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Enzymatic methods for the preparation of enantiopure malic and aspartic acid derivatives in organic solvents

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Abstract

The kinetic resolution of malic and aspartic acid diesters as well as of its *N*-butanoyl dimethyl ester by highly regioselective lipases and acylase I enzymes were studied. *Candida antarctica* lipase A on Celite catalyzed the enantioselective acylation of the hydroxyl and amino groups. Acylase I from *Aspergillus melleus* and *Candida antarctica* lipase B catalyzed the enantioselective alcoholyses of the ester groups at the α- and β-positions, respectively.

1. Introduction

(*S*)-Malic (**1a**, R^1=H) and (*S*)-aspartic acid (**2a**, R^1=H) are multifunctional natural products which belong to the succinic acid family (Scheme 1). Malic acid is widely utilized as a racemate in the food and beverage industry.[1] In synthetic chemistry, the enantiomers of malic and aspartic acids are valuable as reactive synthons for various compounds, such as for the β-lactam precursors of antibiotics and for the enantiomers of carnitine.[2,3] The synthetic dipeptide sweetener, aspartame, is probably the best known of the commercial products needing (*S*)-aspartic acid as a raw material.[4] (*S*)-Aspartic acid is also a chiral synthon for the synthesis of alkaloids, such as (+)-vincamine, which has therapeutic use because of its cerebral vasodilatory effects.[5]

The fumarase-catalyzed hydration of fumaric acid is an industrial process employed for the preparation of (*S*)-malic acid.[1,6,7] The aspartase-catalyzed addition of ammonia to fumaric acid and the L-aspartate β-decarboxylase-catalyzed resolution of racemic aspartic acid have been operated commercially for the production of (*S*)- and (*R*)-aspartic acid, respectively.[4] A method for the industrial production of *(S)*-aspartic acid and *(R)*-alanine by immobilized *Pseudomonas dacunhae* cells has also been described.[8] Microbial reduction of diethyl oxalacetate to diethyl malate has been reported to produce enantiomerically enriched products.[9] Various hydrolytic enzymes in aqueous solutions have also been studied for the

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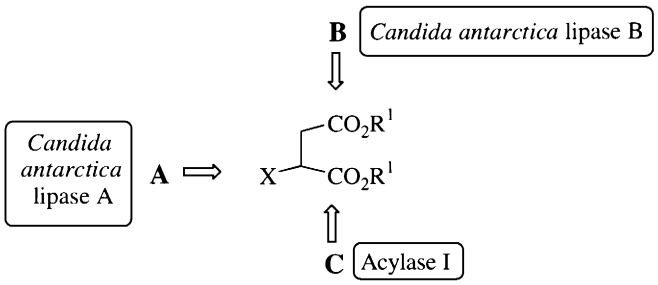


(1); R^1=Me or H
a: X = OH
b: X = $PrCO_2$

(2); R^1 = Me or H
a: X = NH_2
b: X = PrCONH

Scheme 1.

resolution of dialkyl malates.[9–14] These resolution methods have usually provided the less reactive dialkyl esters with the (*R*) absolute configurations and with moderate chemical yields due to only moderately enantioselective hydrolyses.

In this paper, efficient enzymatic kinetic resolutions are described, enabling the preparation of the enantiomers of **1** and **2** (R^1=Me, Scheme 1). The methods exploit the regio- and enantioselectivity of lipases (EC 3.1.1.3) and acylase I enzymes (aminoacylase; EC 3.5.1.14) on racemic or enantiomerically enriched substrates in organic media. Enzymatic acylation of substrates **1a** and **2a** (R^1=Me, site A) by an achiral acyl donor, methanolysis of **1b** (R^1=Me, site A) and alcoholysis of the ester function either at site B or C of **2b** (R^1=Me) by an achiral alcohol are used as the main tools. Alcoholysis by acylase I catalysis in neat alcohols is a novel and highly efficient resolution method exploiting enantioselective acyl transfers from racemic esters (site C) to achiral alcohols.

2. Results and discussion

2.1. Enzyme screening for the selective reactions at sites A, B and C

An enzymatic reaction can take place at three different sites, A, B and C, of dimethyl malate and aspartate (Scheme 1). Initially, 14 lipases or lipase preparations were screened for the selective acylation (site A) of dimethyl malate **1a** (R^1=Me) with vinyl butanoate in diisopropyl ether. In the case of most lipases acylation did not proceed. In the cases of *Pseudomonas* and *C. rugosa* lipases and *Candida antarctica* lipase B (Novozym 435)[15] acylation proceeded smoothly at site A but with only modest enantioselectivity. *Candida antarctica* lipase A (lipase SP 526)[15] when adsorbed on Celite was the only lipase exhibiting a somewhat promising enantioselectivity (*E*=8) for the present substrates. Lipase SP 526 was also the best enzyme in the enzyme screening for dimethyl aspartate **2a** (R^1=Me) with 2,2,2-trifluoroethyl butanoate in acetonitrile (*E*=20). Acetonitrile was used instead of diisopropyl ether because of the solubility problems with **2a** (R^1=Me).

