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Resolution of 2-Methylalkanoic Acids. Enantioselective Esterification with Long Chain Alcohols Catalysed by *Candida rugosa* Lipase*

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Candida rugosa lipase catalysed resolutions of 2-methylalkanoic acids run on a large scale often display decreased enantiomeric rations (*E*-values) and reaction rates compared with small-scale experiments. By continuous control of the water activity (addition of inorganic salt/hydrated salt mixtures) this can be avoided. The importance of the proper choice of alcohol as well as its concentration is demonstrated. (*R*)-2-Methyloctanoic acid (8.6 g) and (*S*)-2-methyl-l-octanol (4.5 g), both in 99.6% *ee* were prepared from 20 g racemic 2-methyloctanoic acid.

Enantiomerically pure 2-methyl-substituted acids are widely used as drugs, pesticides and synthetic building blocks.[1–4] Hence, improved methods for the resolution of such acids are highly desirable. Enzymatic resolutions catalysed by lipase from *Candida rugosa* (CRL) have been widely emplyed in this context, using either hydrolysis of 2-methyl-substituted esters or esterification of the corresponding acids in non-polar solvents.[2–8] We have recently found that esterification (Scheme 1) of long-chain 2-methylalkanoic acids in alkane solvents offers better enantiomeric ratios (*E*-values,[9] see Experimental section) than hydrolysis of the corresponding esters.[3] Proper selection of the chain length of a long-chain alcohol leads to high *E*-values, whereas short-chain alcohols, like ethanol, give poor *E*-values.[2–4] Short-chain acid derivatives (e.g., 2-methylbutanoic) give low *E*-values in both hydrolysis and esterification.[6,7] We have previously shown that by controlling the water activity in the reaction medium increased *E*-values are obtained.[2,4,8] Alternative methods for improving enantiomeric ratios in CRL-catalysed esterifications have been described, such as modification of the commercial enzyme either via purification[10,11] or treatment with certain reagents[10,12] and immobilisation via either adsorption[13] or covalent linking[14] to a carrier. Cross-linking of crystalline purified CRL[15] and its enantioselective inhibition[16,17] lead to increased *E*-values.

We now wish to report some new developments of the kinetic CRL-catalysed resolution of racemic 2-methylalkanoic acids by esterification.

It is not unusual that increasing the scale presents a problem in biocatalysed reactions. Thus, the large-scale esterification earlier described[3] (see Table 1) gives a much lower rate and *E*-value ($E=15$) than those obtained in the small-scale exploratory experiments ($E=37$). Initially, we were interested in improving the reaction conditions so that the *E*-values should be the same, irrespective of scale. We suspected that uncontrolled changes in the water activity before and during reaction were responsible for the variation in the *E*-values.

Addition of inorganic salt/hydrated salt mixtures is known to keep the water activity (a_w) constant in a reaction medium.[18] We have shown that when crude CRL is used in esterifications, the *E*-values and rates are higher at $a_w=0.8$ (using $Na_2SO_4/Na_2SO_4\cdot 10H_2O$) than at lower water activities.[8] Keeping $a_w=0.8$ we then found

Table 1. Decrease of *E*-values on scale-up of esterifications of *rac*-2-methyloctanoic or *rac*-2-methyldecanoic acid (0.15 M) and alcohol (0.9 M). Crude, dry, stored lipase was used.

	Acid		Alcohol,	Conversion			
Entry	*n*	mmol	*m*	(%)	*E*	*t*/h	Ref.
1	8	3.8	12	37	83	15	3
2	8	127	12	44	23	162	This work
3	10	1.5	7	32	37	14	3
4	10	105	7	49	15	330	3

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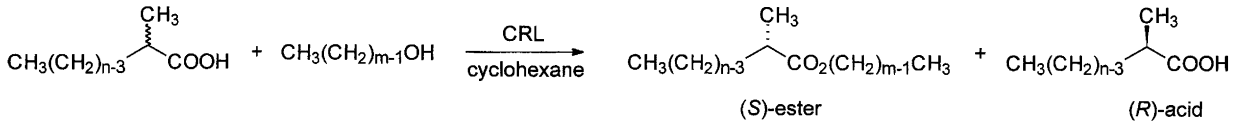


Scheme 1. Esterification of 2-methylalkanoic acids catalysed by *Candida rugosa* lipase (CRL).

that the *E*-values and rates registered were the same irrespective of scale.

