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Enzymatic hydrolytic resolution of (*R*,*S*)-tropic acid esters and (*R*,*S*)-ethyl α-methoxyphenyl acetate in biphasic media

Pei-Yun Wang, Shau-Wei Tsai*

Institute of Biochemical and Biomedical Engineering, Chang Gung University, 259 Wen-Hwa 1st Road, Kwei-Shan, Tao-Yuan 33302, Taiwan

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ABSTRACT

A thermally stable esterase from *Klebsiella oxytoca* is explored as an excellent enantioselective biocatalyst ($E > 100$) for the hydrolytic resolution of (*R*,*S*)-tropic acid esters and (*R*,*S*)-ethyl α-methoxyphenyl acetate in biphasic media. An expanded Michaelis–Menten mechanism for the enzymatic acylation step is adopted for the kinetic analysis, where the structure–enantioselectivity correlations in terms of the logarithms of specificity constants varied with the inductive parameter of leaving alcohol for (*R*,*S*)-tropic acid esters can be employed for interpreting the reaction mechanism and rationalizing the optimal enantioselectivity at the methyl ester. The pH effects on changing the relative specific constants k_{2R}/K_{mR} and k_{2S}/K_{mS} are further applied for estimating the intrinsic specificity constants for both enantiomers. A kinetic analysis among (*R*,*S*)-tropic acid ethyl ester, (*R*,*S*)-ethyl α-methoxyphenyl acetate, (*R*,*S*)-ethyl α-methylphenyl acetate, (*R*,*S*)-ethyl mandelate and (*R*,*S*)-ethyl α-chlorophenyl acetate indicates that the α-substituent has profound influence on the enzyme activity and enantioselectivity, i.e. good ($100 > E > 50$) to excellent ($E > 100$).

1. Introduction

Optically pure α-substituted phenylacetic acids and their aryl analogues are important building blocks for the synthesis of a variety of pharmaceuticals, agrochemicals, resolving agents in resolution processes, and NMR reagents for determining absolute configuration of chiral compounds. For example, α-haloarylacetic acid derivatives are known as important intermediates for synthesizing prostaglandin, prostacyclin, semi-synthetic penicillin and thiazolium salts [1–7]. Esters and amides derivatives of α-aryloxyphenylacetic acids, such as halofenate, are useful in ameliorating various physiological conditions associated with blood lipid deposition [8–10]. α-Alkylarylacetic acids [11], such as α-methylarylacetic acids (profens) [12,13], are marketed as an important class of non-steroidal anti-inflammatory drugs or employed as the intermediates for synthesizing pyrethroid insecticides [14] or chiral dopants for nematic liquid crystals [15]. Several commercially important β-lactam antibiotics contain α-aminophenylacetic acids (e.g. phenylglycine and *p*-hydroxyphenylglycine) as the acyl side-chain of penicillins or cephalosporins [16,17]. α-Hydroxyarylacetic acids, such as mandelic acid, 2-chloromandelic acid or 2-naphthylglycolic acid, are well-known as building blocks for the synthesis of pharmaceuticals and resolving agents [18,19]. Their α-methoxy analogues have been developed as excellent chiral NMR reagents for determining the absolute configuration of secondary alcohols and amines [20–22]. Tropic acid (α-hydroxymethylphenylacetic acid or 2-phenyl-3-hydroxypropionic acid) that is regarded as a special kind of 2-aryl-3-hydroxycarboxylic acids is also an important intermediate for synthesizing biologically active alkaloids such as hyoscyamine and hyoscine [23–26].

Although the biocatalytic resolution processes, using various serine-type hydrolases including esterases, lipases and serine proteases as the versatile biocatalysts, have been employed for the preparation of optically pure α-substituted phenylacetic acids [1–11,13,15–21,24–26], they are scarcely reported in the literature for the resolution of (*R*,*S*)-tropic acid [24–26] or (*R*,*S*)-α-methoxyphenylacetic acid [20]. In the transesterification of (*R*,*S*)-tropic acid ethyl ester with lipase PS and vinyl acetate as an acylation agent in toluene, 39% yield with 87% ee_p of (*S*)-3-acetoxy tropic acid ethyl ester and 42% yield with 94% ee_s of (*R*)-tropic acid ethyl ester was obtained [24]. A dynamic kinetic resolution process using a ruthenium catalyst as the racemization catalyst for the remaining (*R*)-ester was also disclosed, giving 88% yield with 90% ee_p of (*S*)-3-acetoxy tropic acid ethyl ester [26]. Apparently, the problem of separating the resultant (*S*)-ester product from (*R*)-ester substrate and then hydrolysing the former to give desired (*S*)-tropic acid may hinder the process in practical usages.

* Corresponding author. Tel.: +886 3 2118800x3415; fax: +886 3 2118668.
E-mail address: tsai@mail.cgu.edu.tw (S.-W. Tsai).