Due to the low enantioselectivity (*E*=8) for the acylation of **1a** (R^1=Me) at site A the lipases were further screened for the methanolysis of **1b** (R^1=Me) in diisopropyl ether, leading to the formation of dimethyl malate **1a** (R^1=Me). As in the above acylation, enantioselectivity was highest (*E*=22) in the case of lipase SP 526 on Celite. In addition, *C. rugosa* and *Penicillium roqueforti* lipases showed acceptable enantioselectivities (*E*=15–20). It is worth mentioning that *C. rugosa* lipase with the observed

(*R*)-selectivity both in the acylation of **1a** (R′=Me) (*E*=3) and in the methanolysis of **1b** (R′=Me) differs from the (*S*)-selectivity of the other lipases. It was also previously observed that *C. rugosa* lipase does not always conform to the so-called Kazlauskas model of predicting the faster reacting enantiomer.[16] This is especially the case when the group, such as CO_2R, which according to the model should be bound to the smaller hydrophobic pocket of the enzyme, does not contain hydrogen in the carbon atom directly attached to the stereogenic centre.[16] Methanolysis of **2b** (R^1=Me) at site A is not possible by lipases because lipases generally leave the amide bond unreactive.

Possible reaction types to direct the reaction at site B or C are ester alcoholysis and hydrolysis. Reactions directed regioselectively to site B or C are especially important for obtaining the enantiomers of **2b** (R^1=Me). Thus, the lipases were screened for the ethanolysis of **2b** (R^1=Me) in neat ethanol. Among the 14 lipases, Novozym 435 was the only one catalyzing the reaction enantioselectively and exclusively at site B (*E*=35). The corresponding hydrolysis was rejected, because the produced half ester racemized during the enzymatic reaction. Thus, at 50% conversion the Novozym 435-catalyzed hydrolysis of **2b** (R^1=Me) led to the unreacted *(R)*-substrate (ee 98%) whereas the produced *N*-butanoylaspartic acid α-methyl ester was racemic. This is evidently caused by the β-carboxylate acting as an intramolecular base.[17] Similarly, aspartic acid β-methyl ester was shown to racemize.

Enzyme screening with acylases was needed to find a catalyst which selectively directs alcoholysis at site C of **2b** (R^1=Me, Table 1). Acylase I from *Aspergillus melleus* was selected as a catalyst for the further studies at site C.

Table 1
Acylase I-catalyzed butanolysis of **2b** (R^1=Me, 0.1 M) in neat butanol at room temperature[a]

Source of Acylase I	*Aspergillus genus*	*Aspergillus melleus*	*Aspergillus* on Eupergit C	Porcine kidney
Conversion/%	13	23	51	0
E	15	>100	50	-

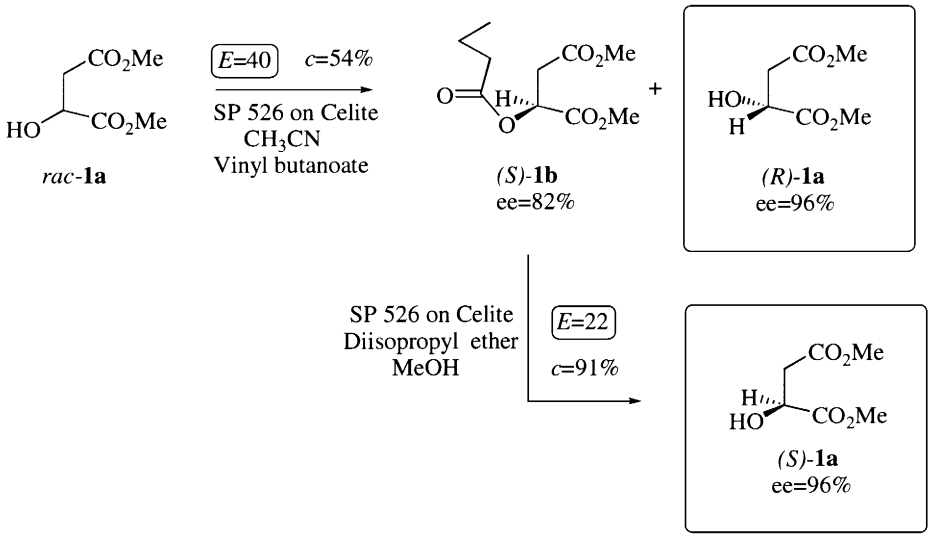
[a]75 mg/ml of the catalyst; reaction time 22 h.

