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Multi-substrate screening for lipase-catalyzed resolution of arylalkylethanols with succinic anhydride as acylating agent

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ABSTRACT

CAL-B lipase-catalyzed resolution of a number of arylalkylethanols using succinic anhydride as acylating agent has been performed to evaluate the corresponding enantioselectivity factors. Then "one-pot multi-substrate screening" reactions involving two- and four-substrate mixtures were carried out and evaluation of the enantioselectivity factors for each alcohol was undertook by a single-run analysis. It was concluded that the alcohols in the mixture behave independently, validating the "one-pot multi-substrate screening" for a rapid evaluation of the enantioselectivity of the kinetic resolution process for each individual substrate.

1. Introduction

The "one-pot multi-substrate screening" [1] has been successfully used for exploration of catalytic activities and enantioselectivities of a given catalyst by performing reaction on a mixture of achiral substrates. Since it is of importance to realize the evaluation of the performance of an enzyme towards several substrates in the shortest time, the multi-substrate screening methodology appears particularly suited. The method requires that the ee's of each unreacted substrate (or product) should be measured in a mixture. The best way is to measure the ee's by a single run on chiral GLC or HPLC, with baseline separation for peaks of all compounds. However, the values measured from multi-substrate reaction are valid for reactions performed onto individual substrates only if these substrates behave independently in the mixture.

Most examples of application of such a methodology involve metal- or organo-catalyzed enantioselective reactions performed onto achiral substrates: oxazaborolidine-catalyzed borane reduction of prochiral ketones [2], diethylzinc addition to aldehydes [3], rhodium-catalyzed hydroformylation of olefins [4], nickel-catalyzed hydrosilylation of ketones [5], copper-catalyzed conjugate addition of diethylzinc to cycloalkenones [6], nitroalkenes [7] and ruthenium-catalyzed reduction of ketones by hydride transfer [8].

To date and to the best of our knowledge, only one example of application of enzyme-catalyzed multi-substrate screening methodology has been reported, dealing with the enzyme-catalyzed hydrolysis of monoesters [9].

These methods allow the evaluation of the stereoselectivities of reactions performed onto a mixture of substrates, to get the stereoselectivity expected on individual substrates.

However, the validation of the methods requires that each substrate reacts in a mixture with the same enantioselectivity as individually under the same conditions, i.e. that the substrates behave independently.

This point needs to be addressed since it has been reported that the stereoselectivity of a lipase-catalyzed acylation of a chiral alcohol may be affected by the presence of another substrate, especially when this latter is of similar structure [10].

In order to have a look to this latter point, we determined the *E*-value for lipase-catalyzed acylation of each individual secondary alcohol, with succinic anhydride as the acylating agent. We then compared with the values obtained in two- and four-substrate acylation reactions performed under the same conditions. Reactions were usually performed twice in order to check their reproducibility.

This work reports our investigation of lipase-catalyzed acylation of several racemic arylalkylethanols with cyclic, achiral, chiral or prochiral anhydrides. Use of cyclic anhydrides as acylating agents has been shown to offer some advantages over the use of vinyl

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esters [11]. The product obtained from acylation is a hemiester which is easily separated from the unreacted alcohol by extraction with an aqueous sodium bicarbonate solution, avoiding the use of a chromatographic separation. Furthermore the hemiester liberates the enantiomeric alcohol through saponification. The enantiomeric purities of both the unreacted alcohol and the produced one were measured by chiral HPLC. Conditions for HPLC analysis were set up to record baseline separations of enantiomers of the mixture of alcohols. Conversions were calculated from these values. Evaluation of the *E*-values for each individual substrate would then be compared with the values recorded for reactions performed onto two- and four-substrate mixtures. This would indicate whether the substrates behave independently, and, accordingly, which substrates show high reactivity and selectivity.

The use of cyclic anhydride in this multi-substrate screening methodology is of interest: (i) the work-up is easily carried out, since it does not require a chromatographic separation; (ii) a single set up of analysis has to be done, since both the unreacted substrates and the products have the same structure (arylalkylethanols); (iii) the accurate measurement (chiral HPLC) of ee's of these alcohols allows to calculate the conversion, and hence the *E* factor for each substrate. We made the assumption that the reactions are irreversible and hence the *E*-values remained constant throughout the reaction [12].