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Nomenclature

ee_p, ee_s	enantiomeric excess for the product and substrate, respectively
E	enantiomeric ratio greater than one, defined as the ratio between k_{2S}/K_{mS} and k_{2R}/K_{mR}
(E_t)	enzyme concentration in aqueous phase (mg/ml)
F	inductive parameter of R_2 moiety in the leaving alcohol
k^*_{2R}, k^*_{2S}, k^{**}_{2R}, k^{**}_{2S}	kinetic parameters for (*R*)- and (*S*)-enantiomers, respectively, in aqueous phase (mmol/g h)
K_{mR}, K_{mS}, K^*_{mR}, K^*_{mS}, K^{**}_{mR}, K^{**}_{mS}	kinetic constants for (*R*)- and (*S*)-enantiomers, respectively, in aqueous phase (mM)
K_p	partition coefficient defined as the ratio of substrate concentration in aqueous phase to that in organic phase
(S_R), (S_S)	(*R*)- and (*S*)-ester concentrations in aqueous phase, respectively (mM)
$(S_R)_{org}$, $(S_S)_{org}$	(*R*)- and (*S*)-ester concentrations in organic phase, respectively (mM)
$(S_R)^*_{org}$, $(S_S)^*_{org}$	initial (*R*)- and (*S*)-ester concentrations in organic phase in equilibrium with those in aqueous phase, respectively (mM)
$(S_{R0})_{org}$, $(S_{S0})_{org}$	initial $(S_R)_{org}$ and $(S_R)_{org}$, respectively (mM)
V	non-enzymatic initial rates for (*R*)- or (*S*)-ester based on aqueous phase (mM/h)
V_{aq}, V_{org}	volumes of aqueous and organic phases, respectively (ml)
V_R, V_S	enzymatic initial rates for (*R*)- and (*S*)-esters based on aqueous phase, respectively (mM/h)
X_R, X_S, X_t	conversions of (*R*)-, (*S*)- and (*R*,*S*)-ester, respectively

Another strategy of using CALB-catalyzed hydrolysis of (*R*,*S*)-tropic acid butyl ester in pH 7 phosphate buffers and transesterification of (*R*,*S*)-tropic acid lactone with butanol in toluene was developed [25]. The 90% ee_p of (*R*)-tropic acid and 99% ee_s of (*S*)-ester for the former, as well as >98% ee_p of (*R*)-butyl ester and >98% ee_s of (*S*)-lactone for the latter, indicate that the lipase has an excellent (*R*)-enantioselectivity for both substrates. However, the same problem of difficult separation of (*S*)-lactone substrate from (*R*)-ester product and then hydrolysing the resultant (*S*)-lactone or (*S*)-tropic acid butyl ester to (*S*)-tropic acid needs to be tackled. Similar arguments for the transesterification of (*R*,*S*)-vinyl α-methoxyphenyl acetate with methanol in isopropyl ether via an *Aspergillus niger* lipase, giving 73% yield with 87% ee_p of (*R*)-methyl ester, can be deduced if (*R*)- or (*S*)-α-methoxyphenylacetic acid is the desired product [20]. Obviously, in order to effectively resolve and separate the desired (*S*)-tropic acid, (*R*)- or (*S*)-α-methoxyphenylacetic acid of high yield and optical purity, an improvement of the enzymatic process is needed.

A thermally stable esterase (named as SNSM-87 from the producer) from *Klebsiella oxytoca* was recently disclosed, showing good to excellent enantioselectivity for the hydrolytic resolution of a variety of carboxylic acid esters [15,27–32]. As a continuation of the previous studies, we here report a successful strategy for the SNSM-87-catalyzed hydrolytic resolution of (*R*,*S*)-tropic acid esters and (*R*,*S*)-ethyl α-methoxyphenyl acetate in biphasic media (Scheme 1, where the configurations assigned as (*R*)- or (*S*)-enantiomers of **1**–**6** different from those of **7**–**10** are due to the priority of the Cahn–Ingold–Prelog rules), in which the acid products and remaining ester substrates can be separated and recovered from the aqueous and organic phases, respectively. The effect of leaving alcohol moiety on the enzyme activity and enantioselectivity using (*R*,*S*)-tropic acid esters as the substrate is first examined. A comparison of the kinetic constants is then made by replacing the α-hydroxymethyl substituent with α-methyl, α-hydroxy, α-methoxy or α-chloro moiety, showing the profound effect of α-substituent on the enzyme activity and enantioselectivity.

2. Materials and methods

2.1. Materials

A *K. oxytoca* esterase (named as SNSM-87 from the producer) was kindly donated from the Research & Development Center, Nagase & Co. Ltd. (Kobe, Japan). 2,2,2-Trifluoroethanol from Aldrich (Milwaukee, WI), (*R*,*S*)-tropic acid ((*R*,*S*)-α-hydroxymethylphenylacetic acid), (*S*)- and (*R*,*S*)-α-methoxyphenylacetic acids from TCI (Tokyo, Japan), ethyleneglycolmonomethyl ether and ethyleneglycolmonoethyl ether from Merck (Darmstadt, Germany), ethanol, isooctane, isopropanol, hexane and methanol from Tedia (Fairfield, OH) were purchased. Other chemicals of analytical grade were commercially available. Citrate, acetate, phosphate and carbonate were employed for the preparation of pH 3, pH 4–5, pH 6–8 and pH 9–10 buffers, respectively.

