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Use of enantio-, chemo- and regioselectivity of acylase I. Resolution of polycarboxylic acid esters

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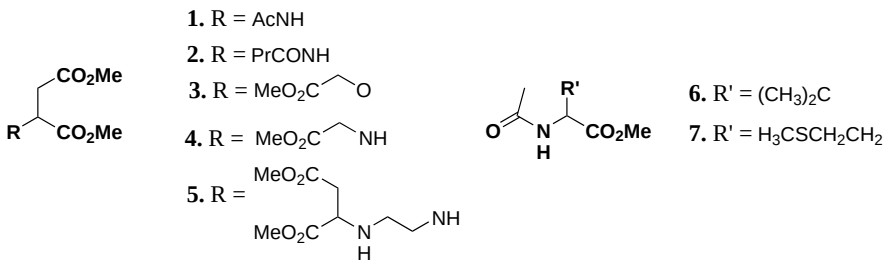
Abstract—Acylase I was used to catalyze the enantioselective butanolysis of trimethyl 2-[(carboxymethyl)oxy]succinate ($E=30$) and *N*-carboxymethylaspartate ($E=9$) exclusively at the most sterically hindered of the three ester groups (the position α to the asymmetric centre). Gram-scale resolution allowed the preparation of the less reactive trimethyl (*S*)-2-[(carboxymethyl)oxy]succinate (96% e.e.), that of the (*R*)-butyldimethyl regioisomer (78% e.e.) at 55% conversion and finally the preparation of the corresponding trisodium carboxylate by saponification. Acylase I was shown to transform (±)-methyl *N*-acetylmethionine and (±)-valine to the corresponding (*S*)-amino acids through ester hydrolysis-*N*-acetyl transfer sequence with absolute chemo- and enantioselectivity. Butanolysis of methyl *N*-acetylmethionine stopped in the formation of the butyl ester ($E=12$), the valine derivative being totally unreactive.

1. Introduction

Commercially available acylase I enzymes (*N*-acylamino acid amidohydrolase, EC 3.5.1.14) include the enzymes purified from porcine kidney and from different *Aspergillus* species. Acylase I from *Aspergillus* on Eupergit C is also available. Acylase I has been shown to exert extremely wide substrate specificity and nearly absolute enantioselectivity toward the hydrolysis of numerous natural as well as unnatural *N*-acyl α-amino acids, leading to the formation of the corresponding α-L-amino acids.[1] In industrial scale resolutions acylase I is widely used for the production of enantiopure α-L-amino acids using fungal *Aspergillus* enzyme with higher stability compared to the renal enzyme.[2] *N*-Acyl-β-amino acids have not been suitable substrates.[1] Previous work under anhydrous conditions have confirmed that the use of acylase I is not restricted to *N*-acyl transfers.[3–12] Thus, acylase I from commercial *Aspergillus* species allows the regioselective alcoholysis of a number of 2-substituted dimethyl succinates including *N*-acylated dimethyl aspartates **1** and **2** (Scheme 1).[3,4] A noteworthy feature of these reactions is that they take place exclusively at the methyl ester function at position 2 (or α to the asymmetric centre) of the succinate even in the presence of the more natural amide functions of **1** and **2**. Acylase I from *Aspergillus melleus* has been useful on enantioselective acylations of primary and secondary alcohols with vinyl esters.[5–10] Acylations of the amino group of α-amino acids have also been performed.[11,12]



Scheme 1.

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We have now studied the regio- and enantioselectivities of acylase I for the butanolysis of tri- and tetracarboxylic acid derivatives **3**–**5** having a 2-substituted succinate backbone (Scheme 1). The aim has been the resolution of these water-soluble chiral chelating agents when reasonable enantioselectivities can be found.[13,14] The fact that ester alcoholysis rather than *N*-acyl transfer takes place in the cases of compounds **1** and **2** awoke interest in chemoselectivity studies for acylase I.[3,4] For that purpose, the hydrolysis and butanolysis of *N*-acetylated methyl esters **6** and **7** were studied (Scheme 1). These natural valine and methionine derivatives were chosen because their free *N*-acylated amino acids are readily hydrolyzed by the enzyme.[1,15,16]