Regioselectivity for the Novozym 435 (site B)- and acylase I (site C)-catalyzed alcoholyses of **2b** (R^1=Me) were determined by performing the enzymatic ethanolyses in neat ethanol. The quality of the two possible regioisomers (α- or β-ethyl ester) was then confirmed by preparing the reference compound, *N*-butanoyl-(*S*)-aspartic acid α-ethyl β-methyl diester ((*S*)-**7**, ethanolysis product for reaction at site C), from commercial (*S*)-aspartic acid β-methyl ester hydrochloride ((*S*)-**5**, see Experimental; Scheme 5). The complete GLC separation of the regioisomers of various alkyl methyl diesters (alkyl=ethyl, propyl, butyl, pentyl or hexyl) made the qualification of the regioisomers easy by assuming that enzymatic regioselectivity is independent of the alcohol used as a medium.

2.2. Optimization for lipase SP 526, Novozym 435 and acylase I catalyses

The lipase SP 526-catalyzed reactions at site A (Scheme 1) predict that the resolution of **1a** (R^1=Me) becomes reasonable through a double resolution technique[18] expecting that the enantioselectivity of the acylation (and/or deacylation) in the butanoylation–methanolysis sequence (Scheme 2) can be enhanced. For that purpose solvent screenings for the lipase SP 526-catalyzed acylation of **1a** (R^1=Me) with vinyl butanoate and for the methanolysis of **1b** (R^1=Me) were performed. The enantioselectivity for these

reactions is clearly dependent on the solvent, acetonitrile with E=40 being the choice for acylation reactions (Table 2). Similarly, acetonitrile is the best solvent in the case of **2a** (R^1=Me, E=20) as a substrate. For the methanolysis of **1b** (R^1=Me), reactions in diisopropyl ether are favourable.



Scheme 2.

Table 2
Solvent effects on the acylation of **1a** (R^1=Me; 0,1 M) with vinyl butanoate (0.4 M) and on the alcoholysis of **1b** (R^1=Me; 0.1 M) with methanol (0.8 M) by lipase SP 526[a]

	Acylation			Methanolysis		
Solvent	Time/h	Conversion / %	*E*	Time/h	Conversion/%	*E*
tert-Amyl alcohol	3	56	7			
Acetonitrile	2	24	40	24	8	2
Toluene	2	52	10			
Diethyl ether	0,75	61	9	2	29	1
Diisopropyl ether	0,25	34	8	1	33[b]	22
THF	24	59	20	24	24	10

[a]75 mg/ml of the enzyme preparation containing 20% (w/w) of the lipase on Celite.

[b]10 mg/ml of the enzyme preparation containing 20% (w/w) of the lipase on Celite.

In order to further enhance enantioselectivity various acyl donors were screened for the lipase SP 526-catalyzed acylation of **1a** and **2a** (R^1=Me) in acetonitrile (Table 3). Vinyl esters clearly result in highest enantioselectivity, the more reactive vinyl butanoate being the preferred acyl donor. Similarly, 2,2,2-trifluoroethyl butanoate was chosen for the lipase SP 526-catalyzed acylation of **2a** (R^1=Me). The only detectable reaction for these acylations proceeded exclusively at site A (Scheme 1) as was expected on the basis of our previous results for the acylation of alicyclic β-amino acid and 3-aminobutanoic acid

Table 3
Effect of an acyl donor (0.2 M) for the acylation of **1a** and **2a** (R^1=Me; 0.1 M) in acetonitrile by lipase SP 526[a]

	Dimethyl malate			Dimethyl aspartate		
Acyl donor	Time/h	Conversion / %	*E*	Time/h	Conversion/%	*E*
2,2,2-Trifluoroethyl chloroacetate	24	44	10	0.5	47	3
Ethyl chloroacetate	24	0	-	24	21	5
2,2,2-Trifluoroethyl butanoate	24	54	8	0.13	51	20
2,2,2-Trifluoroethyl acetate	24	4	30	28	48	20
Vinyl butanoate	1.5	50	40			
Vinyl acetate	24	18	44			
Vinyl decanoate	1.5	42	17			

[a] 75 mg/ml of the enzyme preparation containing 20% (w/w) of the lipase on Celite.

esters.[19,20] This is a clear difference to the behaviour of Novozym 435 which was previously reported to catalyze the simultaneous acylation of the NH_2 group and interesterification between the alcohol part of methyl 3-aminobutanoate and an achiral ester.[20]

The results for the alcohol screening for the Novozym 435- and acylase I-catalyzed alcoholyses of **2b** (R^1=Me) in the neat alcohol are shown in Table 4. Clearly, the lipase leads to higher enantioselectivity and reactivity (in terms of conversion at the certain time) the more hydrophobic the alcohol. The acylase I enzyme is highly enantioselective in every alcohol tested, reactivity decreasing with increasing alcohol chain length. In this work, reactions in butanol were preferred because the separation of the obtained