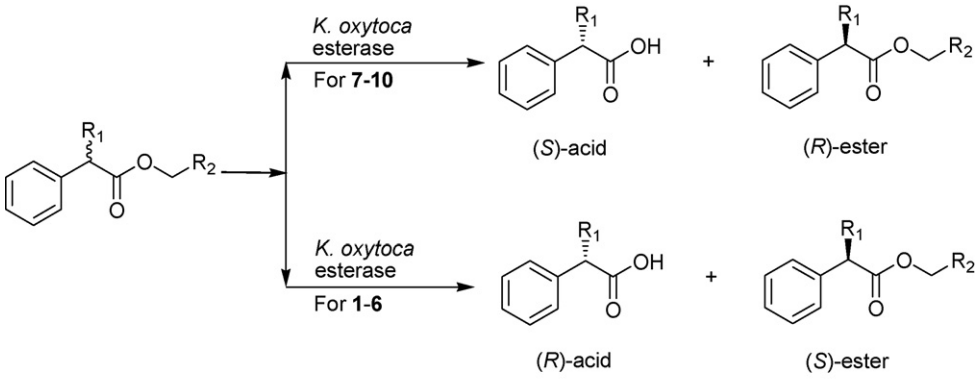
2.2. Synthesis of (R,S)-tropic acid esters, (S)- and (R,S)-ethyl α-methoxyphenyl acetates

To 650 mmol alcohol was added 5.35 mmol (*R*,*S*)-tropic acid (or (*S*)- or (*R*,*S*)-α-methoxyphenylacetic acid) and 5 mmol sulfuric acid and stirred at 55 °C for 6 h. After removing the remaining alcohol under reduced pressure, the residue was dissolved in 20 ml dichloromethane, washed in succession with 30 mM NaOH solution (3× 20 ml) and deionized water (20 ml). The organic phase was separated, dried over anhydrous magnesium sulfate, filtered and concentrated under reduced pressure, giving the desired ester products confirmed by ^{1}H NMR (400 MHz, *d*-DMSO), e.g. for **3**: δ 7.27–7.35 (m, 5H, ArH), 5.09 (t, OH), 3.77–3.81 (m, 3H, CH_2), 3.61–3.65 (m, 4H, CH plus CH_3).

2.3. Analysis

Hydrolysis of (*R*,*S*)-α-substituted phenyl acetates in biphasic media is monitored by HPLC using a chiral column from Daicel (OD-H or OJ-H; Tokyo, Japan) that is capable of separating the internal standard 2-nitrotolune, (*R*)- and (*S*)-ester. Detailed analytical conditions are represented in Table 1. Samples were removed from the organic phase and injected onto the HPLC at different time intervals for analysis, from which the time-course conversion (X_R or X_S) and initial rate for each enantiomer, as well as the racemate conversion (X_t) and enantiomeric excess for the substrate (ee_s) and product (ee_p) were determined.

In order to directly determine the enzyme optical-preference, the organic phase at the end of reaction was separated and concentrated under reduced pressure. Chloroform was then added to dissolve the remaining ester substrate for determining ee_s by injecting the resultant solution onto the HPLC. A Sepa-300 high sensitive polarimeter from Horiba (Tokyo, Japan) was further employed for estimating the angle of rotation: ee_s = 93.5%, $[\alpha]_D^{25}$ = −45.7 (*c* 1.2, $CHCl_3$) for (*S*)-2 in comparison with ee value = 94.0%, $[\alpha]_D^{25}$ = +42.0 (*c* 0.5, $CHCl_3$) for (*R*)-2 from Atuu et al. [24]; ee_s = 80.8%, $[\alpha]_D^{25}$ = −29.7 (*c* 5.5, $CHCl_3$) for (*S*)-4; ee_s = 74.1%,



R_1: CH_2OH; R_2: (**1**) $(CH_2)_4CH_3$, (**2**) CH_3, (**3**) H, (**4**) $CH_2OC_2H_5$, (**5**) CH_2OCH_3, (**6**) CF_3

R_2: CH_3; R_1: (**7**) Cl, (**8**) CH_3, (**9**) OH, (**10**) OCH_3

Scheme 1. Hydrolytic resolution of (*R*,*S*)-α-substituted phenylacetic acid esters in biphasic media via a *Klebsiella oxytoca* esterase.

$[\alpha]_D^{25} = -10.8$ (*c* 2.5, $CHCl_3$) for (*S*)-tropic acid butyl ester in comparison with ee value >98.0%, $[\alpha]_D^{25} = +24.0$ (*c* 4.0, $CHCl_3$) for (*R*)-tropic acid butyl ester from Klomp et al. [25].

2.4. Kinetic resolution of (R,S)-ethyl α-substituted phenyl acetates

Unless specified, a biphasic medium consisting of 2.83 ml pH 6 buffer (300 mM) and 20 ml isooctane containing 1 mM (*R*,*S*)-tropic acid ester (or (*R*,*S*)-ethyl α-methoxyphenyl acetate) were stirred at 55 °C with a magnetic stirrer at 400 rpm. Reaction started when 0.5 ml buffer (pH 6) containing SNSM-87 (33.3 mg for **2** and 16.7 mg for **10**) was added to the resultant solution. Samples were removed from the organic phase and injected onto the HPLC at different time intervals for analysis. Similar experiments without containing the enzyme were performed for estimating the partition coefficient K_p and non-enzymatic initial rate *V* of each substrate. By subtracting *V* from the initial rates, the enzymatic initial rates V_R and V_S were determined. More experiments of varying the substrate concentration in the biphasic media were carried out, from which the kinetic constants were estimated by using an extended Michaelis–Menten kinetics. In order to study aqueous pH effects on the enzyme performance, experiments with or without adding the enzyme to the buffer of a specified pH were also carried out.