The commercially available *Candida antarctica* lipase B (CAL B) was chosen for this study, since it is one of the most used for kinetic resolution of alcohols. Moreover, we showed that it performed better than *Pseudomonas fluorescens* lipase (PFL) [13].

2. Experimental

2.1. Materials and methods

Substrates **1**–**8** and anhydrides **9**–**12** were commercially available and purchased from *Acros Organics* (**1**, **3**, **9**), *Sigma–Aldrich* (**2**, **5**, **11**, **12**), *Fluka* (**4**), *Fluka Chemika* (**6**), *Janssen* (**7**), *Janssen Chemika* (**10**) and *Alfa Aesar* (**8**). They were used as received. Lipase acrylic resin from *C. antarctica* (Novozym, L4777, 3 units/mg) was purchased from *Sigma–Aldrich*.

Reactions were monitored by TLC and the enantiomeric excesses of the alcohols were determined by chiral HPLC on Chiralcel® OD-H column (25 cm × 4.6 mm), with UV-detection at 254 nm.

2.2. General procedure for kinetic resolution

The mixture of equimolecular amounts of the racemic alcohol (1 mmol) and anhydride (1 mmol) in diethyl ether (5 ml) and the lipase (150 mg) was stirred for 24 h at room temperature. After filtration of the enzyme, the ethereal solution was extracted with an aqueous solution of 2 M Na_2CO_3 (2× 2 ml). The combined aqueous extracts were washed with ethyl ether (5 ml) and the combined organic phases were dried ($MgSO_4$) and evaporated to recover the unreacted alcohol. To the combined aqueous phases was added an aqueous NaOH solution (5 ml, 10%). After 2 h the aqueous phase was extracted with ethyl ether (2× 5 ml), the combined ethereal phases were dried ($MgSO_4$) then evaporated to give the alcohol which has reacted in the acylation process.

2.3. HPLC analysis of racemic alcohols *1*–*8*

(1) 1-Indanol (**1**): hexane/*i*-PrOH, 98/2; λ = 254 nm; 0.8 ml/min; $t_{R(\mathbf{1})}$ (*S*) = 20.7 min, $t_{R(\mathbf{1})}$ (*R*) = 23.9 min.
(2) 1,2,3,4-Tetrahydro-1-naphthol (**2**): hexane/EtOH, 98/2; λ = 254 nm; 0.8 ml/min; $t_{R(\mathbf{2})}$ (*S*) = 9.45 min, $t_{R(\mathbf{2})}$ (*R*) = 10.1 min.
(3) 1-(6-Methoxy-2-naphthyl)ethanol (**3**): hexane/*i*-PrOH, 90/10; λ = 254 nm; 1 ml/min; $t_{R(\mathbf{3})}$ (*S*) = 8.70 min, $t_{R(\mathbf{3})}$ (*R*) = 11.85 min.
(4) 1-(2-Naphthyl)ethanol (**4**): hexane/EtOH, 98/2; λ = 254 nm; 0.8 ml/min; $t_{R(\mathbf{4})}$ (*S*) = 28.2 min, $t_{R(\mathbf{4})}$ (*R*) = 30.3 min. [(*S*,*S*)-ulmo-column: hexane/*i*-PrOH, 95/5; λ = 254 nm; 0.5 ml/min; $t_{R(\mathbf{4})}$ (*S*) = 12.6 min, $t_{R(\mathbf{4})}$ (*R*) = 14.5 min].
(5) 1-Acenaphthenol (**5**): hexane/*i*-PrOH, 90/10; λ = 254 nm; 0.5 ml/min; $t_{R(\mathbf{5})}$ (*S*) = 16.0 min, $t_{R(\mathbf{5})}$ (*R*) = 17.0 min.
(6) 1-(1-Naphthyl)ethanol (**6**): hexane/*i*-PrOH, 90/10; λ = 254 nm; 0.9 ml/min; $t_{R(\mathbf{6})}$ (*S*) = 10.5 min, $t_{R(\mathbf{6})}$ (*R*) = 16.6 min.
(7) 1-Phenylethanol (**7**): hexane/*i*-PrOH, 95/5; λ = 254 nm; 1 ml/min; $t_{R(\mathbf{7})}$ (*S*) = 8.37 min, $t_{R(\mathbf{7})}$ (*R*) = 9.67 min.
(8) 1-(4-Methoxyphenyl)ethanol (**8**): hexane/*i*-PrOH, 95/5; λ = 254 nm; 0.8 ml/min; $t_{R(\mathbf{8})}$ (*S*) = 13.5 min, $t_{R(\mathbf{8})}$ (*R*) = 15.1 min.

