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# Efficient enzymatic kinetic resolution of 2-heptylamine with a highly active acyl donor

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ABSTRACT

Novozyme435 facilitated kinetic resolution of 2-heptylamine was here presented. Methyl methoxyacetate was used as acyl donor. A survey of influencing factors including hydrogen bonding effect, solvent effect, steric effect, temperature and the amount of acyl donor were investigated in detail. At the optimum conditions, the enantiomeric separation was successfully obtained within 8 h at 20 °C, and gave high conversion and optical purity of (R)-2-heptylamine, 48.9% and over 99% respectively. The immobilized lipase B was found to be suitable for the enantiomeric separation of aliphatic amines with good recyclability.



## 1. Introduction

Over the last years, single-enantiomer drugs became more and more important in the pharmaceutical industry. Optically active aliphatic amines are a type of compounds for the enantioselective synthesis of various pharmaceuticals [1]. Among these amines, 2-heptylamine (2-HA) is usually used to prepare neurological disease drugs [2]. Enantiomeric forms of these drugs always differ in potency, toxicity and behavior in biological systems. As a result, it is essential to study the resolution methods of 2-HA.

Lipase B from *Candida antarctica* (CALB) has been demonstrated to be a very useful catalyst for the resolution of chiral amines. In order to improve the activity of CALB, a variety of new reaction systems [3–6] and new immobilization methods [7–11] have been reported. Other reports focused their attentions on seeking new acyl donors with high activity, such as ethyl caprylate [12], propylene acetate [13], dibenzyl carbonate [14], capric acid [15], ethyl acetate [16,17] and isopropenyl acetate [18]. However these acyl donors are not suitable for large-scale application in industry because of their low activity. The produced esters or amides were usually difficult to be transferred into corresponding alcohols or amines. In this paper, we reported a highly active acyl donor, methyl methoxyacetate (MMA), which could be supported commercially. Improved condition for enzymatic kinetic resolutions of 2-HA and other aliphatic amines was also provided. Typical reaction was shown in Scheme 1.

## 2. Experimental

### 2.1. General

2, 3, 4, 6-Tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) was synthesized according to the method introduced by Deng and Wan [19]. 2-HA 99%, 2-pentylamine 99%, 4-methyl-2-pentylamine 99%, 2-hexylamine 99%, 3-heptyl amine 99%, 2-nonylamine 99%, α-phenethylamine 98%, methyl methoxyacetate 99%, and methyl dimethoxyacetate 99% were all purchased from J&K Chemical Ltd. Novozyme435 (immobilized lipase B from *C. antarctica*, 4500 LU/g) was purchased from Novozymes (Denmark). All other chemicals and reagents were obtained commercially and were of analytical grade. All solvents were dried by 4 Å molecular sieves before being used.

### 2.2. Lipase-catalyzed acylation of amine

Acyl donor (2 mmol) and amine (2.5 mmol) were dissolved in 10 ml ethyl ether. Novozyme435 (200 mg) was then added. The mixture was incubated at set temperature for a certain time. The reaction was stopped by removal of the enzyme. The unreacted amine was isolated by extraction with hydrochloric acid solution (1.2 N, 10 ml). The organic fraction was concentrated under vacuum and pure amide was obtained. The pH of aqueous solution was adjusted to over 10 with sodium hydroxide solution (1 N). Ethyl ether (15 ml) was introduced and the phases were separated. The unreacted amine was obtained by concentration of organic fraction under vacuum.

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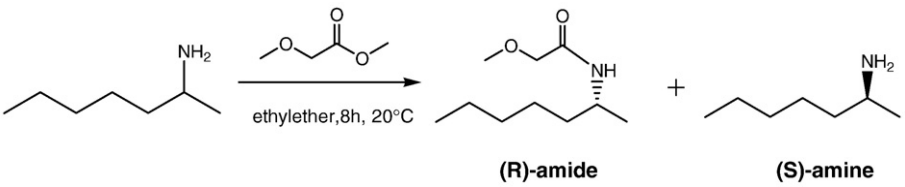



**Scheme 1.** Enzymatic kinetic acylation of amines.

### 2.3. Hydrolysis of amide

A mixture of (R)-amide (2.5 mmol), triethanolamine (200 mg) and 50% sodium hydroxide solution (400 mg) were heated at 120 °C for 6 h. The reaction was quenched by adding 10 ml $H_2O$. The mixture was then cooled to room temperature. Ethyl ether (30 ml) was introduced and the phases were separated. The organic extract was washed with 10 ml $H_2O$. The R-amine was obtained as a colorless liquid by distilling the organic fraction under vacuum.