Table 4
Alcohol effects on the alcoholysis of **2b** (R^1=Me; 0.1 M) in the alcohol by Novozym 435[a] (reaction time 26 h) and acylase I from *A. melleus*[a] (reaction time 22 h)

	Novozym 435		Acylase I	
Alcohol	Conversion / %	*E*	Conversion/%	*E*
EtOH	39	35	41	≈100
PrOH	40	55	39	>100
BuOH	44	55	28	>100
PenOH	49	70	25	>100
HexOH	50	70	25	>100

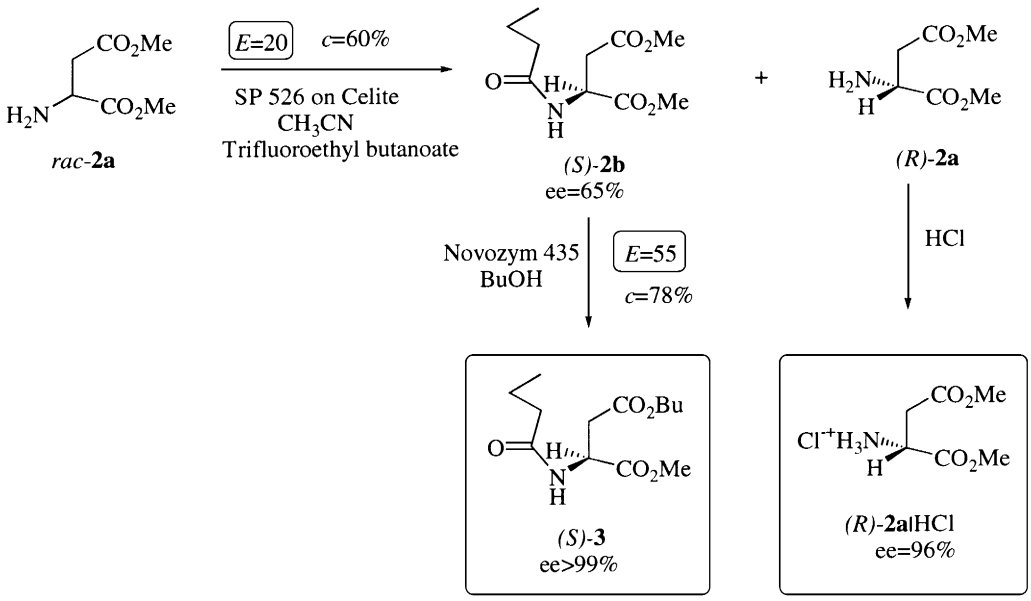
[a] 100 mg/ml of the enzyme preparation

mixed esters from the dimethyl ester by column chromatochraphy is difficult in the case of lower alcohols and the removal of butanol by evaporation takes place easily compared to the higher alcohols.

2.3. Gram-scale preparation of the enantiomers by lipase SP 526, Novozym 435 and acylase I

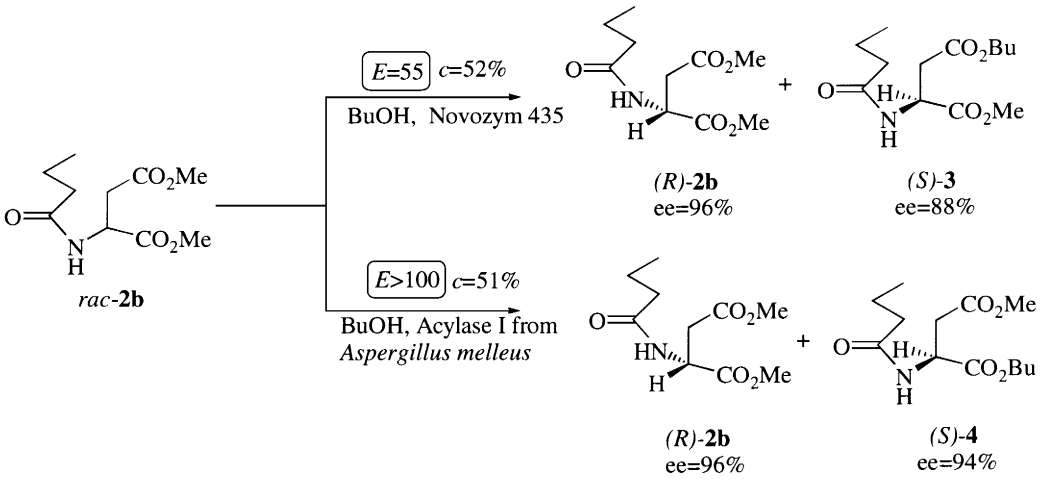
The acylation of **1a** (R^1=Me) with vinyl butanoate in acetonitrile followed by the methanolysis of the produced **1b** (R^1=Me) in diisopropyl ether has been used for the preparation of (*R*)-**1a** and (*S*)-**1a** (R^1=Me) by lipase SP 526 catalysis (Scheme 2). The previously published computer programme for the double resolution technique allows the prediction of the optimal conversions that lead to the desired ee value for the final product when the *E* values are known.[18] When the goal is the preparation of the two enantiomers, the optimal conversions of 54% (ee=95% for the less reactive enantiomer) for the butanoylation and 91% (ee=97% for the reactive enantiomer) for the methanolysis steps were calculated with *E*=40 and 22, respectively. The experimental results in Scheme 2 are in good accordance with the calculated values. The economy of the resolution method is relatively good. Thus, the products (*R*)-**1a** and (*S*)-**1a** were isolated with 34 and 38% chemical yields, respectively, when calculated according to *rac*-**1a** as 100%.