2.4. HPLC analysis of the alcohols in two-substrate mixture

(1) Alcohols [(**1**) + (**6**)]: hexane/EtOH, 98/2; λ = 254 nm; 0.8 ml/min; $t_{R(\mathbf{1})}$ (*S*) = 15.3 min, $t_{R(\mathbf{1})}$ (*R*) = 17.0 min; $t_{R(\mathbf{6})}$ (*S*) = 26.9 min, $t_{R(\mathbf{6})}$ (*R*) = 46.3 min.
(2) Alcohols [(**6**) + (**8**)]: hexane/EtOH, 98/2; λ = 254 nm; 0.8 ml/min; $t_{R(\mathbf{6})}$ (*S*) = 27.3 min, $t_{R(\mathbf{6})}$ (*R*) = 46.7 min; $t_{R(\mathbf{8})}$ (*S*) = 20.0 min, $t_{R(\mathbf{8})}$ (*R*) = 23.8 min.
(3) Alcohols [(**1**) + (**8**)]: hexane/EtOH, 98/2; λ = 254 nm; 0.8 ml/min; $t_{R(\mathbf{1})}$ (*S*) = 15.7 min, $t_{R(\mathbf{1})}$ (*R*) = 17.7 min; $t_{R(\mathbf{8})}$ (*S*) = 20.7 min, $t_{R(\mathbf{8})}$ (*R*) = 24.5 min.
(4) Alcohols [(**2**) + (**3**)]: hexane/EtOH, 98/2; λ = 254 nm; 0.8 ml/min; $t_{R(\mathbf{2})}$ (*S*) = 13.5 min, $t_{R(\mathbf{2})}$ (*R*) = 15.0 min; $t_{R(\mathbf{3})}$ (*S*) = 31.1 min, $t_{R(\mathbf{3})}$ (*R*) = 52.2 min.
(5) Alcohols [(**2**) + (**5**)]: hexane/EtOH, 98/2; λ = 254 nm; 0.8 ml/min; $t_{R(\mathbf{2})}$ (*S*) = 13.7 min, $t_{R(\mathbf{2})}$ (*R*) = 15.2 min; $t_{R(\mathbf{5})}$ (*S*) = 28.7 min, $t_{R(\mathbf{5})}$ (*R*) = 32.9 min.
(6) Alcohols [(**7**) + (**8**)]: hexane/EtOH, 98/2; λ = 254 nm; 0.8 ml/min; $t_{R(\mathbf{7})}$ (*S*) = 13.7 min, $t_{R(\mathbf{7})}$ (*R*) = 17.1 min; $t_{R(\mathbf{8})}$ (*S*) = 19.9 min, $t_{R(\mathbf{8})}$ (*R*) = 21.9 min.
(7) Alcohols [(**1**) + (**7**)]: hexane/EtOH, 98/2; λ = 254 nm; 0.8 ml/min; $t_{R(\mathbf{1})}$ (*S*) = 14.8 min, $t_{R(\mathbf{1})}$ (*R*) = 16.5 min; $t_{R(\mathbf{7})}$ (*S*) = 13.6 min, $t_{R(\mathbf{7})}$ (*R*) = 17.7 min.
(8) Alcohols [(**1**) + (**3**)]: hexane/EtOH, 98/2; λ = 254 nm; 0.8 ml/min; $t_{R(\mathbf{1})}$ (*S*) = 14.9 min, $t_{R(\mathbf{1})}$ (*R*) = 16.9 min; $t_{R(\mathbf{3})}$ (*S*) = 28.9 min, $t_{R(\mathbf{3})}$ (*R*) = 40.6 min.
(9) Alcohols [(**3**) + (**8**)]: hexane/EtOH, 98/2; λ = 254 nm; 0.8 ml/min; $t_{R(\mathbf{3})}$ (*S*) = 31.3 min, $t_{R(\mathbf{3})}$ (*R*) = 54.1 min; $t_{R(\mathbf{8})}$ (*S*) = 21.2 min, $t_{R(\mathbf{8})}$ (*R*) = 23.4 min.
(10) Alcohols [(**2**) + (**7**)]: hexane/EtOH, 98/2; λ = 254 nm; 0.8 ml/min; $t_{R(\mathbf{2})}$ (*S*) = 19.2 min, $t_{R(\mathbf{2})}$ (*R*) = 21.9 min; $t_{R(\mathbf{7})}$ (*S*) = 16.4 min, $t_{R(\mathbf{7})}$ (*R*) = 17.1 min.