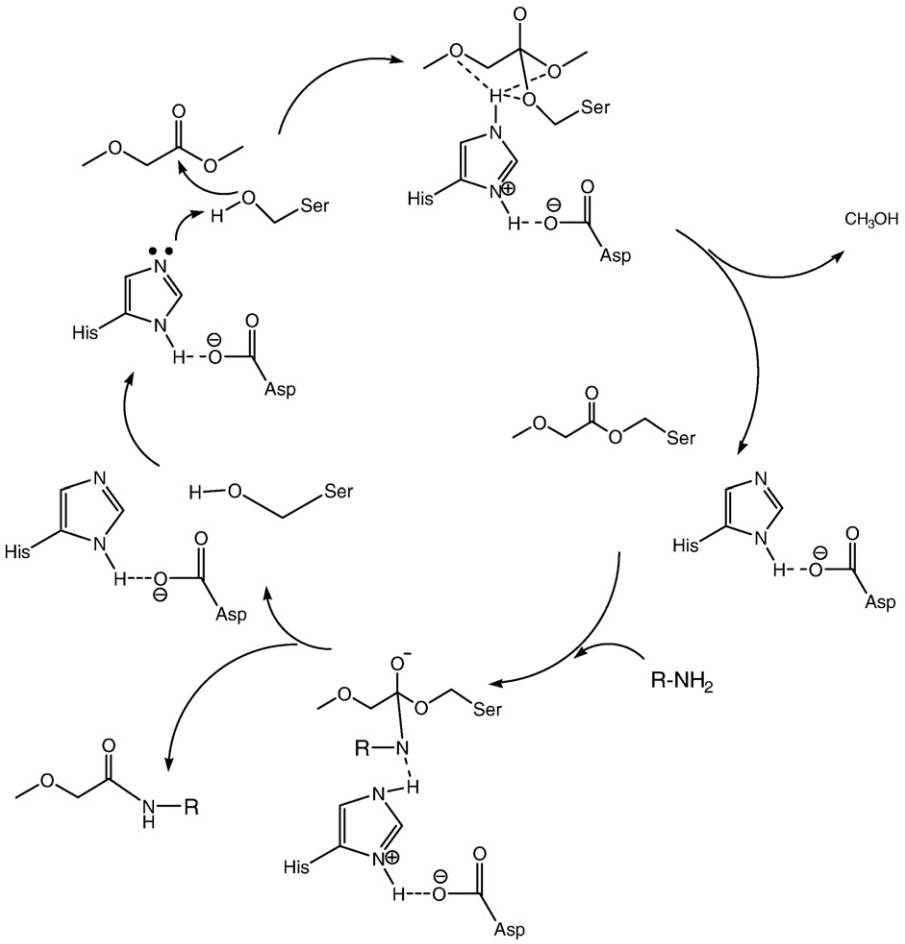
### 2.4. Analysis of the samples

The optical purity and yield measurement were performed on a HPLC system (Shimadzu) by pre-column derivation. Typical derivation of GITC and amine was carried out as followed: a certain amount of GITC was dissolved with acetonitrile to give 2 mol/L solution. Fatty amine (1 mmol) was diluted with a mixed solvent of acetonitrile and three steaming water ($V_{CH3CN}$:$V_{H2O}$ = 50:50), which also contained 1 g/L triethylamine. The final volume of amine solution was 5 ml. GITC solution (50 μl) and amine solution (50 μl) were mixed together with scroll instrument for 10 min. The mixture was placed for 30 min at room temperature.

HPLC separation was achieved on a Diamonsil $C_{18}$ column using an isocratic flow (1 ml/min). The mobile phase consisted of a mixture of TEAA buffer (0.1 M, pH 5.5) and methanol. The ratio of buffer and methanol was 38:62. Twenty microliters sample was injected into HPLC system every time. Wavelength of UV detector was 254 nm.