In our early experiments, we tried to suppress the reversibility of the esterification reaction by using a sixfold molar excess of alcohol over the racemic acid.[3,4,7,8] However, when working on a large scale and using primary C_{16}–C_{20}-alcohols, this excess led to complications during work-up. Therefore, we tried to minimize the amount of alcohol present at the proper end point for a preparative reaction, i.e., just before 50% conversion. Use of 0.75 mol equiv. of the alcohol substrate gave, apart from the anticipated simplification of the work-up procedure, much faster rates and, to our surprise, also to improved enantiomeric ratios (Table 2).

These observations indicated that the alcohols were enantioselective inhibitors of the enzyme. The validity of this hypothesis has been confirmed by some of us and is described elsewhere.[17] In principle, using less than equimolar amounts of alcohol should lead to problems due to the reversibility of the esterification reaction with decreased enantiomeric excesses as the consequence. However, in our case this phenomenon does not influence the course of the reaction until close to 50% conversion, as seen from Fig. 1, which describes the progress of an experiment using 0.6 mol equiv. of alcohol compared with a theoretical irreversible reaction of a 1:1 mixture of the racemic acid and the alcohol with the same *E*-value (described by lines). When working preparatively with reversible reactions, conversions over or close to 50% are in any case not useful for obtaining the enantiomerically pure esters and usually not even for the enantiomerically pure acids. Therefore, when 0.75 equiv. of alcohol was used, the reaction was stopped between 40–45% conversion to achieve a good product *ee* and a reasonable substrate *ee*.

As already mentioned the right chain length of the alcohol is important for achieving high enantioselectivity.[2–4] As a rule of thumb, if $a_w = 0.8$ in the reaction medium, the *E*-values and rates increase with increasing chain length of a primary alcohol from C10 to C18. For 2-methyldecanoic acid, a maximum was obtained for 1-octadecanol using immobilised lipase (Table 3).

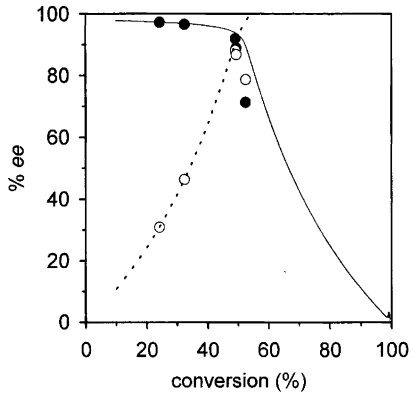


Fig. 1. Esterification of 2-methyldecanoic acid with 1-decanol, alcohol/acid molar ratio = 0.6, *ee* of the product (●) and *ee* of the substrate (○) vs. conversion. The lines represent the computer-simulated theoretical curve according to irreversible uni-uni kinetics (acid/alcohol ratio = 1) for $E = 100$: solid line: product-curve; dotted line: substrate-curve.

Previously, immobilisation on polypropene has been shown not to influence the enantioselectivity appreciably.[2,4] However, we have since found a major difference when 2-methyldecanoic acid is esterified with either 1-hexadecanol, 1-octadecanol or 1-eicosanol (0.75 alcohol/acid molar ratio, $a_w = 0.8$), using either crude or immobilised CRL (Table 3, entries 1, 2 and 3, 4, respectively). The *E*-values were significantly higher when immobilised CRL was used. On the other hand, no significant difference between immobilised or crude CRL was observed for 2-methyloctanoic acid (Table 4). When Celite was used as a polar carrier for immobilisation, the esterifications gave much lower *E*-values (Table 3, entry 7; Table 4, entry 9) compared with those obtained with the non-polar carrier polypropene (Table 3, entry 3; Table 4, entry 5). In addition, 2-methyldecanoic acid

Table 2. rac-2-Methyloctanoic acid and *rac*-2-methyldecanoic acid both (0.15 M) in esterification at various alcohol concentrations, $a_w = 0.8$.