2.5. HPLC analysis of the alcohols in four-substrate mixture

(1) Alcohols [(**1**) + (**3**) + (**6**) + (**8**)]: hexane/EtOH, 98/2; λ = 254 nm; 0.8 ml/min; $t_{R(\mathbf{1})}$ (*S*) = 16.3 min, $t_{R(\mathbf{1})}$ (*R*) = 18.4 min; $t_{R(\mathbf{3})}$ (*S*) = 34.6 min, $t_{R(\mathbf{3})}$ (*R*) = 55.9 min; $t_{R(\mathbf{6})}$ (*S*) = 31.5 min, $t_{R(\mathbf{6})}$ (*R*) = 50.4 min; $t_{R(\mathbf{8})}$ (*S*) = 22.3 min, $t_{R(\mathbf{8})}$ (*R*) = 24.6 min.
(2) Alcohols [(**1**) + (**4**) + (**7**) + (**8**)]: hexane/EtOH, 98/2; λ = 254 nm; 0.8 ml/min; $t_{R(\mathbf{1})}$ (*S*) = 15.9 min, $t_{R(\mathbf{1})}$ (*R*) = 18.0 min; $t_{R(\mathbf{4})}$ (*S*) = 29.7 min, $t_{R(\mathbf{4})}$ (*R*) = 33.4 min; $t_{R(\mathbf{7})}$ (*S*) = 14.3 min, $t_{R(\mathbf{7})}$ (*R*) = 19.0 min; $t_{R(\mathbf{8})}$ (*S*) = 21.0 min, $t_{R(\mathbf{8})}$ (*R*) = 22.9 min.
(3) Alcohols [(**2**) + (**3**) + (**5**) + (**7**)]: hexane/EtOH, 98/2; λ = 254 nm; 0.8 ml/min; $t_{R(\mathbf{2})}$ (*S*) = 10.5 min, $t_{R(\mathbf{2})}$ (*R*) = 11.2 min; $t_{R(\mathbf{3})}$ (*S*) = 21.0 min, $t_{R(\mathbf{3})}$ (*R*) = 33.7 min; $t_{R(\mathbf{5})}$ (*S*) = 23.4 min, $t_{R(\mathbf{5})}$ (*R*) = 25.8 min; $t_{R(\mathbf{7})}$ (*S*) = 11.1 min, $t_{R(\mathbf{7})}$ (*R*) = 14.0 min.

Table 1
CAL-B-catalyzed acylation of alcohols **1**–**8** by cyclic anhydrides[a]: conversions, yields, enantiomeric excesses and *E*-values.

Entry	Substrate	Anhydride[b]	Substrate		Product		c (%)[e]	*E*[e]
			ee_S (%)[c]	Yield (%)[d]	ee_P (%)[c]	Yield (%)[d]		
1		SA	>99	41	>99	26	50	>200
2		GA	47	54	99	15	32	>200
3	**1**	MeSA	75	52	>99	24	43	>200[f]
4		MeGA	30	52	85	16	26	17
5		SA	94	51	>99	26	49	>200
6		GA	24	71	>99	7	20	>200
7	**2**	MeSA	3	66	76	3	4	10[f]
8		MeGA	2	56	97	2	2	70
9		SA	74	52	>99	32	43	>200
10		GA	7	78	74	5	9	8
11	**3**	MeSA	2	85	93	2	2	30[f]
12		MeGA	6	82	98	3	6	>100
13		SA	88	41	>99	30	47	>200
14		GA	31.5	58	61	24	34	6
15	**4**	MeSA	5	78	95	1	3	40[f]
16		MeGA	9	83	87	5	9	15
17		SA	5	56	36	18	12	3
18		GA	<1	84	Nd	1	–	–
19	**5**	MeSA	1	78	44	1	2	4[f]
20		MeGA	0.2	87	Nd	3	–	–
21		SA	5	82	91	5	5	23
22		GA	2	76	71	1	3	7
23	**6**	MeSA	1	88	28	1	2	3[f]
24		MeGA	<1	–	–	–	–	–
25		SA	98	37	99	35	50	>200
26		GA	29	55	>99	8	23	>200
27	**7**	MeSA	11	45	>99	3	9	>200[f]
28		MeGA	16	47	99	7	14	>200
29		SA	97	45	97	30	50	>200
30		GA	42	69	97	18	30	100
31	**8**	MeSA	16	82	98	12	14	>100[f]
32		MeGA	23	46	98	14	19	>100