## 3. Results and discussion

The mechanism of enzymatic kinetic resolution of alcohol has been described by Cygler et al. [20] and Castro and Gago [21]. Cygler and his research group [20] also identified how the enantiomers of menthol, a typical secondary alcohol, bind to lipase B in the transition state using X-ray crystallography. According to the report, mechanism of amine resolution could be summarized by Scheme 2. The overall progress included two defined steps: 1) formation of acyl-enzyme and 2) amination of the acyl-enzyme.




**Scheme 2.** Enzymatic kinetic acylation mechanism of amines.

Hydrogen bonding was supposed to play more important role in the transformation than inductive effect [22]. To confirm this hypothesis, two amines (2-HA and α-phenethylamine) were employed as substrates, and two esters (methyl methoxyacetate and ethyl chloroacetate) were used as acyl donors. A detailed comparison of the effect of acyl donor on conversion of the enzymatic resolution had been described in Fig. 1. Just as shown in Fig. 1, when methyl methoxyacetate was performed as acyl donor, the acylation of 2-HA was faster than the reaction with ethyl chloroacetate as acyl donor. According to previous paper [23], rate of the reaction with methyl methoxyacetate as acyl donor should be slower if the inductive effect of β-position on acyl donor largely affected the acylation. The results in the experiments disagreed with the inductive effect, even though Cl atom is more electronegative than methoxyl group, and a possible explanation is that Cl only forms weaker hydrogen comparing to oxygen [24]. Similar results were observed when α-phenethylamine was employed as substrate, which was shown in Fig. 1. On the base of these results, the rate increase might be attributed to the hydrogen bonding between acyl donor and enzyme. Methyl methoxyacetate was an ideal acyl donor because it could form hydrogen bonding easily with enzyme.

Polarity of organic solvent was major criteria in solvent selection due to its close relationship with the formation of hydrogen bonding. As shown in Fig. 2, an increasing conversion and $ee_S$ with increasing hydrophobicity were observed when ethyl ether, THF and isopropanol were used as solvents. It would appear likely that the differences were due mainly to their effect on the formation of hydrogen bonding. In more hydrophobic solvent, hydrogen bonding between substrate and Novozyme435 could form more easily. In particular, the conversion and $ee_S$ of 2-HA in n-heptane or n-hexane are lower than those in ethyl ether, isopropyl ether or methyl tert-butyl ether, which might be related to the solvent swelling of polyacrylic resin particles where the enzyme is immobilized. The carrier of the enzyme is polyacrylic resin particle, which has certain hydrophilicity. Considering the more conformability the more consistencies, the polyacrylic resin ball is swelling more easily in middle hydrophobic solvents than hydrophobic solvent. Accordingly, the immobilized enzyme cannot be dispersed well in the reaction system using hydrophobic solvent. The same solvent effect was also reported by literature [18]. The results of our experiments suggested that the resolution should be operated in middle hydrophobicity solvents, such as ethyl ether, isopropyl ether and methyl tert-butyl ether.

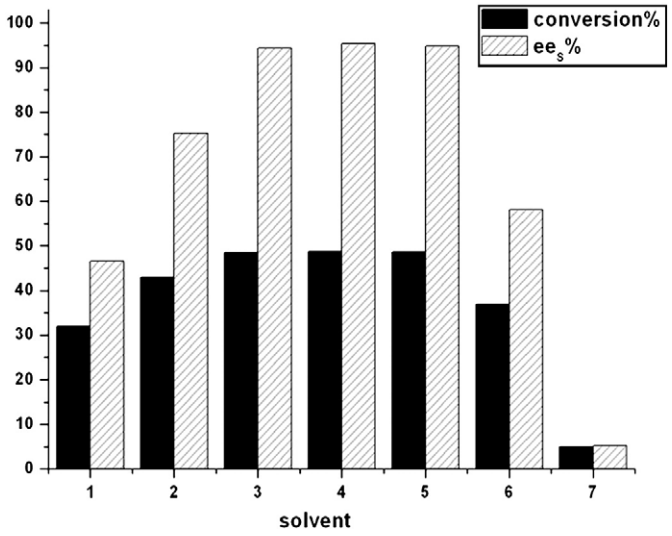



**Fig. 2.** Effect of solvent on the enzymatic acylation conversion and $ee_S$ of 2-HA. Reaction conditions: methyl methoxyacetate, 4 mmol; 2-HA: 5 mmol; Novozyme435: 200 mg; solvent (10 ml): **1** n-heptane, **2** n-hexane, **3** isopropyl ether, **4** methyl tert-butyl ether, **5** ethyl ether, **6** THF, and **7** isopropanol; a slightly elevated temperature 20 °C was used during the reactions.