2. Results and discussion

2.1. Resolution of polycarboxylic acids

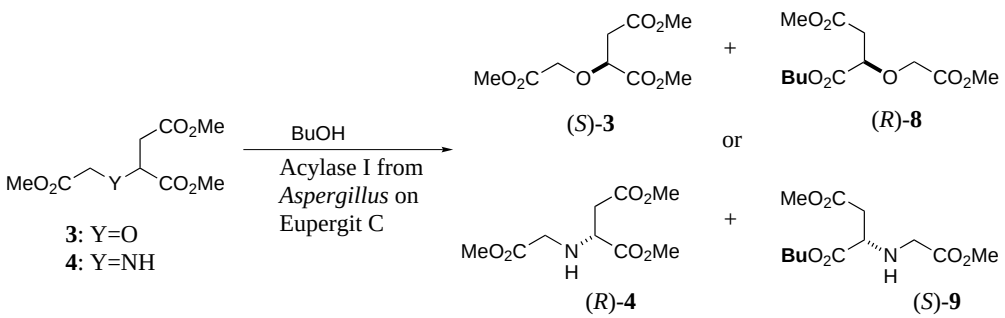
The butanolysis of substrates **3**–**5** in neat butanol has been studied in the presence of fungal acylase I enzymes (Scheme 1). The results in the cases of trimethyl 2-[(carboxymethyl)oxy]succinate **3** and *N*-carboxymethylaspartate **4** together with the previous data[3,4] for the butanolysis of **1** and **2** are given in Table 1. Reactivities (conversion after a certain time) of the three acylase I enzyme preparations clearly differ from each other, the highest reactivity being observed when the enzyme on Eupergit C is used. Interestingly, tricarboxylic acid esters **3** and **4** contain two remote ester groups in addition to that at the sterically hindered position α to the asymmetric centre. The remote ester groups of these triesters were not reactive and the reactions proceeded exclusively at the α-ester group (Scheme 2) in accordance with the results for **1**, **2** and related 2-substituted succinates.[3,4] The strict regioselectivity was confirmed by synthesizing mixed butyl methyl esters (as described in Section 4) and by affirming the identities of the peaks on the GLC chromatograms with those obtained from enzymatic reactions. In spite of similar structural units as in compound **4**, none of the stereoisomers of tetramethyl ethylenediamine-*N*,*N*′-disuccinate **5** was a substrate for acylase I. In this work, the *meso*-isomer was chromatographically separated from the mixture of chemically prepared stereoisomers (as described in Section 4).

Relatively good enantioselectivity ($E=30$; Table 1) was observed for the butanolysis of **3** in the presence of acylase I on Eupergit C, allowing the gram-scale resolution and the preparation of enantiopure (*S*)-**3** (96% e.e.) and enantiomerically enriched (*R*)-**8** (78% e.e.) at 55% conversion as described in Section 4 (Scheme 2). The hydrolysis of the trimethyl ester (*S*)-**3** with aqueous sodium hydroxide yielded trisodium (*S*)-2-[(carboxymethyl)oxy]succinate without racemization. The acylase I-catalyzed methanolysis of enantiomerically enriched (*R*)-**8** to enantiopure (*R*)-**3** in various organic solvents was too slow to be of practical use. On the other hand, the chemical transformation of the mixed ester (*R*)-**8** to the trimethyl ester and repeated enzymatic butanolysis (although not done in the present work) is a potential solution for enhancement of e.e.

Table 1. Acylase I-catalyzed butanolyses of **2**–**4** in neat butanol at room temperature (25°C; $t=24$ h)

Source of acylase I	**1**[a]		**2**[a]		**3**		**4**	
	c/%	*E*	*c*/%	*E*	*c*/%	*E*	*c*/%	*E*
Aspergillus melleus	20	9	23	>100	47	11	<1	3
Aspergillus genus	16	4	13	15	28	8	<1	4
Aspergillus on Eupergit C	46	14	51	50	59	30	30	9
Aspergillus on Eupergit C[b]							23	11
Aspergillus on Eupergit C[c]							23	10
Aspergillus on Eupergit C[d]							20	7

[a] Ref. 3.
[b] In the presence of $CoCl_2$ (0.5 mM).
[c] In the presence of $ZnZl_2$ (0.5 mM).
[d] Temperature 8°C.



Scheme 2.

The enantioselectivity for the acylase I-catalyzed butanolysis of compound **4** was only moderate ($E=9$) and efforts for enantioselectivity enhancement were unsuccessful. Thus, the ability of divalent cations to enhance enantioselectivity was studied in the presence of acylase I on Eupergit C but the addition of $CoCl_2$ and $ZnCl_2$ had no effect (E of the order of 10; Table 1). Decreasing temperature from 25 to 8°C did not improve enantioselectivity ($E=7$; Table 1) either.

We previously observed that the nature of the substrate has a strong effect on the enantiopreference (which enantiomer reacts faster) of acylase I.[4] Thus, the (*R*)-enantiomer of 2-substituted dimethyl succinates reacted faster in the case of halogen, alkoxy and methyl substituents at position 2 (R in Scheme 1), while (*S*)-enantiopreference was observed with hydroxyl, amino, ester and amido substituents. In accordance with these structural effects, (*R*)-enantiopreference was observed in the case of compound **3** with an alkoxy (OCH_2CO_2Me) substituent and (*S*)-enantiopreference for compound **4** with an amino ($NHCH_2CO_2Me$) substituent at the position 2 of the succinate backbone (Scheme 2).

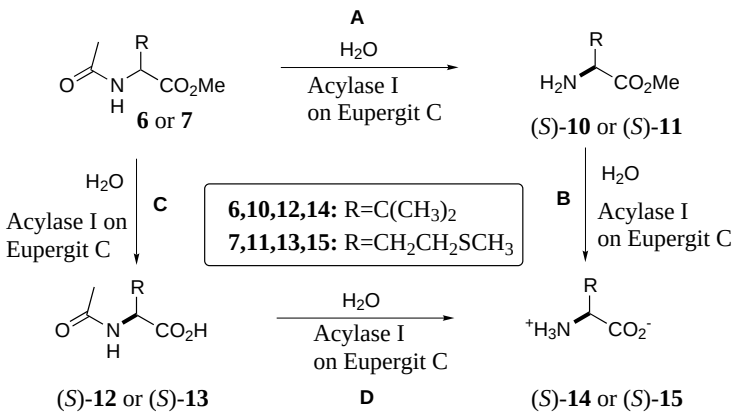
The acylase I enzyme is well characterized as a Zn(II)-containing enzyme (1–3 zinc ions per subunit).[1,15] In spite of that, the exact structure of the active site is not known and the reaction mechanism is simply proposed on the basis of the mechanisms of Zn(II)-containing carboxypeptidases.[17] Accordingly, it is difficult to interpret the above-described dependence of enantiodiscrimination on substrate structure. In the previous paper two different binding modes for chiral ester substrates were suggested.[4] The possibility for a second catalytic site with an esterase-like activity in the enzyme has also been suggested.[5,7] The present results, including the chemoselectivity results below, do not bring light to this question. However, the present results clearly emphasize that for ester substrates acylase I is a transesterification catalyst rather than catalyzing *N*-acyl transfers.