2.5. Estimation of kinetic constants

A generalized expanded Michaelis–Menten mechanism for the rate-limiting acylation step is employed to describe the enzymatic kinetics of the esterase-catalyzed hydrolysis. By using the pseudo-steady-state approximation for all Michaelis complexes and tetrahedral intermediates and assuming an equilibrium partitioning for each substrate between aqueous and organic phases, the enzymatic initial rates based on the aqueous phase are derived as [30–32]:

$$V_R = \frac{k_{2R}^{**}(S_R)(E_t)}{K_{mR}^{**} + (S_R)} \quad (1)$$

$$V_S = \frac{k_{2S}^{**}(S_S)(E_t)}{K_{mS}^{**} + (S_S)} \quad (2)$$

Notations (E_t), (S_R) and (S_S) denote the initial enzyme, (*R*)- and (*S*)-ester concentrations in the aqueous phase, respectively. The latter two concentrations can be related to the substrate concentration in the organic phase $(S_{R0})_{org}$ or $(S_{S0})_{org}$ as $(S_R) = (S_S) = K_p(S_{R0})_{org}/(1 + K_p V_{aq}/V_{org})$. Detailed relationships between the kinetic parameters can be found elsewhere [30–32]. When the enzyme has high enantioselectivity for the (*R*)-antipode, the (*R*)-ester concentration may be negligible in comparison with the (*S*)-ester concentration in determining the initial rate V_S. One can estimate $K_{mS}^{**} = K_{mS}^{*}$ and $k_{2S}^{**} = k_{2S}^{*}$ from Eq. (2), and then K_{mR}^{**} and k_{2R}^{**} (and hence K_{mR}^{*} and k_{2R}^{*}) from Eq. (1). Therefore, the variations of specificity constants $k_{2R}/K_{mR} (= k_{2R}^{*}/K_{mR}^{*})$ and $k_{2S}/K_{mS} (= k_{2S}^{*}/K_{mS}^{*})$, and hence the enantiomeric ratio E $(= k_{2R}K_{mS}/k_{2S}K_{mR})$, with the inductive parameter of leaving alcohol moiety are obtainable. Moreover for the case $K_{mR}^{**} \gg (S_R)$ and $K_{mS}^{**} \gg (S_S)$, k_{2R}/K_{mR} and k_{2S}/K_{mS} can be determined from $V_R/(E_t)/(S_R)$ and $V_S/(E_t)/(S_S)$, respectively.

3. Results and discussion

3.1. Effects of leaving alcohol of (R,S)-tropic acid esters

The time-course concentrations of (*R*)- and (*S*)-tropic acid esters in the organic phase can be employed for calculating the initial rates of enzymatic and non-enzymatic reactions, and the substrate partition coefficient. Table 2 demonstrates effects of changing the

Table 1
HPLC analytical conditions

Ester	Chiral column	Mobile phase (hexane/IPA, v/v)	Flow rate (ml/min)	Retention time for (IS)/(*R*)-ester/(*S*)-ester (min)
1	OD-H	97/3	2.0	2.4/5.3/6.3
2	OD-H	97/3	2.0	2.4/6.7/7.2
3	OD-H	97/3	1.5	3.8/10.1/11.6
4	OJ-H	97/3	2.0	3.2/9.5/8.8
5	OJ-H	97/3	2.0	3.2/9.2/8.3
6	OJ-H	97/3	2.0	3.6/19.0/17.5
10	OD-H	97/3	1.5	3.8/8.1/4.2

Conditions: 2-nitrotolune as internal standard (IS); IPA as isopropanol; UV detection at 220 nm; column temperature at 25 °C.

Table 2
Effects of leaving group on partition coefficient K_p, specific initial rates, V_R/V_S, conversion X_t and ee_s for (*R*,*S*)-tropic acid esters

Ester	*R*	Inductive parameter	K_p	V (mM/h)	$V_R/(E_t)$ (mmol/h g)	$V_S/(E_t)$ (mmol/h g)	V_R/V_S	(E_t) (mg/ml)	Time (h)	X_t (%)	ee_s (%)
1	$(CH_2)_4CH_3$	−0.01	5.61(E−2)	5.82(E−5)	7.23(E−3)	2.85(E−5)	2.54(E+2)	10	48	51.1	100.0
2	CH_3	0.01	4.34(E−1)	6.30(E−4)	9.74(E−2)	2.16(E−4)	4.51(E+2)	5	48	51.5	100.0
3	H	0.03	1.54	1.74(E−3)	8.22(E−1)	1.30(E−3)	6.32(E+2)	5	10	54.8	100.0
4	$CH_2OC_2H_5$	0.11	1.12	7.80(E−3)	1.11	1.92(E−3)	5.78(E+2)	5	3	53.2	100.0
5	CH_2OCH_3	0.13	2.11	1.74(E−2)	2.81	5.40(E−3)	5.21(E+2)	1	8	52.5	100.0
6	CF_3	0.38	1.55(E−1)	5.70(E−3)	2.94(E−1)	1.92(E−3)	1.53(E+2)	5	24	53.0	100.0