Entry	Acid, *n*	Alcohol, *m*	[Alcohol]/M	Immob. enzyme	(*S*)-Ester (% *ee*)	(*R*)-acid (% *ee*)	*E*	Initial rate (% conv. h^{-1})	Ref.
1	8	16	0.9	No	97.0	34.7	92	14.5	2
2	8	16	0.15	No	97.3	39.1	109	30	2
3	10	7	0.9	Yes	93.2	—	37	1	4
4	10	7	0.09	Yes	96.9	24.9	81	6	4
5	10	10	0.9	Yes	96.0	32.4	67	3	17
6	10	10	0.15	Yes	97.1	28.2	90	9	17
7	10	10	0.09	Yes	97.3	31.0	100	11	17

Table 3. Effect of immobilisation of CRL for *rac*-2-methyldecanoic acid (0.15 M) in esterification with long chain alcohols (0.11 M), $a_w = 0.8$.

Entry	Alcohol, *m*	Immob. enzyme	(*S*)-ester (% *ee*)	(*R*)-acid (% *ee*)	*E*
1	16	Yes	90.5	33.9	28
2	16	No	81.1	39.1	14
3[a]	18	Yes	97.9±0.2	31.4–37.0[c]	120–150
4[b]	18	No	90.0±0.3	18.9–23.0[c]	23–24
5	18	Yes (A-fraction)	88.1	20.2	19
6	18	No (A-fraction)	87.3	18.6	18
7	18	Yes (Celite)	89.9	34.4	26
8	20	Yes	91.4	49.3	36
9	20	No	90.0	25.9	25
10	22	No	89.5	22.0	22

[a] Three replicates. Variation in *ee* of remaining substrate due to different final conversions. [b] Two replicates [c] Variation in *ee* of remaining substrate are due to different final conversions.

gave a low *E*-value using crude CRL, similar to that obtained for Celite-immobilised lipase (Table 3, entry 4). It is interesting to note that the enantioselectivity of the immobilised enzyme is much less sensitive to changes in water activity than the crude enzyme (Table 5).

Crude CRL yields two major active hydrolases when the proteins in the commercially available preparation are separated on an SP-Sephadex C-50 column.[10] They probably represent two stable conformations, the A- and B-form of the same protein, which are not readily interconvertible under the conditions normally used for esterifications or hydrolyses. The A-form can be obtained by treatment of the B-form with different reagents[10,12] and the process is irreversible.[10] Preparations probably containing only the A-form have been found to be much more enantioselective than the crude enzyme in the hydrolyses of methyl arylpropanoates[10,12] and chloroethyl arylpropanoates.[12] However, when esterifying 2-methylalkanoic acids we found that the commercial A-form preparation, either crude or immobilised on polypropene, gave lower *E*-values than the crude commercial preparation (Table 3, entries 3–6; Table 4, entries 5–8).

Table 4. Effect of immobilisation of CRL for *rac*-2-methyloctanoic acid (0.15 M) in esterification with long chain alcohols (0.11–0.15 M), $a_w = 0.8$.

Entry	Alcohol, *m*	[Alcohol]/M	Immob. enzyme	(*S*)-ester (% *ee*)	(*R*)-acid (% *ee*)	*E*
1	16	0.11	Yes	97.6	31.5	91
2	16	0.15	No	97.3	39.1	109[a]
3	18	0.11	Yes	96.7	34.3	84
4	18	0.15	No	96.8	27.3	81
5	20	0.11	Yes	97.5	38.6	115
6	20	0.15	No	97.4	67.9	154[a]
7	20	0.11	Yes (A-fraction)	96.9	20.8	78
8	20	0.11	No (A-fraction)	96.4	25.4	70
9	20	0.11	Yes (Celite)	96.6	31.4	79
10	22	0.12	No	96.4	19.9	66[a]

[a]Ref 2.

Table 5. Effect of water activity on the *E*-value. Crude compared with immobilised CRL in esterifications with 0.15 M acid and 0.9 M alcohol.

Entry	Acid, *n*	Alcohol, *m*	Immob. enzyme	a_w	Initial rate/μmol g^{-1} min^{-1}	*E*	Ref.
1	8	12	No	—	0.92	83	3
2	8	12	No	a	—	7	7
3	8	12	No	0.16	—	23	7
4	8	12	No	0.80	—	95	7
5	8	12	Yes	0.16	1.46	43	This work
6	8	12	Yes	0.80	2.06	50	This work
7	10	10	No	—	0.69	10	3
8	10	10	No	*a*	0.06	2.5	4
9	10	10	No	0.16	0.56	37	This work
10	10	10	No	0.80	1.52	81	This work
11	10	10	Yes	0.16	0.85	52	17
12	10	10	Yes	0.80	3.9	66	4

[a]Dried enzyme.