2.2. Chemoselectivity of acylase I

Acylase I was reported to catalyze the enantioselective hydrolysis of *N*-acyl α-L-amino acids, the terminal carboxylate group being essential for productive binding and turnover of the substrates.[1,18] Ester hydrolysis as a minor side reaction was reported in the case of *N*-chloroacetylalanine ethyl ester as a substrate.[18] In contrast, the previous results for the ester alcoholysis of *N*-acylated amino acid esters, such as that of **1** and **2** (Table 1), proceeded without *N*-acyl transfer by acylase I.[3,4]

In the present work, we found it reasonable to study the chemoselectivity of acylase I on Eupergit C by subjecting the methyl esters of (±)-*N*-acetylvaline **6** and (±)-*N*-acetylmethionine **7** to hydrolysis in water and butanolysis in neat butanol. The hydrolysis reactions of the starting materials **6** and **7** to the products **14** and **15**, respectively, can proceed through two different ways (A+B) and/or (C+D) depending on the enzymatic chemoselectivity (Scheme 3). Similar schemes can be drawn for butanolysis reactions. The results for the acylase I-catalyzed hydrolysis of **6** and **7** reveal absolute chemo- and enantioselectivity for the reactions at the ester function (route C) in the formation of (*S*)-**12** and (*S*)-**13** (●, Figs. 1 and 2, respectively). This reaction is followed by the subsequent hydrolysis of the amide bond in the obtained (*S*)-acid (route D), leading to the formation of (*S*)-**14** and (*S*)-**15** (▲). The hydrolysis of **6** is slower than that of **7**. Thus, unreacted (*R*)-**6** and (*R*)-**7** were obtained at 95% e.e. after 27 and 1.5 h, respectively. The hydrolysis of chemically prepared (±)-**10** and (±)-**11** (route B) proceeds with poor enantioselectivities ($E=2$ and 4, respectively) and relatively slowly (conversion 30% after 36 and 1 h, respectively), further implying that *N*-acyl transfer through route A is impossible.

Butanolysis of **7** in the presence of acylase I proceeds with low (*S*)-enantioselectivity ($E=12$, $c=15\%$, $t=24$ h). As in the above hydrolysis reactions, butanolysis starts with the reaction at the methyl ester function (route C with butanol in the place of water). The difference to the hydrolysis is that the second step, *N*-acyl transfer to butanol, does not occur, just as we observed for the butanolysis of **1** and **2** (Table 1).[3,4] Another difference is that the ester hydrolysis of **6** and **7** proceeds with absolute enantioselectivity, whereas the



Scheme 3.

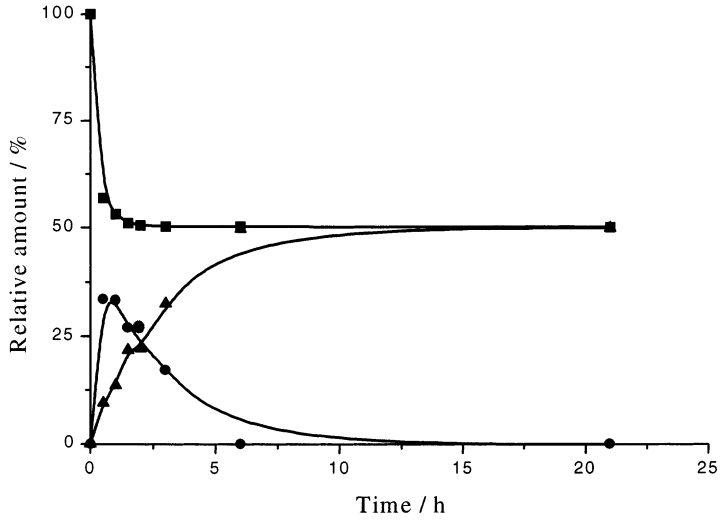


Figure 1. Progress of the hydrolysis of **7** (0.05 M) containing 40 mg/mL acylase I on Eupergit C, (■ **7**, ● **13**, ▲ **15**).

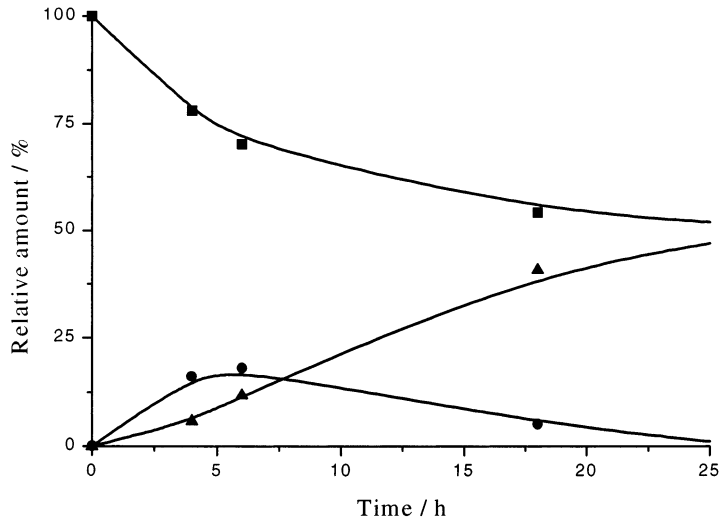


Figure 2. Progress of the hydrolysis of **6** (0.05 M) containing 40 mg/mL acylase I on Eupergit C, (■ **6**, ● **12**, ▲ **14**).

ester butanolysis of **1**, **2** and **7** ($E=14$, 50 and 12, respectively; Table 1) is of moderate to good enantioselectivity. There is no alcoholysis in the case of the valine ester **6**.