Conditions: 20 ml isooctane containing 1 mM (*R*,*S*)-tropic acid ester and 3.33 ml pH 6 buffer (300 mM) containing SNSM-87 for all esters at 55 °C and 400 rpm. Values in the parentheses are exponents, e.g. (E−1) = 10^{-1}.

leaving alcohol on K_p, V, specific initial rates $V_R/(E_t)$, $V_S/(E_t)$ and V_R/V_S ratio, X_t, and ee_s. With (*R*,*S*)-methyl tropic acid ester (**3**) as the reference, increasing the carbon chain of leaving alcohol except for **5** containing a polar methoxy group results in the reduction of K_p. This will decrease the substrate solubility and generally the enzymatic rate in the aqueous phase. A comparison of the non-enzymatic initial rates for **2** and **6** indicates that an electro-withdrawing 2,2,2-trifluoro moiety, but not methyl group, in the leaving alcohol is advantageous for the hydrolysis. However, this will deteriorate the optical purity of the product or remaining substrate and should be suppressed. The inductive parameter *F* for the leaving alcohol can provide a measure of the relative effect of chain substituent on the electron density and nucleophilic ability of the hydroxyl group [33]. Increasing *F* yields maximum $V_R/(E_t)$ and $V_S/(E_t)$ at **5**, but the highest V_R/V_S acting as an index of enzyme enantioselectivity at **3**. As V_R/V_S values in Table 2 are all greater than 100, SNSM-87 is regarded as an excellent biocatalyst for resolving (*R*,*S*)-tropic acid esters. Moreover, compound **5** is selected as the best ester for the production of (*S*)-tropic acid when considering the highest K_p and $V_R/(E_t)$.

In order to examine the intrinsic enzymatic kinetic behavior, the kinetic parameters k_{2R}/K_{mR} and k_{2S}/K_{mS} for each substrate have been estimated from $V_R/(E_t)/(S_R)$ and $V_S/(E_t)/(S_S)$, respectively. Fig. 1 illustrates the logarithms of k_{2R}/K_{mR}, k_{2S}/K_{mS} and *E* varied with *F*, where about 15-fold enhancement of k_{2R}/K_{mR} (or 25-fold increase of k_{2S}/K_{mS}) by changing **1** to **6** is found, implying that the acylation step is the rate-limiting step for all substrates. A two-stage Brønsted slope with the breaking point at $F = 0.03$ for the fast-reacting (*R*)-esters is shown, indicating that breakdown of the tetrahedral adduct to the acyl-enzyme intermediate is rate-limiting for the (*R*)-ester containing a difficult leaving alcohol moiety. However, it changes to formation of the tetrahedral adduct when (*R*)-esters contain an easy leaving alcohol group. Only one Brønsted slope for all (*S*)-esters in the whole range of inductive parameters is depicted, indicating that breakdown of the tetrahedral adduct is rate-limiting and can be attributed a concerted but inefficient proton transfer from the catalytic imidazolium to the leaving alcohol moiety. Therefore, a maximum enantioselectivity at $F = 0.03$ for (*R*,*S*)-tropic acid methyl ester is rationalized. Similar kinetic behaviors for the esterase-catalyzed hydrolysis of (*R*,*S*)-α-chlorophenyl acetates, (*R*,*S*)-mandelates and (*R*,*S*)-2-chloromandelates have been reported [30–32].

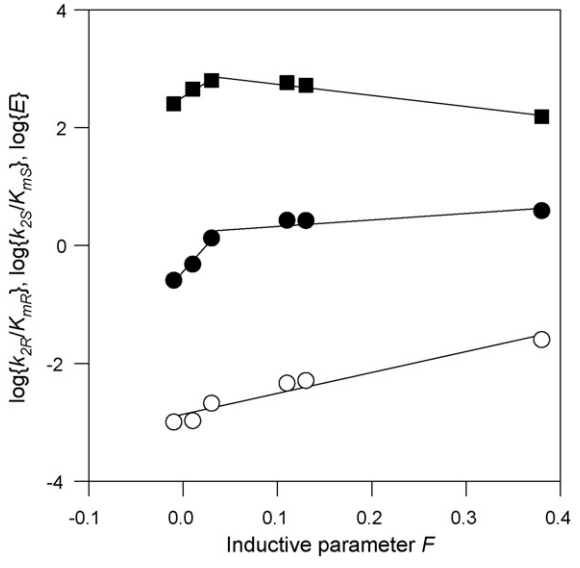


Fig. 1. Variations of (●) $\log\{k_{2R}/K_{mR}\}$, (○) $\log\{k_{2S}/K_{mS}\}$, and (■) $\log\{E\}$with inductive parameter *F* of R_2 moiety for (*R*,*S*)-tropic acid esters.

