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Acylase I in the alcoholysis of α-substituted dicarboxylic acid esters and derivatives: enantio- and regioselectivity

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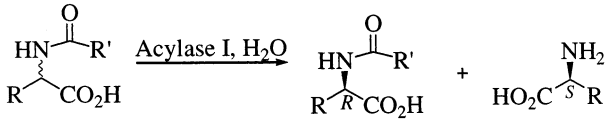
Abstract

Enantio- and regioselective butanolyses of α-substituted dimethyl succinates (substituents: Me–, MeO_2CCH_2–, NH_2–, AcHN–, PrCOHN–, HO–, MeO–, PrO–, AcO–, $PrCO_2$–, $HepCO_2$–, Cl– and Br–) and glutarates (substituents: PrCONH– and CbzNH–) and that of methyl pyroglutamate with acylase I enzymes have been studied. Acylase I-catalyzed reactions were totally regioselective proceeding exclusively at the sterically more hindered methyl ester group α to the substituent. High enantioselectivities (*E* from 50 to ≫100) were observed only for the substrates containing CONH functionality in the substituent although the C–N bond was unreactive. The nature of the substituent influenced which of the two enantiomers reacted faster.

1. Introduction

Acylases are readily available—although synthetically seldom studied—hydrolytic enzymes. Especially interesting are the acylase I enzymes (*N*-acylamino acid amidohydrolases; EC 3.5.1.14) as they hydrolyze neutral aliphatic *N*-acyl α-amino acids, including many xenobiotic-derived mercapturates (*S*-substituted *N*-acetyl-L-cysteine conjugates) and nonproteinogenic amino acids, to give the corresponding L-amino acids (usually the (*S*) absolute configuration) and fatty acids (Scheme 1).[1–4] Accordingly, acylase I from *Aspergillus melleus* is reported to catalyze the enantioselective hydrolysis of numerous natural as well as non-natural *N*-acyl



Scheme 1.

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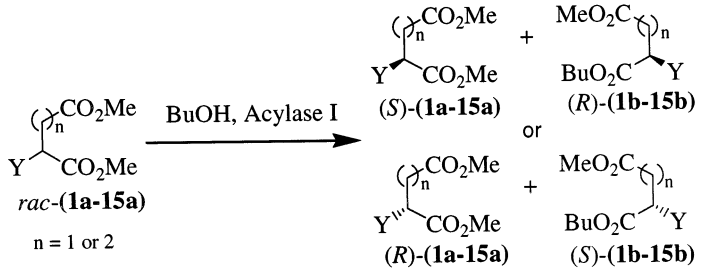
amino acids, the terminal carboxylate group being important for productive binding and turnover of the substrates.[3] The highly enantioselective hydrolysis of methyl *cis*-3-chloromethyl-2-tetrahydrofuran carboxylate reveals that the enzyme is capable of cleaving not only C–N bonds but also ester bonds.[5] In accordance with the C–O bond cleaving capability, the acylase I enzymes from commercial *Aspergillus* species previously allowed the (*S*)-enantioselective reactions of *N*-butanoylated dimethyl aspartate as novel alcoholysis reactions (Scheme 2).[6] These reactions are noteworthy as they proceed exclusively at the sterically more hindered α-methyl ester function, leaving the more natural amide bond as well as the β-methyl ester function unreacted. Also noteworthy is that the enzymes accept dimethyl aspartate, a substrate for acylase II (aspartoacylase, EC 3.5.1.15):[1,2] *N*-acyl derivatives of aspartic acid were previously reported as non-substrates for hydrolysis with acylase I (Scheme 1).[3] Acylase I from *Aspergillus melleus* is also useful for enantioselective acylations of primary and secondary alcohols by vinyl esters.[7–11]



Scheme 2.

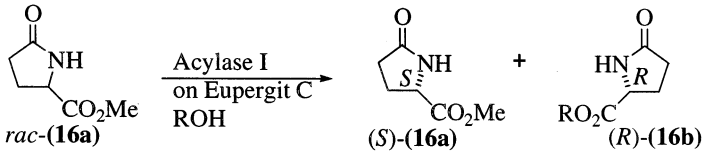
The mechanism of acylase I-catalyzed acyl transfers is not known. Some mechanistic features can be proposed on the basis of the mechanisms of Zn^{2+}-containing carboxypeptidases.[12] The enzymes such as zinc metalloproteins utilize Zn^{2+} as a cofactor and a Lewis acid. Thus, for the hydrolysis of *N*-acyl α-amino acids (Scheme 1) the metal cation is expected to coordinate to nucleophilic water, making it more acidic, and/or to the carbonyl group of the scissile C–N bond, increasing its susceptibility toward nucleophilic attack. For the hydrolysis of alkyl esters by acylase I, the ester carbonyl can coordinate in the place of the amide carbonyl. For alcoholysis, the alcohol as a nucleophile takes the place of water.

The existing data clearly show that the acylase I enzymes are capable of acting on the C–O bonds of carboxylic acid esters and thus function chemo- and regioselectively recognizing chirality at the acid part of their substrates.[5–11] This property made us interested in widening the scope of dicarboxylic acid substrates for acylase I-catalyzed alcoholyses in neat alcohols. Thus, the regio- and enantioselective butanolyses of α-substituted dimethyl succinates and glutarates **1a**–**15a** have now been studied paying attention to the nature of the α-substituent Y (Scheme 2; Table 1). Methyl pyroglutamate **16a** has served as a cyclic bifunctional substrate (Scheme 3).