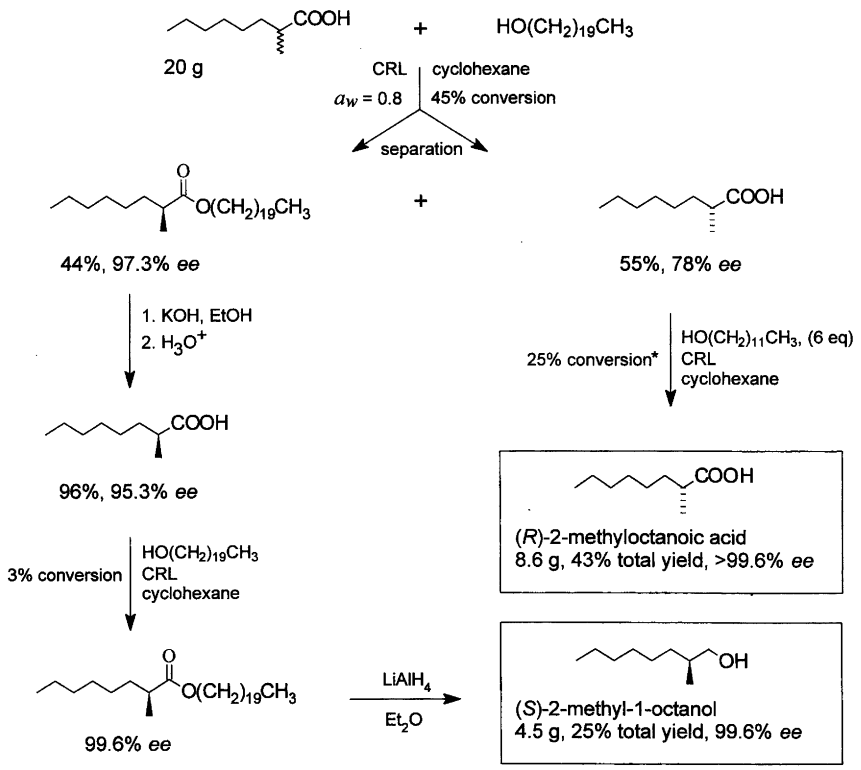
It is thus possible that immobilisation on polypropene changes the enzyme conformation into a more enantioselective form which is, obviously, different from the commercial A-form described above.

The conclusions drawn from the results and discussion above helped us to design an efficient resolution procedure for 2-methylalkanoic acids which includes three resolution steps on a preparative scale (Scheme 2). Thus, we recommend that one uses immobilised enzyme [although in some cases the crude enzyme should work equally well (Table 4)]. The water activity should be controlled ($a_w = 0.8$) and 0.75 molar equiv. of a long chain primary alcohol (C18 or C20), as the cosubstrate in cyclohexane, should be used. The first reaction with racemic acid should be run to slightly below 50% conversion. After the remaining (*R*)-acid (75–90% *ee*) has been extracted into a carbonate solution, the resulting (*S*)-ester/alcohol mixture should be hydrolysed in aqueous methanolic potassium hydroxide solution (despite attempted improvements, this hydrolysis invariably led to a 1–2% loss of *ee*). After extractive removal of the alcohol formed, the (*S*)-acid obtained on acidification should be reesterified to ≈60–70% conversion which should yield an ester of >99.5% *ee*. This can then be transformed into the (*S*)-2-methyl-1-alkanol in unchanged[3] *ee* using lithium aluminium hydride. The (*R*)-acid fraction from above should then be re-esterified with a molar excess of a shorter alcohol (6 equiv. to shift the equilibrium towards esterification) to a predetermined conversion (which varies depending on the starting *ee*). This should give the enantiopure (*R*)-acid (>99.5% *ee*). Using this protocol (Scheme 2) and starting from 20 g of racemic 2-methyloctanoic acid we obtained 4.5 g (*S*)-2-methyl-1-octanol (>99.6% *ee*) and 8.6 g (*R*)-2-methyloctanoic acid (>99.6% *ee*) and about 5.5 g of nearly racemic acid, which can be reused. If needed, the (*S*)-acid can be obtained in high yield and unchanged *ee* via oxidation of the (*S*)-alcohol with Jones reagent.[3,19]