3. Conclusions

Butanolysis of carboxylic acid esters **3**–**5** in the presence of acylase I enzymes from *Aspergillus* species were studied, the enzyme on Eupergit C being most appropriate in the present work (Table 1, Scheme 1). Reactions of triesters **3** and **4** exclusively proceeded at the sterically more hindered α-methyl ester group with (*R*)-enantioselectivity (with $E=30$) for **3** and (*S*)-enantioselectivity (with $E=9$) for **4** (Scheme 2). Tetramethyl ethylenediamine-*N*,*N*′-disuccinate **5** was not a substrate for acylase I. On the basis of this information together with the previous results for 15 structurally different dicarboxylic acid esters[3,4] it is justifiable to predict that acylase I is highly selective for the position of the ester bond (must be α to the asymmetric center) and that enantiodiscrimination depends on the chemical nature of substrate structure.

Chemoselectivity of acylase I was studied using natural substrates, the racemic methyl esters of *N*-acetylvaline **6** and *N*-acetylmethionine **7**. The reactions followed chemo- and enantiospecifically ester hydrolysis—amide hydrolysis route (C+D; Scheme 3). This, together with the fact that *N*-acyl transfers for butanolysis reactions were not detected, reveals that a free α-carboxyl group is essential for *N*-acyl transfers and that a reaction at an α-ester function is not a side reaction.

4. Experimental

4.1. Materials

Acylase I from *Aspergillus melleus* and *Aspergillus genus* were obtained from Sigma (Deisenhofen, Germany) and Tokyo Kasei (Tokyo, Japan), respectively. Acylase I from *Aspergillus* on Eupergit C (388.4 U/g)

was obtained from Fluka (Buchs, Switzerland). The more precise origin of the immobilized enzyme is not reported by the producer.

Dimethyl malate, triethylamine and diethylazodicarboxylate were products of Tokyo Kasei, J. T. Baker and Fluka, respectively. Dimethyl maleate, (*S*)-dimethyl malate, trifluoroacetic anhydride, triphenylphosphine, silver(I) oxide, bromoacetic acid, (±)-*N*-acetylmethionine, (*S*)-methionine, (±)-valine, (*S*)-valine methyl ester hydrochloride and 4-(*N*,*N*-dimethylamino)pyridine (DMAP) were obtained from Aldrich. Trisodium 2-[(carboxymethyl)oxy]succinate, prepared via a La(III)-catalyzed Michael addition of glycolic acid to maleate[19] and (±)-*N*-carboxymethylaspartate, prepared via Michael addition of glycine to maleate[20] were obtained from Kemira laboratories. Methyl bromoacetate and sodium iodide were purchased from Acros. Thionyl chloride was a product of Merck. (±)-*N*-acetylvaline and the hydrochlorides of (*S*)-aspartic acid β-methyl ester and dimethyl aspartate were from Sigma. The solvents were of the highest analytical grade and obtained from Lab Scan LTD or Aldrich.

4.2. Methods

The progress of the enzymatic reactions of **3**–**5** was followed by taking samples (100 μL) at intervals, filtering off the enzyme and analyzing the samples by GLC on Astec Chiraldex G-TA or on Chrompack CP-Chirasil-L-Valine columns. Conversions were calculated according to the equation: $c = \text{e.e.}_S/(\text{e.e.}_S+\text{e.e.}_P)$ or by using dihexyl ether as an internal standard. The determination of E was based on equation $E = \ln[(1-c)(1-\text{e.e.}_S)]/\ln[(1-c)(1+\text{e.e.}_S)]$.[21] Using linear regression E is achieved as the slope of a line. The progress of the hydrolysis of **6** and **7** was followed by taking samples (400 μL) and filtering off the enzyme with 10K Nanosep centrifugal tubes. Chrompack CP-Chirasil-DEX CB was used for the analysis of **6**, **10**, **7** and **11**. HPLC with Chirex D-penicillamine column (Phenomenex) was employed for detecting the enantiomers of **12**–**15**. ^{1}H NMR spectra were measured in $CDCl_3$ on a Jeol Lambda 400 or Bruker 200 (compounds **5**, **6** and **23**) spectrometer, tetramethylsilane being 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.

4.3. Synthesis of racemic starting materials

4.3.1. Synthesis of 3. Trisodium 2-[(carboxymethyl)-oxy]succinate (32.2 g, 125 mmol) was added in methanol (300 mL). Addition of sulphuric acid (30 mL) started the reaction. After refluxing for 5 h the reaction was stopped by adding triethylamine (30 mL). Methanol was evaporated in vacuo. The mixture was purified twice by column chromatography using acetone: petroleum ether (gradient 1:9–1:1) as an eluent, yielding **3** (14.5 g, 62 mmol).