Steric effect was also studied by the comparison between the reactions with methyl methoxyacetate and methyl dimethoxyacetate as acyl donors. The reactions were carried out in ethyl ether system at 20 °C. Methyl dimethoxyacetate was supposed to form hydrogen bonding with enzyme more easily than methyl methoxyacetate because it had two methoxyl groups. However, it could be clearly seen from Fig. 3 that the conversion of 2-HA declined largely when methyl dimethoxyacetate was used as acyl donor. The result might be attributed to another influencing factor (steric effect). Two methoxyl groups on methyl dimethoxyacetate configuration made the combination of enzyme and acyl donor unstable, which led to lower conversion. These results showed that straight chain acyl donors were desirable.

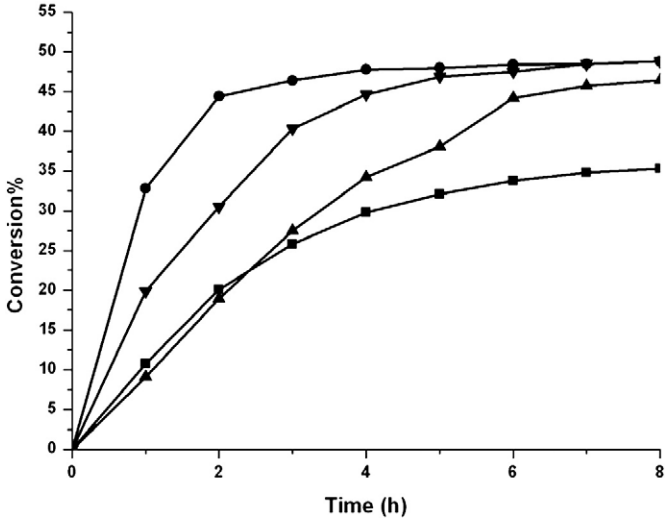



**Fig. 1.** Effect of acyl donor on the enzymatic acylation conversion of 2-HA and α-phenethylamine. Reaction conditions: (●) methyl methoxyacetate: 4 mmol, 2-HA: 5 mmol (■) ethyl chloroacetate:4 mmol, 2-HA: 5 mmol (►) methyl methoxyacetate: 4 mmol, α-phenethylamine: 5 mmol (◄) ethyl chloroacetate: 4 mmol, α-phenethylamine: 5 mmol; Novozyme435, 200 mg; 10 ml ethylether was used as solvent. A slightly elevated temperature 20 °C was used during the reactions.

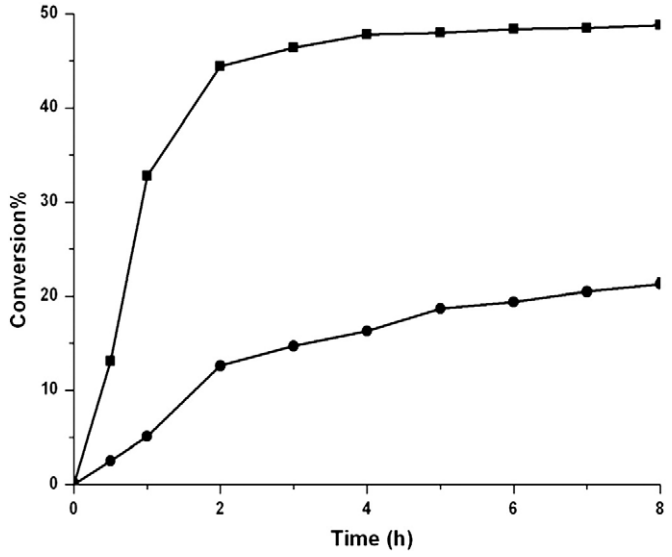



**Fig. 3.** Effect of acyl donor on the enzymatic acylation conversion of 2-HA. Reaction conditions: (■) methyl methoxyacetate, 4 mmol (●) methyl dimethoxyacetate, 4 mmol; 2-HA: 5 mmol; Novozyme435: 200 mg; 10 ml ethyl ether was used as solvent. A slightly elevated temperature 20 °C was used during the reaction.