In conclusion we have shown that it is possible to obtain fair amounts of both the pure enantiomers of 2-methylalkanoic acids using CRL-catalysed resolutions. This method should be competitive with the best procedures using chiral catalysis or chiral auxiliaries. The enzymatic method is easy to perform and no expensive auxiliaries or heavy-metal catalysts are used. The reactions are simple and the waste produced is essentially harmless. If immobilised enzyme is used this can easily be recovered from the reaction mixture.[4]

Experimental

Silica gel 60, 230–400 mesh, was used for MPLC using cyclohexane with a gradient of ethyl acetate as the eluent. Racemic 2-methylalkanoic acids were prepared as previously described.[3] The starting materials were commercially available and were used without further purification unless otherwise stated. Macroporous polypropene



Scheme 2. Resolution of 2-methyloctanoic acid by esterification with 1-eicosanol (0.75 equiv.) and 1-dodecanol (6 equiv.). Chemical yields in each step are shown. *Estimation based on an (*R*)-acid (70.4% *ee*) run to 36% conversion.

Accurel EP 100, 350–1000 μm was obtained from Akzo Faser AG, Obernburg, Germany.

Enzyme. Commercial extracellular lipase (EC 3.1.1.3) from *Candida rugosa* was obtained from Sigma (St. Louis, MO, USA). The specific activity was 900 units mg^{-1} solid and 4865 units mg^{-1} protein. One unit will hydrolyse 1.0 mmol of fatty acid from a triglyceride in 1 h at pH 7.2 and 37 °C using olive oil (incubation time: 30 min). Commercial Lipase-CR Analytical Grade (AGCR-350) derived from *Candida rugosa* was obtained from Altus Biologics Inc. (Cambridge, MA, USA). 600 units per 350 mg dry weight. One unit will hydrolyse 1.0 mmol of acetic acid from triacetin per minute at pH 7.0 and 25 °C in 1 mM $CaCl_2$.

Immobilisation. The crude lipase from *Candida rugosa* (Sigma) was immobilised on polypropene as previously described.[4] Lipase-CR derived from *Candida rugosa* (Altus, 50 mg) was dissolved in sodium phosphate buffer (10 ml, 20 mM, pH 7.0). Macroporous polypropene, Accurel EP100, 350–1000 μm (50 mg) was mixed with ethanol (1.5 ml) and the mixture was degassed under vacuum. To this mixture was added the enzyme suspension followed by the treatment previously described.[4] Immobilisation on Celite was carried out as described by Bovara *et al.*[20]

Esterification reactions. A cyclohexane solution (1 vol) containing a 2-methylalkanoic acid (0.15 M), an alcohol (0.075–0.9 M), an internal standard (eicosane or tetracosane, 6.1 mg ml^{-1} solution) and $Na_2SO_4/Na_2SO_4 \cdot 10H_2O$ (for $a_w = 0.8$; 0.2 and 0.1 mmol ml^{-1} of solution) or $Na_2HPO_4/Na_2HPO_4 \cdot 2H_2O$ (for $a_w = 0.15$; 0.2 and 0.1 mmol ml^{-1} of solution) was stirred for 15 min in a sealed flask at room temperature. The lipase (16.9–67.5 mg crude, unmodified enzyme per ml solution or 2 mg unmodified enzyme of the A-fraction per ml solution and when immobilised as indicated in Tables 2–5; 16.9 mg particles per ml solution or 1.5 mg particles per ml of the immobilised A-fraction) was added and the mixture was stirred with a magnetic stirring bar at 700 rpm. The esterification reactions were followed by GC and stopped at the appropriate stages of conversion by filtering off the enzyme and washing it with cyclohexane (0.5 vol) and pentane (0.5 vol) For the small-scale esterifications the solvent was then evaporated off and the ester and remaining acid were purified by MPLC. When dealing with the large-scale esterifications the remaining acid was extracted from the neutral components before purification by MPLC. Thus, the combined organic phase was extracted with aqueous sodium carbonate (10%, 5×0.2 vol) and sat. sodium chloride solution (0.2 vol). The organic phase containing the (*S*)-ester, alcohol and internal standard was dried ($MgSO_4$). The solvent was evaporated off and the residue was purified by MPLC. The combined water phase containing the carboxylate salt and some alcohol was acidified to pH 1 with 6 M HCl and extracted with diethyl ether (5×0.5 vol). The combined ether phase was washed with sat. sodium chloride solution (0.2 vol), dried ($MgSO_4$) and evaporated to dryness yielding the (*R*)-acid. At 27% conversion (*R*)-2-methyldecanoic acid (13 g, 67% yield, 36.2% ee) and octadecyl (*S*)-2-methyldecanoate (approx. 11 g, 24% yield, 97.9% *ee*) was obtained from 19.5 g racemic acid. At 45% conversion (*R*)-2-methyloctanoic acid (12 g, 55% yield, 78% *ee*) and (*S*)-eicosyl 2-methyloctanoate (24 g, 44% yield, 97.3% *ee*) was obtained from 20 g racemic acid.