The optimized conditions for the double resolution of **2a** (R^1=Me) are shown in Scheme 3. This time the second resolution step is directed to site B (or C) rather than A because neither lipase- nor acylase-catalyzed alcoholyses of the amide bond of **2b** (R^1=Me) were successful. The calculated conversions of 59% (ee=97% for the less reactive enantiomer) for the lipase SP 526-catalyzed butanoylation at site A and that of 83% (ee=97% for the reactive enantiomer) for the following Novozym 435-catalyzed alcoholysis at site B are in good accordance with the experimental data shown in Scheme 3. The isolated chemical yields of (*S*)-**3** (33%) and (*R*)-**2a**·HCl (32%) as calculated according to *rac*-**2a** are well accepted.



Scheme 3.

Novozym 435-catalyzed resolution was also performed with *rac*-**2b** (R′=Me) (Scheme 4). The reaction gave (*R*)-**2b** (ee 96%) and (*S*)-**3** (ee 88%) at 52% conversion with the isolated yields of 45 and 50% (calculated from the racemic starting material), respectively.



Scheme 4.

The most valuable hydrolytic enzyme in our present work is acylase I from *A. melleus*. Thus, the butanolysis of **2b** (R^1=Me) in neat butanol leads to the mixture containing (*R*)-**2b** (R^1=Me) and (*S*)-**4** after a single resolution step (Scheme 4). These constituents were then isolated in chemical yields of 48 and 46%, respectively. Acylase I typically catalyzes the hydrolysis of amide bonds in aqueous solutions. Kinetic resolutions of other reaction types are relatively rarely studied. They are restricted to the acylation of alcohols with vinyl esters and to the hydrolysis of methyl esters.[21–26] The present results show for the first time the value of acylase I for the resolutions exploiting enantioselective ester alcoholysis of racemic esters rather than ester or amide hydrolysis.

3. Conclusions

In this work, the regio- and enantioselectivity of lipase SP 526, Novozym 435 and acylase I from *A. melleus* has been exploited in the gram-scale resolution of dimethyl malate and aspartate as well as of some other derivatives (Scheme 1). Although the regioselectivity of the enzymes at sites A, B and C, respectively, is excellent the enantioselectivity of the lipases calls for a double resolution technique[18] in order to get the more reactive (*S*)-enantiomers in highly enantiopure forms. Thus, the butanoylation–methanolysis sequence at site A allows the resolution of *rac*-**1a** (R^1=Me) to (*R*)-**1a** (R^1=Me) and (*S*)-**1a** (R^1=Me) by lipase SP 526, resulting in 96% ee for the two enantiomers. The butanoylation of **2a** (R^1=Me) at site A by lipase SP 526 followed by butanolysis at site B by Novozym 435 yields (*R*)-**2a** (R^1=Me; ee 96%) and (*S*)-**3** (ee>99%) (Scheme 3). A novel acylase I-catalyzed butanolysis at site C is the most convenient method for the resolution of **2b** (R^1=Me), allowing the preparation of the two enantiomers in a single resolution step (Scheme 4).

4. Experimental

4.1. Materials

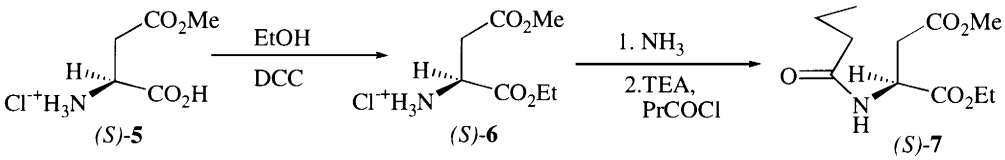
Lipases from *Pseudomonas cepacia, Pseudomonas fluorescence, Mucor javanicus, Penicillium camemberti, Aspergillus niger, Rhizopus* sp., *Candida lipolytica, C. rugosa* and *P. roqueforti* were pur-

chased from Amano Pharmaceutical Co., Ltd (Nagoya, Japan). *Candida antarctica* lipase A (lipase SP 526) and B (lipase SP 525) as well as the corresponding immobilized *Candida antarctica* lipase B (Novozym 435) were the products of Novo Nordisk (Bagsvaerd, Denmark).[15] Before use, lipase SP 526 was adsorbed on Celite in the presence of sucrose, as previously described.[27] Lipases from *C. rugosa* (CRL) and porcine pancreas as well as acylase I from porcine kidney and *A. melleus* were obtained from Sigma (Deisenhofen, Germany). Acylase I from the *Aspergillus* genus and from *Aspergillus* on Eupergit C were the products of Tokyo Kasei (Tokyo, Japan) and Fluka (Buchs, Switzerland), respectively.