The structure–reactivity correlations are further represented in Table 3, showing a steep ascent Brønsted slope of 17.89 for the fast-reacting esters containing a difficult leaving alcohol. This slope levels off to 1.10 if the inductive parameter further increases from 0.03 to 0.38, indicating that the rate-limiting step has shifted from breakdown to formation of the tetrahedral intermediate. However, only one Brønsted slope of 3.54 for all (*S*)-esters in the whole range of inductive parameter is shown, implying the rate-limiting breakdown of tetrahedral intermediate to the acyl-enzyme intermediate. Therefore from the structure–enantioselectivity correlation, it is rational to obtain the maximum enantioselectivity at $F = 0.03$ for **3**. Similar structure–reactivity correlations for the esterase-catalyzed hydrolysis of (*R*,*S*)-α-chlorophenyl acetates and (*R*,*S*)-mandelates are also tabulated. Yet, the Brønsted slope for the fast-reacting esters containing a difficult leaving alcohol moiety greatly increases from 17.89 to 36.34 and 57.97 when the α-hydroxymethyl substituent is changed to α-hydroxy and α-chloro moiety, respectively.

Table 3
Structure–reactivity correlations for SNSM-87-catalyzed hydrolysis of (*R*,*S*)-tropic acid esters, (*R*,*S*)-α-chlorophenyl acetates and (*R*,*S*)-mandelates in biphasic media

Substrates	Structure–reactivity correlations	Constrains
(*R*,*S*)-Tropic acid esters		
(*R*)	$\log(k_{2R}/K_{mR}) = -0.44 + 17.89F$, $r^2 = 0.98$	$-0.01 \le F \le 0.03$
	$\log(k_{2R}/K_{mR}) = 0.21 + 1.10F$, $r^2 = 0.74$	$0.03 \le F \le 0.38$
(*S*)	$\log(k_{2S}/K_{mS}) = -2.86 + 3.54F$, $r^2 = 0.95$	$-0.01 \le F \le 0.38$
(*R*,*S*)-α-Chlorophenyl acetates[a]		
(*R*)	$\log(k_{2R}/K_{mR}) = -1.02 + 4.15F$, $r^2 = 0.96$	$-0.01 \le F \le 0.38$
(*S*)	$\log(k_{2S}/K_{mS}) = 0.27 + 57.97F$, $r^2 = 1.0$	$-0.01 \le F \le 0.01$
	$\log(k_{2S}/K_{mS}) = 0.87 + 2.28F$, $r^2 = 0.99$	$0.01 \le F \le 0.38$
(*R*,*S*)-Mandelates[a]		
(*R*)	$\log(k_{2R}/K_{mR}) = -0.27 + 2.88F$, $r^2 = 0.98$	$0.01 \le F \le 0.40$
(*S*)	$\log(k_{2S}/K_{mS}) = 0.93 + 36.34F$, $r^2 = 1.0$	$0.01 \le F \le 0.03$
	$\log(k_{2S}/K_{mS}) = 1.98 + 1.01F$, $r^2 = 0.92$	$0.03 \le F \le 0.40$

[a] From [31], with the enzyme optical-preference for (*S*)-α-chlorophenyl acetates is corrected.

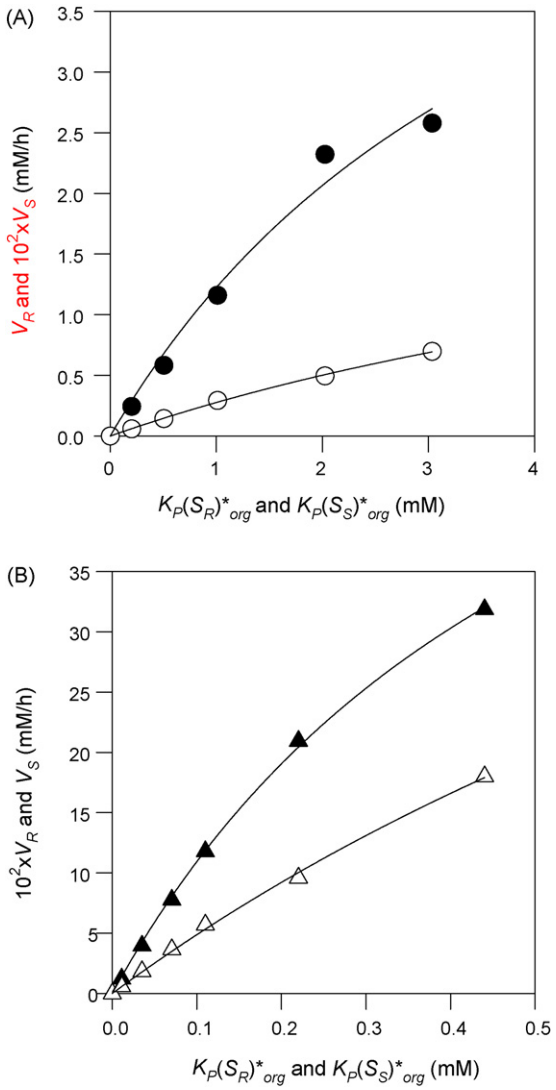


Fig. 2. Variations of initial rate with substrate concentration of aqueous phase ($K_P(S_R)^*_{org}$ or $K_P(S_S)^*_{org}$). (A) (●) for (*R*)-**2** and (○) for (*S*)-**2**; (B) (△) for (*R*)-**10** and (▲) (*S*)-**10**; (—) best-fit results. Conditions: 20 ml isooctane containing 1 mM racemate and 3.33 ml buffer (300 mM) containing SNSM-87 (33.3 mg for **2** and 16.7 mg for **10**) at 55 °C and 400 rpm.