(±)-**3**: ^{1}H NMR: δ (ppm) 2.90 (2×q, 2H, C$\underline{H}_2$CO$_2$Me), 3.72 (s, 3H, C$\underline{H}_3$), 3.75 (s, 3H, C$\underline{H}_3$), 3.79 (s, 3H, C$\underline{H}_3$), 4.30 (q, 2H, C$\underline{H}_2$O), 4.43 (m, 1H, C$\underline{H}$), ^{13}C NMR: δ (ppm) 37.5 (CH$\underline{C}$H$_2$CO), 51.9 ($\underline{C}$H$_3$), 52.0 ($\underline{C}$H$_3$), 52.3 ($\underline{C}$H$_3$), 68.0 (O$\underline{C}$H$_2$CO$_2$Me), 75.4 ($\underline{C}$H), 170.0 ($\underline{C}$O$_2$Me), 170.3 ($\underline{C}$O$_2$Me), 171.0 ($\underline{C}$O$_2$Me). Elemental analysis: obs. 46.15%C 5.89%H, calcd C, 46.15%; H, 6.03% for $C_9H_{14}O_7$. $[M+H]^+ = 235$.

4.3.2. Synthesis of 4. Trisodium *N*-carboxymethylaspartate (1.46 g, 5.7 mmol) was added in methanol (150 mL). Hydrochloride was bubbled into the solution and the reaction mixture was refluxed for 12 h. Methanol was evaporated in vacuo. The crude mixture was dissolved in chloroform and sodium chloride was filtered off. Ammonia was then bubbled for 1 h. The precipitated ammonium chloride was filtered off and solvent evaporated. After purification by column chromatography as above trimethyl *N*-carboxymethylaspartate was isolated (800 mg, 3.4 mmol). (±)-**4**: ^{1}H NMR: δ (ppm) 2.24 (s, 1H, N$\underline{H}$), 2.70 (2×q, 2H, CHC$\underline{H}_2$CO$_2$Me), 3.43 (q, 2H, C$\underline{H}_2$NH), 3.62 (m, 1H, C$\underline{H}$), 3.63 (s, 3H, C$\underline{H}_3$), 3.66 (s, 3H, C$\underline{H}_3$), 3.68 (s, 3H, C$\underline{H}_3$), ^{13}C NMR: δ (ppm) 37.5 (CH$\underline{C}$H$_2$CO), 49.0 (NH$\underline{C}$H$_2$CO$_2$Me), 51.8 (2×$\underline{C}$H$_3$), 52.2 ($\underline{C}$H$_3$), 56.9 ($\underline{C}$H), 171.1 ($\underline{C}$O$_2$Me), 172.0 ($\underline{C}$O$_2$Me), 173.2 ($\underline{C}$O$_2$Me). Elemental analysis: obs. C, 46.63%; H, 6.38%; N, 5.83%, calcd C, 46.35%; H, 6.48%; N, 6.01% for $C_9H_{15}NO_6$. $M^+ = 233$.

4.3.3. Synthesis of 5. Ethylenediamine monohydrate (5.00 g, 64 mmol) was dissolved in acetonitrile (25 mL). Dimethyl maleate (16.00 mL, 128 mmol) was also dissolved in acetonitrile, and the former solution was added dropwise into the latter. The reaction took place for 20 h before the solvent was evaporated in vacuo. Purification by column chromatography as described above yielded tetramethyl *meso*-ethylenediamine-*N*,*N*′-disuccinate (6.72 g, 19 mmol). Tetramethyl (±)-ethylenediamine-*N*,*N*′-disuccinate came out from the column together with (3-oxopiperazinyl)-2-acetic acid methyl ester as a side product. Separation of these two compounds failed. Enzymatic reactions were performed with a fraction containing 10% of the side product. *meso*-**5**: ^{1}H NMR: δ (ppm) 1.99 (s, 2H, 2×N$\underline{H}$), 2.60 (m, 4H, HNC$\underline{H}_2$C$\underline{H}_2$NH), 2.70 (m, 4H, 2×CHC$\underline{H}_2$CO$_2$Me), 3.65 (m, 2H, 2×C$\underline{H}$), 3.69 (2×s, 6H, 2×C$\underline{H}_3$), 3.74 (2×s, 6H, 2×C$\underline{H}_3$), ^{13}C NMR: δ (ppm) 37.9 (2×CH$\underline{C}$H$_2$CO), 47.1 (NH$\underline{C}$H$_2$CH$_2$NH), 47.6 (NH$\underline{C}$H$_2$CH$_2$NH), 51.8 (2×$\underline{C}$H$_3$), 52.1 (2×$\underline{C}$H$_3$), 57.3 ($\underline{C}$H), 57.6 ($\underline{C}$H), 171.3 (2×$\underline{C}$O$_2$Me), 174.0 (2×$\underline{C}$O$_2$Me). Elemental analysis: obs. C, 45.02%; H, 7.10%; N, 7.38%, calculated C, 46.70%; H, 6.63%; N, 8.38% for $C_{14}H_{24}N_2O_8$.

4.3.4. Synthesis of 6. Compound **6** was synthesized from (±)-valine (1.957 g, 16.7 mmol) by thionyl chloride (1.212 mL, 16.7 mmol) in methanol (100 mL). After evaporation the crude product was dissolved in dichloromethane. Triethylamine (11.6 mL, 83 mmol) and acetic anhydride (2.37 mL, 25 mmol) were added. Purification by column chromatography using acetone:petroleum ether (3:7) yielded **6** (2.462 g, 14.2 mmol).