The esters of malic acid were products of Tokyo Kasei except (*S*)-dimethyl malate, which was obtained from Aldrich. The esters of aspartic acid hydrochloride were purchased from Sigma. Vinyl acetate, butanoate and decanoate were obtained from Riedel de Haën, Fluka and Aldrich, respectively. The solvents were of the highest analytical grade and obtained from Lab Scan LTD or Aldrich. The other esters were synthesized from the corresponding alcohol and acid chloride or anhydride by the usual procedures.

4.2. Methods

The progress of the reactions was followed by taking samples (100 μl) at intervals, filtering off the enzyme and analyzing the sample by GLC on permethylated β-cyclodextrin or Chirasil-L-valine capillary columns. The determination of E was based on ee_S and ee_P or on ee_S and conversion c according to the equation $E = \ln[(1 - ee_1)/(1 - ee_1/ee_P)]/\ln[(1 - ee_1)/(1 + ee_1/ee_P)]$ with $c = ee_1/(ee_1+ee_P)$ and on linear regression.[28] The absolute configurations were determined by referring the retention times in the gas chromatograms and the specific rotations to those of the commercial compounds or derivatives prepared from them. The regioselectivity in the case of **2b** (R^1=Me) was determined as shown later (Scheme 5). ^{1}H NMR spectra were measured in $CDCl_3$ on a Jeol Lambda 400 spectrometer, with tetramethylsilane as an internal standard. MS spectra were recorded on a VG Analytical 7070E instrument equipped with a VAXstation 3100 M76 computer. Optical rotations were measured using a Jasco DIP-360 polarimeter. Elemental analyses were performed using a Perkin–Elmer CHNS-2400 Ser II Elemental Analyzer.



Scheme 5.

4.3. Gram-scale resolution of dimethyl malate (*1a*, R^1=Me)

Dimethyl malate (2.50 g, 15 mmol) and vinyl butanoate (3.52 g, 31 mmol) were dissolved in acetonitrile (154 ml) and lipase SP 526 (11.55 g; 20% (w/w) of the enzyme on Celite) was added. At 54% conversion after 5.5 h the enzyme was filtered off and the solvent evaporated. Purification by column chromatography (elution with 3:7 acetone:hexane) yielded the unreacted (*R*)-**1a** [R^1=Me; 0.84 g, 5.2 mmol, ee 96%, $[\alpha]_D^{20}$ +8.3 (*c*=1.0, MeOH)] and the formed (*S*)-**1b** [R^1=Me; 1.71 g, 7.4 mmol, ee 82%, $[\alpha]_D^{20}$ –21.1 (*c*=1.0, MeOH)]. For ee determination, the formed (*S*)-**1b** (R^1=Me) was transformed to (*S*)-dimethyl malate by methanolysis with Amberlyst 15 ion-exchange resin as an acid catalyst.

Compound (S)-**1b** (R^1=Me; 1.08 g, 4.7 mmol, ee 82%) and methanol (1.19 g, 37 mmol) were dissolved in diisopropyl ether, and lipase SP 526 (0.46g; 20% (w/w) of the enzyme on Celite) was added. After 5

h the enzyme was filtered off at 91% conversion and the solvent evaporated. Purification by column chromatography as above yielded (*S*)-**1a** [R^1=Me; 0.600 g, 3.7 mmol, ee 96%, $[\alpha]_D^{20}$ –8.2 (*c*=1.0, MeOH)] and the unreacted (*R*)-**1a** [R^1=Me; 0.099 g, 0.43 mmol, ee 44%, $[\alpha]_D^{20}$ +11.2 (*c*=1.0, MeOH)].

^{1}H NMR, (*R*)-**1a** (R^1=Me): δ 2.85 (m, 2H, C*H*$_2$, J=4.4; 6.1 and 16.4 Hz), 3.37 (s, 1H, O*H*), 3.72 (s, 3H, C*H*$_3$), 3.82 (s, 3H, C*H*$_3$), 4.52 (m, 1H, C*H*). Elemental analysis: obs. 43.85% C, 6.19% H, calcd for $C_6H_{10}O_5$ 44.45% C, 6.22% H.