Moreover for a given inductive parameter, more than an order-of-magnitude of k_{2R}/K_{mR} or k_{2S}/K_{mS} is obtainable when (*R*,*S*)-tropic acid esters are replaced by (*R*,*S*)-α-chlorophenyl acetates or (*R*,*S*)-mandelates. Therefore, the strong influence of α-substituent on the enzyme activity and enantioselectivity is evident, and needs further kinetic analysis for rationalizing the correlations.

3.2. Kinetic analysis for (R,S)-ethyl α-substituted phenyl acetates

The kinetic constants and *E* value using (*R*,*S*)-tropic acid ethyl ester or (*R*,*S*)-ethyl α-methoxyphenyl acetate as the substrate were estimated from Eqs. (1) and (2) coupled with the initial rates varied with substrate concentrations (Fig. 2). As demonstrated in Table 4, almost the same K^*_{mR} and K^*_{mS} for **7**–**9** were found, implying that all (*R*)- or (*S*)-esters containing an α-hydroxy, α-chloro or α-methyl moiety have the same affinity on combining the free enzyme to either the enzyme-substrate complex or tetrahedral intermediate. There is still no explanations for the slight decrease of K^*_{mR} and K^*_{mS} for **10**, and an enhancement of the kinetic constants for **2**, although α-hydroxymethyl and α-methoxy moieties have similar sizes and capability on forming a hydrogen bonding with amino acid residues in the active site.

A detailed analysis in Table 4 indicates that an electronegative atom attached to the α-carbon of **7**, **9** and **10**, but not **2** and **8,** is advantageous for giving higher k^*_{2R} and k^*_{2S} and hence the specificity constants. For example, about 53-fold decrease of k_{2R}/K_{mR} of (*R*)-**2** in comparison with k_{2S}/K_{mS} of (*S*)-**9** for the fast-reacting enantiomer as well as 516-fold decrease of k_{2S}/K_{mS} of (*S*)-**2** in comparison with k_{2R}/K_{mR} of (*R*)-**9** for the slow-reacting antipode are found. These results indicate that the amino acid residues in the active site do exert different non-covalent bonding to attract or expel the α-substituent. This may lead to a miniature change of the transition states of **7**, **9** and **10**, but not **2** and **8**, on enhancing the proton transfer from the catalytic imidazolium to break the tetrahedral intermediate. Apparently if the 3D enzyme crystal structure is disclosed in the future, the molecular modeling technique by docking various substrates to the active site will be helpful for elucidating the α-substituent effect.

As the imidazole moiety of catalytic histidine acting as a general acid-base catalyst is directly involved in the reaction, it must be uncharged for catalysis and may play an essential role on creating the chiral discrimination ability for all esters. Therefore, an estimation of the ionization constant of the imidazolium moiety at the presence of substrate is needed in order to evaluate the contribution of uncharged imidazole moiety on the specificity constants. Fig. 3 illustrates the pH effects on changing the relative enzyme activity for **2** and **9**. Since $K^{**}_{mR} \gg (S_R)$ and $K^{**}_{mS} \gg (S_S)$ at the present reaction conditions (Table 4), this figure can be regarded as the pH effects on varying the relative k_{2R}/K_{mR} and k_{2S}/K_{mS}. An empirical equation $k_{2i}/K_{mi} = (k_{2i}/K_{mi})_{int}/[1 + K_{1i}/(H^+) + (H^+)/K_{2i}]$ ($i = R$ and *S*) with (H^+) as the proton concentration has been employed for estimating the intrinsic specificity constants $(k_{2i}/K_{mi})_{int}$, ionization constants for the catalytic imidazolium moiety K_{2i} and other acidic or basic groups perturbing the activity, respectively [32]. Unfortunately, the curve fitting of giving $pK_{1S} = 9.31$ and $pK_{2S} = 5.60$ for (*S*)-**2**, as well as $pK_{1R} = 9.22$ and $pK_{2R} = 3.46$ for (*R*)-**2**, are not satisfactory (best-fit curves not shown). Yet, a shift of the left-hand-side bell-shape curve of (*S*)-**2** in comparison with that of (*R*)-**9** (or (*R*)-**2**

Table 4
Partitioning coefficient, kinetic constants and *E* for SNSM-87-catalyzed hydrolysis of (*R*,*S*)-ethyl α-substituted phenyl acetates

Ester	R_1	K_p	k^*_{2R} (mmol/h g)	K^*_{mR} (mM)	k_{2R}/K_{mR} (l/h g)	k^*_{2S} (mmol/h g)	K^*_{mS} (mM)	k_{2S}/K_{mS} (l/h g)	*E*
2	CH_2OH	4.3(E−1)	2.7	8.9	3.0(E−1)	5.4(E−3)	8.7	6.2(E−4)	487
7[a]	Cl	9.8(E−2)	1.9(E−1)	2.2	8.7(E−2)	2.7(E+1)	3.3	8.3	95
8[a]	CH_3	9.5(E−2)	4.8(E−3)	2.4	2.0(E−3)	1.8	2.9	6.4(E−1)	317
9[a]	OH	1.2(E−1)	8.6(E−1)	2.7	3.2(E−1)	4.1(E+1)	2.5	1.6(E+1)	51
10	OCH_3	2.2(E−2)	1.7(E−1)	1.6	1.1(E−1)	2.3(E+1)	9.1(E−1)	2.5(E+1)	227

Conditions as given in Fig. 2. Values in the parentheses are exponents, e.g. (E−1) = 10^{-1}.