[a] Reactions were performed onto 1 mmol of alcohol and 1 equiv. of anhydride in diethyl ether (5 ml) in the presence of 150 mg lipase for 24 h.
[b] SA: succinic anhydride; GA: glutaric anhydride; MeSA: 2-methylsuccinic anhydride; MeGA: 3-methylglutaric anhydride.
[c] ee_s and ee_p were measured by chiral HPLC on Chiralcel® OD-H column.
[d] Isolated yield.
[e] Conversion c and enantioselectivity *E* were calculated from relationships: $c = ee_S/(ee_S + ee_P)$; $E = \ln[1 - c(1 + ee_P)]/\ln[1 - c(1 - ee_P)]$ [14].
[f] Calculated with the assumption that the stereoselectivity of attack of the alcohol onto the diastereomeric acylenzymes solely arises from asymmetric induction of the enzyme chirality.

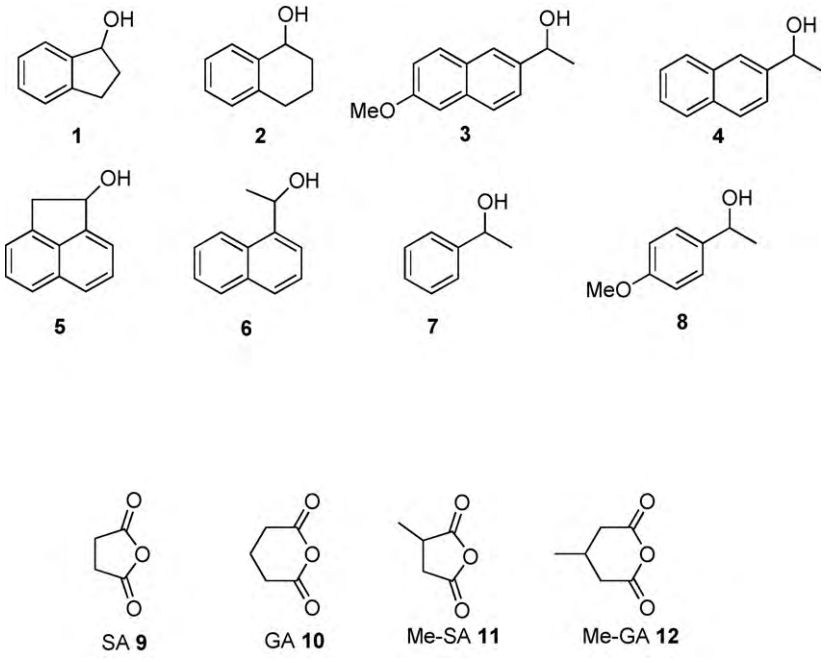

Fig. 1. Arylalkyl alcohols to be resolved and cyclic anhydrides used as acylating agents.

Table 2
Acylation of a two-substrate mixture of alcohols: conversions, yields, enantiomeric excesses and *E*-values.