^{1}H NMR, (*S*)-**1b** (R^1=Me): δ 0.99 (t, 3H, C*H*$_3$CH$_2$, J=7.3 Hz), 1.68 (m, 2H, C*H*$_2$CH$_3$, J=7.3 Hz), 2.37 (td, 2H, C*H*$_2$CO$_2$, J=7.3 and 2.9 Hz), 2.89 (d, 2H, CHC*H*$_2$CO$_2$, J=6.1 Hz), 3.72 (s, 3H, OC*H*$_3$), 3.77 (s, 3H, OC*H*$_3$), 5.49 (t, 1H, C*H*, J=6.1 Hz). Elemental analysis: obs. 51.60% C, 6.91% H, calcd for $C_{10}H_{16}O_6$ 51.72% C, 6.94% H.

*4.4. Gram-scale resolution of dimethyl aspartate (**2a**, R^1=Me)*

Fresh dimethyl aspartate was first prepared by bubbling ammonia through its hydrochloride solution in chloroform. Lipase SP 526 (1.00 g; 20% (w/w) of the enzyme on Celite) was added to a solution of dimethyl aspartate (1.86 g, 12 mmol) and trifluoroethyl butanoate (3.94 g, 23 mmol) in acetonitrile (100 ml). The reaction was stopped at 60% conversion after 6 h by filtering off the enzyme. The solvent was evaporated and the products were dissolved in ethyl acetate. Bubbling with hydrochloric acid caused the precipitation of the unreacted (*R*)-**2a** [R^1=Me; 0.74 g, 4.6 mmol, ee 96%, $[\alpha]_D^{20}$ –15.5 (*c*=1.0, MeOH)]. Evaporation of ethyl acetate gave the produced (*S*)-**2b** [R^1=Me; 1.29 g, 5.6 mmol, ee 65%, $[\alpha]_D^{20}$ –12.7 (*c*=1.0, MeOH)].

Compound (*S*)-**2b** [R^1=Me; 0.55 g, 2.4 mmol, ee 65%] in butanol (25 ml) was added on Novozym 435 (1.98g). After 163 h the reaction was stopped by filtering off the enzyme. The reaction yielded (*S*)-**3** [0.35 g, 1.3 mmol, ee>99%, $[\alpha]_D^{20}$ −13.5 (*c*=1.0, MeOH)] and (*R*)-**2b** [R^1=Me; 86 mg, 0.37 mmol, ee 47%, $[\alpha]_D^{20}$+8.5 (*c*=1.0, MeOH)].

^{1}H NMR, (*R*)-**2a**·HCl (R^1=Me): δ 3.28 (d, 2H, C*H*$_2$, J=17.8 Hz), 3.71 (s, 3H, C*H*$_3$), 3.80 (s, 3H, C*H*$_3$), 4.61 (s, 1H, C*H*), 8.75 (s, 3H, N*H*$_3^+$). Elemental analysis: obs. 36.11% C, 6.21% H, 7.01% N, calcd for $C_6H_{12}NO_4Cl$: 36.48% C, 6.08% H, 7.09% N.

^{1}H NMR, (*S*)-**2b** (R^1=Me): δ 0.91 (t, 3H, C*H*$_3$CH$_2$, J=7.6 Hz), 1.62 (m, 2H, C*H*$_2$CH$_3$, J=7.6 Hz), 2.15 (t, 2H, C*H*$_2$CONH, J=7.6 Hz), 2.90 (m, 2H, C*H*$_2$CO$_2$, J=17.1 and 4.4 Hz), 3.65 (s, 3H, OC*H*$_3$), 3.72 (s, 3H, OC*H*$_3$), 4.84 (m, 1H, C*H*, J=4.4 Hz), 6,47 (s, 1H, N*H*). Elemental analysis: obs. 51.07% C, 7.60% H, 5.95% N, calcd for $C_{10}H_{17}NO_5$ 51.94% C, 7.41% H, 6.06% N.

^{1}H NMR, (*S*)-**3**: δ 0.93 and 0.95 (t, 6H, C*H*$_3$, J=7.6 Hz), 1.36 (m, 2H, C*H*$_2$, J=7.6Hz), 1.61 (p, 2H, C*H*$_2$, J=7.6 Hz), 1.67 (m, 2H, C*H*$_2$, J=7.6 Hz), 2.22 (t, 2H, C*H*$_2$CONH, J=7.6 Hz), 2.95 (dd, 2H, C*H*$_2$CO$_2$, J=4.6, 4.4 and 17.1 Hz), 3.76 (s, 3H, OC*H*$_3$), 4.09 (t, 2H, C*H*$_2$O$_2$C, J=6.6 Hz), 4.88 (m, 1H, C*H*, J=4.4, 4.6 and 7.8 Hz), 6.52 (d, 1H, N*H*, J=7.8 Hz). Elemental analysis: obs. 56.39% C, 8.73% H, 5.08% N, calcd for $C_{13}H_{23}NO_5$ 57.13% C, 8.48% H, 5.12% N.