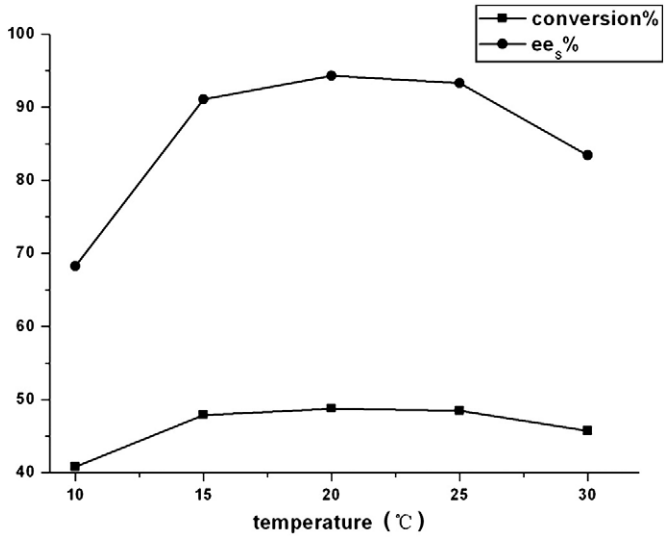



**Fig. 4.** Effect of reaction temperature on the enzymatic acylation conversion and $ee_S$ of 2-HA. Reaction conditions, varying temperature from 10 °C to 30 °C; Methyl methoxyacetate, 4 mmol; 2-HA: 5 mmol; Novozyme435: 200 mg; 10 ml ethyl ether was used as solvent.

The choice of temperature was of great importance for the asymmetric acylation of 2-HA. A survey of temperatures below 30 °C were investigated because the reactions were operated in ethyl ether system. As shown in Fig. 4, the conversion and $ee_S$ of 2-HA increased when temperature was increased from 10 °C to 20 °C. The highest conversion and $ee_S$ came close to 50% and 95%. But when temperature was increased sequentially to 30 °C, the conversion and $ee_S$ declined to 46% and 85%. The results showed that asymmetric acylation of 2-HA should be carried out at 20 °C.

The effect of amount of acyl donor on the asymmetric acylation was investigated to confirm acyl donor inhibition. The results were shown in Fig. 5. When the amount of methyl methoxyacetate was lower than 4 mmol, the conversion and $ee_S$ increased with the increased amount of acyl donor. When the amount of acyl donor was increased from 4 mmol to 30 mmol, the conversion and $ee_S$ did not show significant change. The results indicate that acyl donor has no

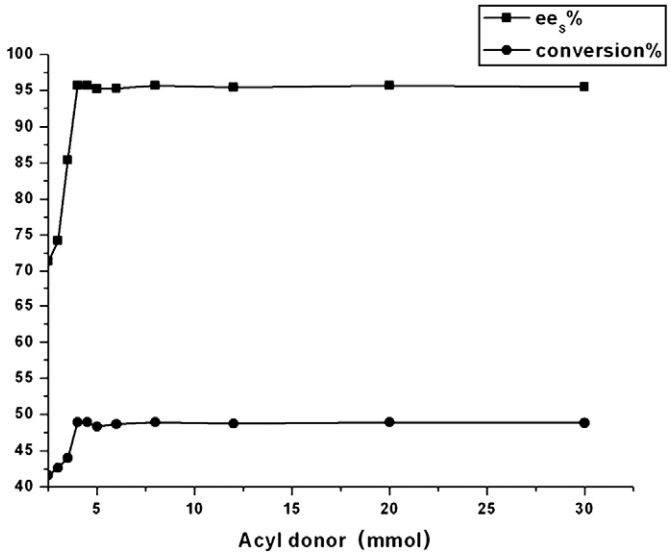



**Fig. 5.** Effect of amount of acyl donor on the enzymatic acylation conversion and $ee_S$ of 2-HA. Reaction conditions, varying the amount of methyl methoxyacetate from 2.5 mmol to 30 mmol; 2-HA: 5 mmol; Novozyme435: 200 mg; 10 ml ethyl ether was used as solvent. A slightly elevated temperature 20 °C was used during the reaction.

