supercritical $CO_2$. Compounds **27** are used for the kinetic resolution in the hydrolysis of nitrile and amide, and compound **28** is for the reaction in the hydrolytic ring opening of epoxide. These examples show the utility of the low-temperature method; however, some examples show rather negative temperature effect as shown in Figure 10. These compounds exhibit the better $E$ values at higher temperatures. The latter examples are also interesting, and may be due to the entropy-driven reactions.[13]

## 8. Conclusion

Recently, temperature control in the enzymatic reaction is actively studied for fine tuning of the enantioselectivity,[15,47–50] although the method had not been considered to be practical before our report.[2–4] Beside the synthetic utility, the physical and theoretical aspects are also interesting, and will be further studied for understanding the enzymatic reaction.

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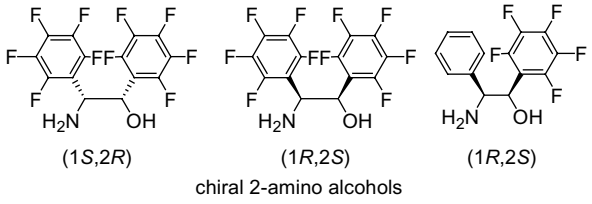
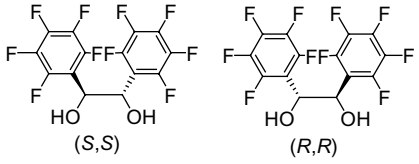



chiral 2-amino alcohols




chiral 1,2-diols


19. Toyonite type 200 without organic bridges on the surface: a new type of spherical porous ceramics support having 155±5 μm average diameter and 60 nm average sized pores (Yamashita, Y.; Kamori, M.; Takenaka, H.; Takahashi, J. *Jpn. Kokai Tokkyo Koho*, JP 09313179 (1997); U.S. Patent 6,004,786). Commercially available Toyonite 200M is the one that 3-(2-methylpropenoyloxy)propylsilanetrioxyl groups are attached on Toyonite 200. Lipase PS immobilized on Toyonite 200M is also available from Amano Pharmaceutical Co., Ltd or Wako Pure Chemical Industries, Ltd as Lipase PS-C 'Amano' II. See also Ref. 2c.
20. For current examples: (a) Palomo, J. M.; Fernández-Lorente, G.; Mateo, C.; Fuentes, M.; Fernández-Lorente, R.; Guisan, J. M. *Tetrahedron: Asymmetry* **2002**, *13*, 1337–1345; (b) Terreni, M.; Mateo, C.; Bastida, A.; Dalmases, P.; Hugust, L.; Guisan, J. M. *Enzyme Microb. Technol.* **2001**, *28*, 389.
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24. Toyonite D-M (porous ceramic support, Toyo Denka Kogyo Co., Ltd) has 120 nm of mean pore size having 3-(2-methylpropenoyloxy)propylsilanetrioxyl bridges on the surface.
25. Recent reports for the enzymatic resolution with various acylating reagents: (a) Kawasaki, M.; Goto, M.; Kawabata, S.; Kodama, T.; Kometani, T. *Tetrahedron Lett.* **1999**, *40*, 5223–5226; (b) Hirose, K.; Naka, H.; Yano, M.; Ohashi, S.; Naemura, K.; Tobe, Y. *Tetrahedron: Asymmetry* **2000**, *11*, 1199–1215; (c) Kita, Y.; Takebe, Y.; Murata, K.; Naka, T.; Akai, S. *J. Org. Chem.* **2000**, *65*, 83–88; (d) Ottosson, J.; Hult, K. *J. Mol. Catal. B: Enzym.* **2001**, *11*, 1025; (e) Reaction in ionic solvent: Itoh, T.; Akasaki, E.; Nishimura, Y. *Chem. Lett.* **2002**, 154–155; (f) Akai, S.; Naka, T.; Fujita, T.; Takebe, Y.; Tsujino, T.; Kita, Y. *J. Org. Chem.* **2002**, *67*, 411–419.
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