4.5. Gram-scale resolution of N*-butanoyl dimethyl aspartate (**2b**, R^1=Me) by Novozym 435*

Dimethyl aspartate hydrochloride (10.7 g, 54 mmol) was first transformed to dimethyl aspartate (R=H) and the product was dissolved in chloroform. Triethyl amine (23 ml, 166 mmol) and butanoyl chloride (7.37 ml, 70 mmol) in chloroform were added. After 24 h the reaction was stopped with methanol (25 ml). After evaporation the crude product was dissolved in diisopropyl ether and the precipitated salt was filtered off. Purification by column chromatography (acetone:hexane, 3:7) gave *rac*-**2b** (R^1=Me, R=PrCO; 9.1 g, 39 mmol).

Novozym 435 (8.56 g) was added to **2b** (R^1=Me; 2.49g, 11 mmol) in butanol (107 ml). After 124 h the enzyme was filtered off at 52% conversion. Purification by column chromatography (acetone:hexane, 2:8) led to (*R*)-**2b** [R^1=Me; 1.12 g, 4.8 mmol, ee 95%, $[\alpha]_D^{20}$ +18.9 (*c*=1.0, MeOH)] and (*S*)-**3** [1.36 g, 5.0 mmol, ee 87%, $[\alpha]_D^{20}$ –12.6 (*c*=1.0, MeOH)].

4.6. Gram-scale resolution of N*-butanoyl dimethyl aspartate (**2b**, R^1=Me) by acylase I*

Acylase I (8.80 g) was added to **2b** (R^1=Me; 2.56g, 11 mmol) in butanol (110 ml). After 152 h the enzyme was filtered off at 51% conversion. Purification by column chromatography (acetone:hexane, 2:8) yielded the unreacted (*R*)-**2b** [R^1=Me; 1.22 g, 5.3 mmol, ee 96%, $[\alpha]_D^{20}$ +19.3 (*c*=1.0, MeOH)] and the produced (*S*)-**4** [1.39 g, 5.1 mmol, ee 94%, $[\alpha]_D^{20}$ –23.2 (*c*=1.0, MeOH)].

^{1}H NMR, (*S*)-**4**: δ 0.93 and 0.96 (t, 6H, CH_3, J=7.6 Hz), 1.36 (m, 2H, CH_2, J=7.6 Hz), 1.61 (p, 2H, CH_2, J=7.6 Hz), 1.67 (m, 2H, CH_2, J=7.6 Hz), 2.21 (t, 2H, CH_2CONH, J=7.6 Hz), 2.94 (dd, 2H, CH_2CO$_2$, J=4.6, 4.4 and 17.1 Hz), 3.69 (s, 3H, OCH_3), 4.16 (t, 2H, CH_2O$_2$C, J=7.6 Hz), 4.86 (m, 1H, C*H*, J=3.9, 4.6 and 7.6 Hz), 6.48 (d, 1H, N*H*, J=7.6 Hz). Elemental analysis: obs. 56.26% C, 8.63% H, 5.09% N, calcd for $C_{13}H_{23}NO_5$: 57.13% C, 8.48% H, 5.12% N.

4.7. Determination of the enzymatic regioselectivity at sites B and C

The method for determining the regioselectivity of the enzymatic reactions is shown in Scheme 5. Dicyclohexyl carbodiimide (4.23 g, 21 mmol) was added to (*S*)-aspartic acid β-methyl ester hydrochloride (*S*)-**5** (1.51 g, 8.2 mmol) in ethanol (100 ml). After 72 h the ethanol was evaporated off. The crude product (*S*)-**6** was dissolved in chloroform. Bubbling with ammonia released amino group from its ammonium salt. After filtering off the precipitated ammonium chloride triethyl amine (3.41 ml, 25 mmol) and butanoic acid chloride (1.12 ml, 11 mmol) were added. Purification by column chromatography (acetone:hexane, 3:7) gave (*S*)-**7** (0.49 g, 2.0 mmol, ee 94%). Optical rotation was not determined because of minor impurities among (*S*)-**7**.

^{1}H NMR: δ 0.95 (t, 3H, CH_3, J=7.3 Hz), 1.27 (t, 3H, CH_3, J=7.1 Hz), 1.68 (m, 2H, CH_2, J=7.3 Hz), 2.22 (t, 2H, CH_2CONH, 7.3 Hz), 2.95 (dd, 2H, CH_2CO$_2$, J=4.4, 4.6 and 18.0 Hz), 3.70 (s, 3H, OCH_3), 4.22 (m, 2H, CH_2O$_2$C, J=7.1 Hz), 4.88 (m, 1H, C*H*, J= 4.4 and 4.6 Hz), 6.56 (s, 1H, N*H*). Mass spectrum: M^+=246.

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