Entry	Alcohol[a]		Unreacted alcohol		Recovered alcohol		c (%)[e]	*E*[e]
			ee_S [d] (%)	Yield[c] (%)	ee_P[d] (%)	Yield[c] (%)		
1	(1)		>99	41	>99	26	50	>200
2	(2)		94	51	>99	26	49	>200
3	(3)		74	52	>99	32	43	>200
4	(4)		88	42	>99	25	47	>200
5	(5)		5	56	36	18	12	3
6	(6)		5	82	91	5	5	23
7	(7)		98	37	99	35	50	>200
8	(8)		97	45	97	30	50	>200
9	(1)+(6)	(1)	>99		>99		50	>200
		(6)	1		72		2	7
10	(6)+(8)	(6)	4		62		6	5
		(8)	88		95		48	110
11	(1)+(8)	(1)	80		97		45	160
		(8)	56		95		37	70
12	(2)+(3)	(2)	75		>99		43	>200
		(3)	68		99		41	>200
13	(2)+(5)	(2)	41		95		30	60
		(5)	11		70		14	7
14	(7)+(8)	(7)	17		>99		15	>200
		(8)	23		98		19	120
15	(1)+(7)	(1)	36		96		27	70
		(7)	14		89		14	20
16	(1)+(3)	(1)	93		89		51	60
		(3)	20		>99		17	>200
17	(3)+(8)	(3)	89		89		50	50
		(8)	84		>99		46	>200
18	(2)+(7)	(2)	6		69		8	7
		(7)	21		85		20	16
19	(7)+(8)	(7)	17		>99		15	>200
		(8)	23		98		19	120
20[b]	[7+Enz]+8	(7)	59		75		44	15
		(8)	74		74		50	15
21[b]	[8+Enz[c]]+7	(7)	60		95		39	70
		(8)	76		91		46	50

[a] Entries 1–8: alcohol (1 mmol), SA (1 equiv.), lipase (150 mg), diethyl ether (5 ml), 24 h; entries 9–21: under the same conditions but with 0.5 mmol for each alcohol.
[b] The first alcohol and the enzyme were stirred for 10 min before the addition of the second alcohol.
[c] Isolated yield.
[d] ee_s and ee_p were measured by chiral HPLC on Chiralcel® OD-H column;.
[e] Conversion c and enantioselectivity *E* were calculated from relationships: $c = ee_S/(ee_S + ee_P)$; $E = \ln[1 - c(1 + ee_P)]/\ln[1 - c(1 - ee_P)]$ [14].

3. Results and discussion

3.1. CAL-B-catalyzed acylation of benzylic-type alcohols

The kinetic resolution of arylalkyl alcohols **1**–**8** was carried out with four different cyclic anhydrides **9**–**12** under *CAL-B* catalysis (Fig. 1).

Each alcohol was subjected to acylation according to the reported procedure, in diethyl ether as the solvent. Enantiomeric excesses of the unreacted alcohol and the alcohol obtained from the hemiester produced were measured by chiral HPLC. The conversion was calculated from these values, together with the enantioselectivity factor *E*. Table 1

With succinic anhydride as the acylating agent, the conversion was over 43% and the *E*-value > 200, except for 1-acenaphthenol **5** and 1-(1-naphthyl)-ethanol **6**, which show both low reactivity and enantioselectivity. Glutaric anhydride provided less satisfactory results, although enantioselectivity remained high for substrates **1**, **2**, **7** and **8**. Methylglutaric anhydride afforded high enantioselectivities for the same substrates as glutaric anhydride did (with the exception of **1** and **4**), albeit with lower activities.

Acylation of the enzyme by MeSa could result in the formation of regio- and diastereoisomeric acylenzymes. However, the *E*-value was estimated on the assumption that the stereoselection solely arose from the enzyme chirality. Since conversions of most of the alcohol substrates, with the exception of **1**, were low, results recorded from acylation with MeSA-**11** will not be further discussed.

Low values for activity and enantioselectivity were recorded for alcohols **5** and **6** whatever the acylating agent was.

On the basis of these results, succinic anhydride was then selected to carry out the multi-substrate assays.

3.2. CAL-B-catalyzed acylation of a two-substrate mixture of alcohols

In order to check whether the acylation of both substrates proceeded independently, we ran two-substrate experiments under analogous conditions than those established in single-substrate assays. A number of two-substrate mixtures were subjected to *CAL-B*-catalyzed acylation with succinic anhydride under the same experimental procedure as the one used in single-substrate assay.

Table 3
CAL-B-catalyzed acylation of a four-substrate mixture of alcohols.