(±)-**6**: ^{1}H NMR: δ (ppm) 0.93 (t, 6H, 2×C$\underline{H}_3$), 2.05 (s, 3H, C$\underline{H}_3$CONH), 2.15 (m, 1H, C$\underline{H}$(CH$_3$)$_2$), 3.75 (s, 3H, CO$_2$CH$_3$), 4.57 (dd, 1H, C$\underline{H}$CO$_2$Me), 6.15 (d, 1H, N$\underline{H}$), ^{13}C NMR: δ (ppm) 17.8 (CH($\underline{C}$H$_3$)$_2$), 18.8 (CH($\underline{C}$H$_3$)$_2$), 23.1 ($\underline{C}$H$_3$CONH), 31.2 ($\underline{C}$H(CH$_3$)$_2$), 52.0 ($\underline{C}$H$_3$), 57.0 ($\underline{C}$HCO$_2$Me), 170.0 ($\underline{C}$O$_2$Me), 172.7 ($\underline{C}$O$_2$Me). Elemental analysis: obs. C, 55.52%; H, 8.88%; N, 8.05%, calcd C, 55.47%; H, 8.73%; N, 8.09% for $C_8H_{15}NO_3$. $M^+=173$.

4.3.5. Synthesis of 7. Compound **7** (4.557 g, 22.2 mmol) was prepared by esterifying (±)-*N*-acetylmethionine (5.402 g, 28.2 mmol) with thionyl chloride (2.46 mL, 33.7 mmol) in methanol (75 mL) and purifying in the same manner as compound **6**. (±)-**7**: ^{1}H NMR: δ (ppm) 1.96 (s, 3H, C$\underline{H}_3$CONH), 2.00 (m, 2H, C$\underline{H}_2$CH$_2$S) 2.04 (s, 3H, C$\underline{H}_3$S), 2.46 (t, 2H, CH$_2$C$\underline{H}_2$S), 3.71 (s, 3H, CO$_2$CH$_3$), 4.67 (m, 1H, C$\underline{H}$CO$_2$Me), 6.40 (s, 1H, N$\underline{H}$), ^{13}C NMR: δ (ppm) 15.4 ($\underline{C}$H$_3$S), 23.0 ($\underline{C}$H$_3$CONH), 29.9 ($\underline{C}$H$_2$S), 31.6 ($\underline{C}$H$_2$CH$_2$S) 51.4 ($\underline{C}$H$_3$), 52.4 ($\underline{C}$HCO$_2$Me), 169.9 ($\underline{C}$O$_2$Me), 172.5 ($\underline{C}$O$_2$Me). Elemental analysis: obs. C, 46.31%; H, 7.42%; N, 6.91%, calculated C, 46.81%; H, 7.37%; N, 6.82% for $C_8H_{15}NO_3S$. $M^+=205$.

4.4. Enzymatic reactions

The reactions were typically performed as small-scale experiments where one of the substrates **3**–**7** (**3**–**5**: 0.1 M, **6** and **7**: 0.05 M) was dissolved in butanol or water (2 mL). The enzyme preparation (as a best compromise 75 mg/mL for **3**–**5** and 40 mg/mL for **6** and **7**) was added in order to start the reaction and the mixture was shaken at room temperature (25°C).

For gram-scale resolution, acylase I on Eupergit C (4.5 g) was added on **3** (1.375 g, 5.9 mmol) in butanol (129 mL) and dihexyl ether (1.379 mL, 5.9 mmol). After 53 h the enzyme was filtered off at 55% conversion. Purification by column chromatography using acetone:petroleum ether (2:8) yielded the unreacted trimethyl ester (*S*)-**3** (0.479 g, 2.1 mmol, e.e. 96%, $[\alpha]_D^{20}$ −63.0 ($c=1$, MeOH)) and α-butyl ester (*R*)-**8** (0.575 g, 2.1 mmol, e.e. 78%, $[\alpha]_D^{20}$ +49.0 ($c=1$, MeOH)). Spectroscopic data for (*S*)-**3** are in accordance with those for (±)-**3**. The hydrolysis of (*S*)-**3** (243 mg, 1.04 mmol) with sodium hydroxide (125 mg, 3.13 mmol) yielded trisodium (*S*)-2-[(carboxymethyl)oxy]succinate (258 mg, 1.00 mmol, e.e. 96%, $[\alpha]_D^{20}$ −20.6 ($c=0.2$, H$_2$O)). Enantiopurity was determined by esterifying the product back to (*S*)-**3**.