**Table 1**
Enzyme catalyzed resolution of aliphatic amines[a].

| Entry | Amine | Time (h) | $ee_S$% | $ee_P$% | $E$[b] | Conversion% |
|---|---|---|---|---|---|---|
| 1 | 2-Hexylamine | 8 | 96.9 | >99 | >1000 | 49.5 |
| 2 | 4-Methyl-2-pentylamine | 8 | 97.7 | >99 | >1000 | 49.7 |
| 3 | 2-Heptylamine | 8 | 95.1 | >99 | >1000 | 48.9 |
| 4 | 3-Heptylamine | 2 | 96.3 | >99 | >1000 | 49.3 |
| 5 | 2-Pentylamine | 2 | 96.4 | >99 | >1000 | 49.3 |
| 6 | 2-Heptylamine | 2 | 80.5 | >99 | >611 | 44.8 |
| 7 | 2-Nonylamine | 8 | – | – | – | n.d. |

[a] All reactions were conducted in dry ether using 5 mmol amine, 4 mmol methyl methoxyacetate and 200 mg. Novozym 435 at 20 °C for 8 h. Conversion and ee were determined by HPLC.
[b] $E = \ln[(1-C)(1-ee_S)]/\ln[(1-C)(1+ee_S)]$ [25].

inhibition to the activity of Novozyme435 obviously. In order to reduce the cost, Novozyme435-catalyzed resolution would better be carried out at the molar ratio of acyl donor and 2-HA, 4:5.

A remaining task of the overall process was the hydrolysis of the R-configured amide. Based on the above results, 2-HA was firstly separated to give R-configured amide. Hydrolysis of (R)-amide to (R)-amine was operated in NaOH-triethanolamine system. Optical purity of (R)-2-HA was determined on HPLC with the method of pre-column derivation. The final optical purity was 99.6% ee, and conversion of (R)-2-HA was 48.9%, which was calculated according to literature [25].

To test the recyclability, Novozyme435 was repeatedly recovered from the reaction mixture, washed with ethyl ether, and reused. After just eight cycles at 20 °C, the conversion of 2-HA was still over 48%. These experiments showed that this immobilized CALB showed a good stability in our conditions. The slight decline was mainly due to the leakage of the enzyme from polyacrylic resin balls on which the enzyme was non-covalently absorbed. Accordingly, wild stirring, which might shatter the polyacrylic resin ball, should be avoided. In the experiments, the reaction system was incubated by rotating the reactor.

By the enzymatic procedure, various racemic aliphatic amines (Table 1, Entry 1–5) were successfully isolated into optically pure amines in high convention and high enantiomeric excess, over 48.9% and 99% respectively. It was observed that for hydrophilic amines, such as 2-pentylamine (Table 1, Entry 5), 3-heptylamine (Table 1, Entry 4), the enantiomers were obtained within 2 h, but at the same conditions, the conversion and $ee_S$ of the amines with longer carbon chain, such as 2-HA (Table 1, Entry 6) reduced to 44.8% and 80.5%. When 2-nonylamine (Table 1, Entry 7) was used as a substrate, the enantiomeric separation did not proceed, even though the reaction time was extended to 8 h. These results suggested that increase of hydrophilic nature in amines might increase the reaction activity, and finally lead to better enantiomeric separation. Comparing previous studies [12–18], an ideal acyl donor was used for the enantiomeric resolution of aliphatic amines, and thus high conversion and optical purity of (R)-amines were achieved.

## 4. Conclusions

Novozyme435-catalyzed asymmetric acylation of 2-HA was successfully carried out in ethyl ether system. Methyl methoxyacetate was found to be an ideal acyl donor for this enzymatic resolution, and high conversion and optical purity of enantiomers of amines were obtained. It somehow suggested that hydrogen bonding and steric effect might play important roles in the reaction. Finally, the enzyme and the solvent could be reused in the next run, which significantly reduced the cost of the process. In summary, a highly efficient protocol for asymmetric resolution of 2-HA and its homologous compounds was developed.

## Acknowledgments

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