[a] Data from [31], with the enzyme optical-preference corrected for (*S*)-**7** is corrected. A Sepa-300 high sensitive polarimeter from Horiba was employed for measuring angles of rotation for products in the aqueous phase and remaining substrates in the organic phase at the end of reaction: $ee_s = 94.0\%$, $[\alpha]_D^{25} = -90.4$ (*c* 9.0, $CHCl_3$) for (*R*)-7 and $ee_p = 98.0\%$, $[\alpha]_D^{25} = +13.3$ (*c* 0.15, $CHCl_3$) for (*S*)-α-chlorophenylacetic acid in comparison with ee value = 19.0%, $[\alpha]_D^{26} = -26.0$ (*c* 3.3, C_6H_6) and $[\alpha]_D = +132.13$ (*c* 3.33, C_6H_6) for (*R*)- and (*S*)-α-chlorophenylacetic acids, respectively, from Yamazaki et al. [34].

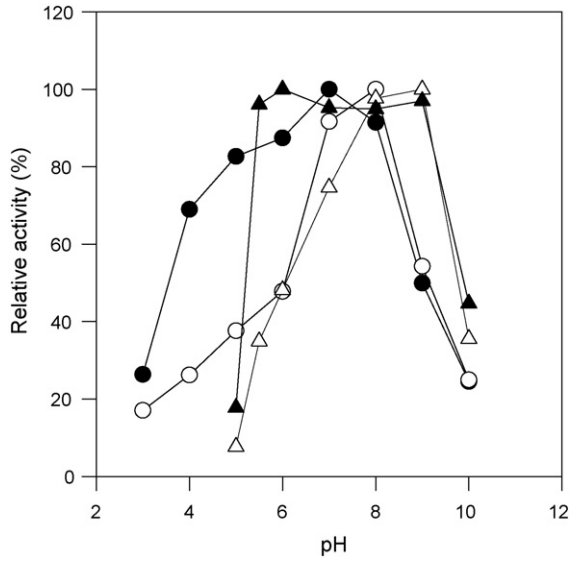


Fig. 3. Relative activity varied with aqueous pH; (●) for (*R*)-**2**, (○) for (*S*)-**2**, (△) for (*R*)-**9** and (▲) for (*S*)-**9**; 100% specific initial rates of 9.45×10^{-2} mmol/h g for (*R*)-**2**, 4.81×10^{-4} mmol/h g for (*S*)-**2**, 9.54×10^{-2} mmol/h g for (*R*)-**9**, and 1.62 mmol/h g for (*S*)-**9**. Conditions: 20 ml isooctane containing 1 mM ester and 3.33 ml buffer (300 mM) containing 333 mg SNSM-87 for **2** at 55 °C and 400 rpm. Data for **9** from [32].

with (*S*)-**9**) indicates that more fraction of the uncharged catalytic imidazole moiety in pH 6 buffers must exist when using **2** as the substrate. Therefore, about 60-fold difference between $(k_{2R}/K_{mR})_{int}$ of (*R*)-**2** and $(k_{2S}/K_{mS})_{int}$ of (*S*)-**9** as well as 705-fold difference between $(k_{2S}/K_{mS})_{int}$ of (*S*)-**2** and $(k_{2R}/K_{mR})_{int}$ (*R*)-**9** are calculated if $pK_{1R} = 9.87$ and $pK_{2R} = 5.96$ for (*R*)-**9** as well as $pK_{1S} = 9.31$ and $pK_{2S} = 5.60$ for (*S*)-9 are adopted [32]. Then, the intrinsic higher reactivity of the enzyme to the substrate, such as **9** but not **2,** containing an electronegative atom to the α-carbon is concluded. Moreover, Table 4 indicates that the enzyme enantioselectivity is good ($100 > E > 50$) to excellent ($E > 100$) for the hydrolytic resolution of (*R*,*S*)-ethyl α-substituted phenyl acetates.

4. Conclusions

A thermally stable *K. oxytoca* esterase has been employed for the hydrolytic resolution of (*R*,*S*)-tropic acid esters and (*R*,*S*)-ethyl α-methoxyphenyl acetate in biphasic media, where excellent enzyme enantioselectivity ($E > 100$) was obtained for all substrates. By altering the leaving alcohol moiety of (*R*,*S*)-tropic acid esters, an expanded Michaelis–Menten mechanism was first adopted for interpreting the enzyme kinetic behavior of the rate-limiting acylation step. For the fast-reacting (*R*)-esters containing a difficult leaving alcohol moiety, the two-stage Brønsted slope indicates that the rate-limiting breakdown of tetrahedral intermediate is valid for the substrate containing a difficult leaving alcohol, and shifts to formation of tetrahedral intermediate if the substrate contains an easy leaving alcohol. However for all (*S*)-esters, only one Brønsted slope and hence the rate-limiting breakdown of tetrahedral intermediate is found, and consequently leads to an optimal enantioselectivity for (*R*,*S*)-tropic acid methyl ester. Comparisons and elucidation of the kinetic constants and *E* values for various (*R*,*S*)-ethyl α-substituted phenyl acetates indicate that the α-substituent has profound influence on changing the enzyme activity and enantioselectivity.

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