(*R*)-**8**: ^{1}H NMR: δ (ppm) 0.94 (t, 3H, C$\underline{H}_3$CH$_2$), 1.38 (m, 2H, CH$_3$C$\underline{H}_2$), 1.64 (m, 2H, CH$_3$CH$_2$C$\underline{H}_2$), 2.89 (2×q, 2H, C$\underline{H}_2$CO$_2$Me), 3.72 (s, 3H, C$\underline{H}_3$), 3.75 (s, 3H, C$\underline{H}_3$), 4.18 (m, 2H, CO$_2$C$\underline{H}_2$), 4.31 (q, 2H, C$\underline{H}_2$O), 4.41 (m, 1H, C$\underline{H}$), ^{13}C NMR: δ (ppm) 13.7 ($\underline{C}$H$_3$CH$_2$), 19.1 (CH$_3$$\underline{C}H_2$), 30.5 (CH$_3CH_2$$\underline{C}H_2$), 37.6 (CH$\underline{C}H_2$CO), 51.9 ($\underline{C}H_3$), 52.0 ($\underline{C}H_3$), 65.4 (CO$_2$$\underline{C}H_2$), 68.1 (O$\underline{C}H_2CO_2$Me), 75.5 ($\underline{C}$H), 170.1 ($\underline{C}O_2$), 170.4 ($\underline{C}O_2$), 170.6 ($\underline{C}O_2$). Elemental analysis: obs. C, 52.37%; H, 7.24%, C, calcd 52.17%; H, 7.30% for $C_{12}H_{20}O_7$. $[M+H]^+=277$.

Trisodium 2-[(carboxymethyl)oxy]succinate: ^{1}H NMR: δ (ppm) 2.38 (2×q, 2H, CHC$\underline{H}_2$CO$_2$), 3.75 (q, 2H, C$\underline{H}_2$O), 3.92 (dd, 1H, C$\underline{H}$), ^{13}C NMR: δ (ppm) 42.6 (CH$\underline{C}$H$_2$CO), 69.9 (O$\underline{C}$H$_2$), 79.9 ($\underline{C}$H), 178.7 ($\underline{C}$O$_2$), 180.4 ($\underline{C}$O$_2$), 180.6 ($\underline{C}$O$_2$). $M^+=258$.

4.5. Determination of enzymatic regioselectivity

The mixtures of the trimethyl ester and three dimethyl monobutyl regioisomers in addition to three dibutyl monomethyl regioisomers and tributyl ester were obtained when **3** was dissolved in butanol in the presence of sulfuric acid as a catalyst and the solution was stirred at room temperature for 1 h. Analysis by GLC showed the retention times for the components in the reaction mixture. Regioselectivity of the enzymatic reaction was then confirmed by preparing chemically all three monobutyl regioisomers (**8**, **24** and **25**, Scheme 4) and comparing the place of the peaks in the chromatogram with those of the enzymatic reaction.

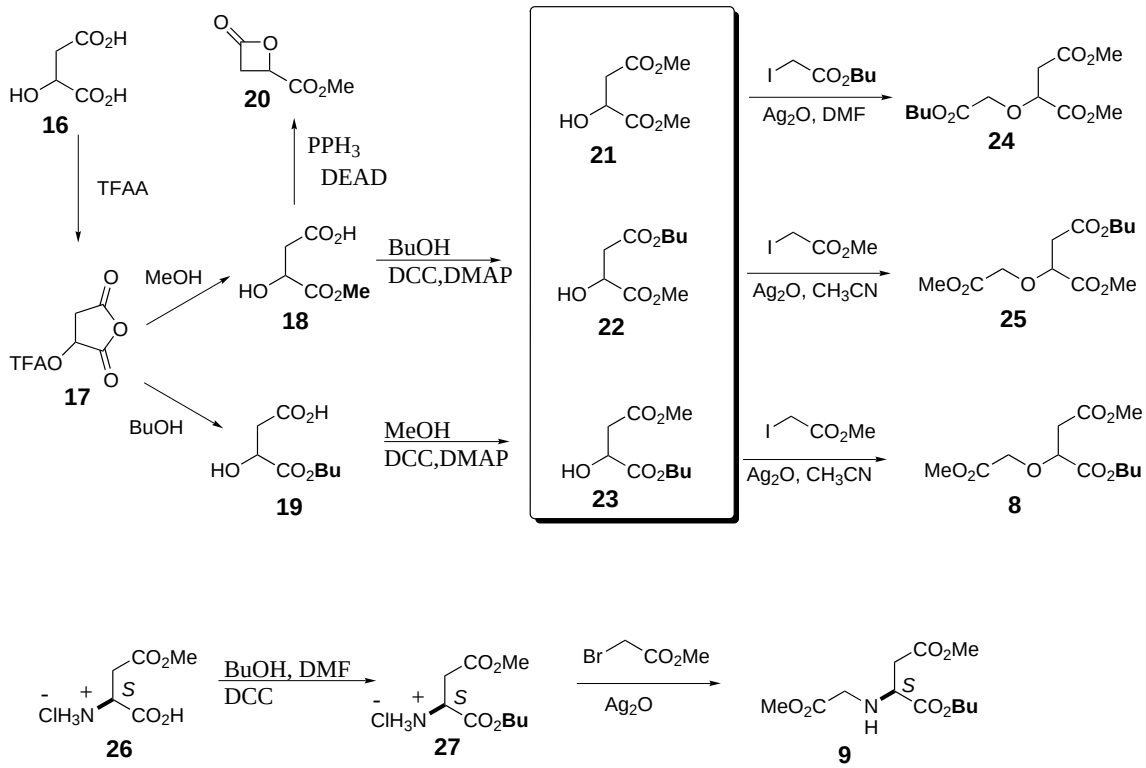
Synthetic routes to compounds **8**, **24** and **25** are depicted in Scheme 4. α-Methyl malic acid **18** was synthesized from **16** and the structure confirmed by preparing intramolecular lactone **20** by known methods.[22] α-Butyl malic acid **19** was synthesized in a similar manner. Esterifications to methyl butyl esters **22** and **23** were performed in neat alcohol with dicyclohexylcarbodiimide (DCC, 1 equiv.) and DMAP (0.05 equiv.). Compounds were purified by column chromatography as described in Section 4.3.