Chemical hydrolysis. The (*S*)-ester was dissolved in 95% EtOH containing 10% KOH (0.33 g ester/ml). The mixture was stirred at room temperature for 3 h and the reaction was stopped before 100% conversion. The mixture was diluted with water and then acidified with hydrochloric acid and partitioned between diethyl ether and water. The water phase was extracted with five portions of diethyl ether. The combined organic phase was extracted with aqueous sodium carbonate solution (10%, five portions). The pooled carbonate solution was acidified to pH 1 (6 M HCl) and extracted with five portions of diethyl ether. The ether phase was dried ($MgSO_4$) and concentrated to give (*S*)-2-methyldecanoic acid in 94% yield or (*S*)-2-methyloctanoic acid in 96% yield, calculated on the esters. The loss in *ee* was 1 and 2% *ee*, respectively. If the hydrolysis is interrupted before the reaction is complete, the remaining trace of ester displays a more significant loss of *ee*, indicating as expected, that racemisation takes place in the ester via base-catalysed enolisation. The crude acid obtained was used directly in an additional esterification reaction with a long-chain alcohol as described in the procedure above.

Reduction procedure. The ester obtained after MPLC was 100% chemically pure (GC). The ester was dissolved in diethyl ether (≈ 5 ml g^{-1} ester) and reduced by slowly adding it to a solution of $LiAlH_4$ (1 mol equiv., 8 mg ml^{-1}) in anhydrous diethyl ether under argon. The mixture was stirred at 20 °C for 2 h and quenched with water (0.5 ml g^{-1} $LiAlH_4$), 15% NaOH (0.5 ml g^{-1} $LiAlH_4$) and water (1.0 ml g^{-1} $LiAlH_4$). After reflux for 1 h the mixture was filtered and dried ($MgSO_4$). After evaporation of the diethyl ether, hexadecane was added to the mixture in order to prevent simultaneous distillation of the two alcohols produced. Thus, distillation at reduced pressure (2 mmHg) furnished the (*S*)-alcohol together with hexadecane free from the long chain primary alcohol also produced from the ester.

Hexadecane was easily removed by MPLC to give the pure (*S*)-alcohol with no loss in *ee*. From (*S*)-octadecyl-2-methyldecanoate (4.9 g), obtained from a second esterification to 79% conversion, (*S*)-2-methyl-1-decanol (approx. 1.9 g, 99.1% *ee*, 11% total yield) was obtained. (*S*)-eicosyl (*S*)-2-methyloctanoate ca. 10 g), obtained from (*S*)-2-methyloctanoic acid (95.3% *ee*) in a second

esterification to 63% conversion, furnished (*S*)-2-methyl-1-octanol (4.5 g, 99.6% *ee*, 25% total yield).

Determination of enantiomeric excess. The *ee* of the acids and esters were determined by GC after derivatisation to the corresponding phenylethyl amides obtained as described[3] from the enantiomerically pure 1-phenylethylamines. The diastereomeric ratios were determined using a Varian 3300 gas chromatograph equipped with a 30 m × 0.32 mm I.D. capillary column coated with cross-linked Carbowax 20 M, $d_f = 0.25$ μm; carrier gas He, 15 psi, split 1:20.

Determination of E-*values.* The enantiomeric ratio or *E*-value is defined[9] as the ratio between the specificity constants for the two enantiomers

$$E = (k^S_{cat}/K^S_M)/(k^R_{cat}/K^R_M)$$

which is a concentration-independent measure of the initial rate ratios of the enantiomers in a racemic mixture. The *E*-values were calculated from the ee of the product (ee_p) and the conversion (ξ) using the equation deduced by Chen *et al.*[9] The conversion ξ at each point was obtained from ee_p and that of the substrate (ee_s) according to $\xi = ee_s/(ee_p + ee_s)$.

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