Entry	Alcohol[a]		Unreacted alcohol		Recovered alcohol		c (%)	E^c
			ee_S (%)[c]	Yield (%)	ee_P (%)[c]	Yield (%)		
1	(1)		>99	41	>99	26	50	>200
2	(**2**)		94	51	>99	26	49	>200
3	(**3**)		74	52	>99	32	43	>200
4	(**4**)		88	42	>99	30	47	>200
5	(**5**)		5	56	36	18	12	3
6	(**6**)		5	82	91	5	5	20
7	(**7**)		98	37	99	35	50	>200
8	(**8**)		96	45	97	30	50	>200
9	(**1**+**3**+**6**+**8**)	(**1**)	>99		>99		50	>200
		(**3**)	33		99		25	>200
		(**6**)	1		61		2	5
		(**8**)	>99		>99		50	>200
10	(**1**+**4**+**7**+**8**)[b]	(**1**)	93		>99		48	>200
		(**4**)	25		99		20	>200
		(**7**)	69		94		42	66
		(**8**)	67		98		41	>200
11	(**2**+**3**+**5**+**7**)	(**2**)	13		>99		42	>200
		(**3**)	34		79		26	90
		(**5**)	3		90		3	20
		(**7**)	37		99		27	>200

Conversion c and enantioselectivity E were calculated from relationships: $c = ee_S/(ee_S + ee_P)$; E =conversion c and enantioselectivity E were calculated from relationships: $c = ee_S/(ee_S + ee_P)$; $E = \ln[1 - c(1 + ee_P)]/\ln[1 - c(1 - ee_P)]$ [14]. $\ln[1 - c(1 + ee_P)]/\ln[1 - c(1 - ee_P)]$ [14].

[a] Reactions were performed onto 0.5 mmol of each alcohol in the presence of 150 mg lipase/mmol in diethylether for 24 h.

[b] Reaction carried out with 75 mg lipase.

[c] ee_s and ee_p were measured by chiral HPLC on Chiralcel® OD-H column.

The results collected in Table 2 show the following main features: for runs involving a "reactive" substrate and a "poorly reactive" one, results – in terms of reactivity and selectivity – are not so affected by the presence of the poor substrate and are closed to those obtained in single-substrate runs. Hence the alcohols appear to react independently.

However, for reaction involving a 2-"good"-substrate mixture, the conversion appears sometimes depleted although the enantioselectivity is hardly affected (run **14**, **7** and **8**; run **15**, **1** and **3**).

We may tentatively offer an explanation for the depleted conversion observed when using mixtures of several – however active – substrates compared to the rates observed for individual substrates.

As mentioned in previous reports, acylation of the enzyme by an acylating agent of structure similar to the nucleophile involved in the deacylation process would result in an imprinting process which would favor the following reaction of the nucleophile. Conversely, it may disfavor the reaction of other nucleophiles.

So that for multi-alcohol reactions, we may tentatively suggest that each alcohol would imprint, in the deacylation process a number of enzyme sites favoring the further reactions of this alcohol with these sites. This would result in "specialization" of the sites of an enzyme towards one particular alcohol of the mixture. Hence, in addition of being in lower molecular ratio, each alcohol would have access to a limited number of sites with a good reactivity, resulting in a lower overall rate of the process.

The order of introduction of the reagents (enzyme added to a mixture of the 2 alcohols) has some influence on the reactivity and selectivity.

For the mixture of substrates **7** and **8**, the enzyme added to the mixture of alcohols resulted in a reduction of the reactivity although the enantioselectivities for both substrates were preserved. However, addition of the enzyme to the first alcohol before addition of the second substrate restored the reactivity, albeit with depletion of the enantioselectivity.

The hemiester produced from reaction of one alcohol could be an acylating agent to provide new acylenzymes which would be deacylated by the same or other alcohol, with different stereoselectivities than when reacting onto the primary acylenzyme, affording diesters. This could explain the altered enantioselectivity recorded. However, we did not detect significant amounts of diesters in the reaction product.

In most cases, the multi-substrate screening would afford stereoselectivity figures generally lower or similar to those shown for reactions performed with individual substrates.

3.3. *CAL-B*-catalyzed acylation of a four-substrate mixture of alcohols

Some experiments have been conducted with a four-substrate mixture (Table 3). The results show that enantioselectivities are not greatly affected, although for some combinations the conversion of some alcohol may be reduced, as for 3 in the mixture (**1**+**3**+**6**+**8**) or (**2**+**3**+**5**+**7**) and **4** in the mixture (**1**+**4**+**7**+**8**).

4. Conclusion

As a conclusion, the use of a multi-substrate procedure in the *CAL-B*-catalyzed acylation by succinic anhydride allows an evaluation of the enantioselectivity displayed by the enzyme for the single substrate. The evaluation may however be underestimated. The reactivity of some substrates might be affected. The order of addition of the alcohols and enzyme may also influence both parameters.

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