^{1}H NMR **22**: δ (ppm) 0.88 (t, 3H, C$\underline{H}_3$CH$_2$), 1.33 (m, 2H, CH$_3$C$\underline{H}_2$), 1.56 (m, 2H, CH$_3$CH$_2$C$\underline{H}_2$), 2.77 (2×q, 2H, CHC$\underline{H}_2$CO$_2$Me), 3.76 (s, 3H, C$\underline{H}_3$), 4.06 (t, 2H, CO$_2$C$\underline{H}_2$CH$_2$), 4.46 (dd, 1H, C$\underline{H}$), ^{13}C NMR: δ (ppm) 13.6 ($\underline{C}$H$_3$CH$_2$), 19.0 (CH$_3$$\underline{C}H_2$), 30.4 (CH$_3CH_2$$\underline{C}H_2$), 38.6 (C$\underline{H}_2CO_2$Me), 52.7 ($\underline{C}H_3$), 64.8 (CO$_2$$\underline{C}H_2$), 67.2 ($\underline{C}$H), 170.6 ($\underline{C}O_2$Me), 173.7 ($\underline{C}O_2$Me). $[M+H]^+=205$.

^{1}H NMR **23**: δ (ppm) 0.94 (t, 3H, C$\underline{H}_3$CH$_2$), 1.36 (m, 2H, CH$_3$C$\underline{H}_2$), 1.66 (m, 2H, CH$_3$CH$_2$C$\underline{H}_2$), 2.83 (2×q, 2H, CHC$\underline{H}_2$CO$_2$Me), 3.25 (d, 1H, C$\underline{H}$), 3.72 (s, 3H, C$\underline{H}_3$), 4.25 (t, 2H, CO$_2$C$\underline{H}_2$CH$_2$), 4.51 (dd, 1H, C$\underline{H}$), ^{13}C NMR: δ (ppm) 13.5 ($\underline{C}$H$_3$CH$_2$), 18.9 (CH$_3$$\underline{C}H_2$), 30.4 (CH$_3CH_2$$\underline{C}H_2$), 38.5 (C$\underline{H}_2CO_2$Me), 51.9 ($\underline{C}H_3$), 65.8 (CO$_2$$\underline{C}H_2$), 67.2 ($\underline{C}$H), 170.6 ($\underline{C}O_2$Me), 173.7 ($\underline{C}O_2$Me). Elemental analysis: obs. C, 53.17%; H, 7.92%, calcd C, 52.93%; H, 7.90% for $C_9H_{16}O_5$.

Monobutyl esters **24**, **25** and **8** were synthesized from **21** (13.3 μL, 0.10 mmol), **22** (20 mg, 0.10 mmol) and **23** (20 mg, 0.10 mmol), respectively, with the aid of silver(I) oxide (70 mg, 0.30 mmol) in given organic solvents. The amounts of butyl and methyl 2-iodoacetates were 97 mg (0.40 mmol) and 60 mg (0.30 mmol), respectively. Reactions took place for 4 days and the products were detected by the GLC method and confirmed by GLC–MS. The products were not isolated.

Methyl 2-iodoacetate was synthesized from methyl 2-bromoacetate (6.4 mL, 68 mmol) by dissolving it into acetone (75 mL) and adding sodium iodide (10.17 g, 68



Scheme 4.

mmol). The solvent was evaporated and the product was dissolved in chloroform. The precipitated sodium bromide was filtered off. Evaporation of chloroform yielded methyl 2-iodoacetate (11.02 g, 55 mmol). Butyl 2-iodoacetate (0.96 g, 4 mmol) was synthesized by esterifing 2-bromoacetic acid (1.62 g, 12 mmol) with thionyl chloride (1 mL, 14 mmol) in butanol (30 mL). After evaporation bromine was replaced with iodine as in the synthesis of methyl 2-bromoacetate.

Dimethyl monobutyl regioisomers of the aspartate were obtained by refluxing **4** in butanol for 24 h. All three regioisomers were separated from each other and identified by GC–MS. The assumed product of the enzymatic reaction, α-butyl ester **9** (Scheme 4), was synthesized from commercially available (*S*)-aspartic acid β-methyl ester hydrochloride **26** (417 mg, 2.3 mmol) by dissolving it in *N*,*N*-dimethylformamide (12 mL). BuOH (5 mL) and DCC (470 mg, 2.3 mmol) were dissolved in DMF (12 mL) and added dropwise to the previous solution. The reaction took place overnight. Dicyclohexylurea was filtered off and the solvent evaporated. The crude product was dissolved in acetonitrile (3 mL) and triethylamine (1.6 mL, 11.3 mmol) and methyl bromoacetate (0.86 mL, 9.1 mmol) were added. Product **9** was detected by GLC and identified by GLC–MS without purification.

4.6. Determination of absolute configurations

The absolute configuration of **3** was confirmed by synthesizing (*S*)-**3** from (*S*)-dimethyl malate and methyl 2-iodoacetate by the aid of silver(I) oxide as described in Section 4.5. (*S*)-**4** was prepared by esterifying L-aspartic acid by thionyl chloride in methanol. Addition of triethylamine and methyl bromoacetate in chloroform yielded (*S*)-**4**. (*S*)-**6** was synthesized from L-valine methyl ester hydrochloride and (*S*)-**7** from L-methionine by standard procedures described in Section 4.3.

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