Table 1
Acylase I-catalyzed butanolysis of α-substituted dimethyl succinates and glutarates ($t=22$ h) at room temperature

Compound	Y	Reactive enantiomer	Acylase I from *Aspergillus genus*		Acylase I from *Aspergillus melleus*		Acylase I on Eupergit C	
			c (%)	E	c (%)	E	c (%)	E
1a ($n=1$)	Me	*R*	1	2	2	2	5	2
2a ($n=1$)	MeO_2CCH_2[a]	–	1	–	2	–	10	–
3a ($n=1$)	H_2N	*S*	35	5	47	13	85	7
4a ($n=1$)	AcNH	*S*	16	4	20	9	46	14
5a ($n=1$)[b]	PrCOHN	*S*	13	15	23	>100	51	50
6a ($n=2$)	PrCONH	*S*	–	–	–	–	44	>100
7a ($n=2$)	CbzNH	*S*	–	–	–	–	49	≫100
8a ($n=1$)	HO	*S*	8	3	15	7	37	3
9a ($n=1$)	MeO	*R*	4	5	8	7	29	17
10a ($n=1$)	PrO	*R*	1	4	1	4	7	9
11a ($n=1$)	AcO	*S*	4	3	7	3	24	2
12a ($n=1$)	$PrCO_2$	*S*	11	–[c]	13	–[c]	24	7
13a ($n=1$)	$HepCO_2$	*S*	2	2	2	2	7	3
14a ($n=1$)	Cl	*R*	23	2	27	2	45	3
15a ($n=1$)	Br	*R*	13	3	22	3	38	4

[a] Both the substrate and the reaction product are prochiral.
[b] Ref. 6.
[c] Mixture of products observed.



Scheme 3.

2. Results and discussion

The results for the acylase I-catalyzed butanolyses of α-substituted dimethyl succinates **1a**–**5a**, **8a**–**15a** and glutarates **6a** and **7a** by fungal acylase I enzymes are given in Table 1. Reactivities (conversion at a certain time) of the three enzyme preparations clearly differ from each other, highest reactivity always being observed when the enzyme on Eupergit C is used. There is hardly any reaction with Y = Me or CH_2CO_2Me (compounds **1a** and **2a**). In contrast, the substrates **3a**–**9a**, **11a**, **12a**, **14a** and **15a** show from reasonable to high reactivities. In these cases Y is attached to the succinate or glutarate backbone through an electronegative oxygen, nitrogen or halogen atom which all contain free electron pairs. If electronic properties alone are important for reactivity it is difficult to explain why compounds **10a** and **13a** react slowly.

Acylase I-catalyzed reactions of compounds **1a**–**15a** with butanol all proceed exclusively at the sterically more hindered α-ester group of a molecule even though the presence of the β- or γ-methyl ester and amide functions (Scheme 2). There is no sign of the formation of the dibutyl esters. The strict chemo- and regioselectivity was confirmed by preparing mixed butyl methyl esters from the corresponding commercial half esters and confirming the identities of the GLC

chromatograms with those obtained from enzymatic reactions. In the case of compound **12a**, however, the reaction proceeds at the α-butanoyl group in addition to the α-methyl ester group, leading to the mixture of products. This is taken to be indicative of at least two different ways of substrate binding at the active site of the enzyme. The corresponding acyl transfers from the *O*-acylated dimethyl malates **11a** and **13a** are not observed. This is in accordance with the previous observations that butanoate is favored over acetate, hexanoate or higher carboxylates for the acylase I-catalyzed reactions of vinyl esters with alcohols.[9,11]

The enantioselectivities of the acylase I enzymes for substrates **1a**–**15a** are generally from negligible to moderate except when *N*-substituted amino acid esters **5a**–**7a** serve as substrates (Table 1). Clearly, acylase I on Eupergit C best suits the enantioselective butanolyses in neat butanol. Thus, the resolutions of *N*-butanoylated esters **5a** and **6a** result in excellent enantioselectivities, the reaction in the case of **7a** being practically completely enantiospecific (both the unreacted (*R*)-enantiomer and the obtained α-butyl β-methyl ester with the (*S*) absolute configuration are obtained with >99% ee at 50% conversion). Moderate enantioselectivity is observed for the *N*-acetylated compound **4a**.

It is clear according to the results in Table 1 that good reactivity does not necessarily correlate with good enantioselectivity (*E* values) for the acylase I-catalyzed alcoholyses of compounds **3a**–**9a**, **11a**, **12a**, **14a** and **15a**. Noteworthy amongst the results is the dependence of the observed enantiopreference (which enantiomer reacts faster) on the structure of Y. Thus, the (*R*)-enantiomer reacts faster in the case of halogen, alkoxy and methyl substituted compounds **1a**, **9a**, **10a**, **14a** and **15a**, while the (*S*)-enantiopreference is observed in all the other cases. Two different binding possibilities at the active site of the enzyme are proposed, leading from excellent (*S*)-selectivity ($E \gg 100$) in the case of *N*-substituted glutarate **7a** to acceptable (*R*)-selectivity ($E = 17$) in the case of **9a** and finally to good (*R*)-enantioselectivity ($E = 35$) in the case of pyroglutamate **16a** as shown in Tables 2 and 3 (Scheme 3). As a possible binding mode, the change from *S* to *R* takes place when the substituent Y competes from the subsite which in the case of (*S*)-selectivity tends to be occupied by the succinate or glutarate backbone and vice versa.

Table 2
Acylase I (on Eupergit C)-catalyzed butanolysis of glutamic acid derivatives at room temperature

Compound	Time (h)	*c* (%)	ee_S	ee_P
6a	66	50	96	95
7a	24	50	>99	>99
16a	20	53	92	83

Table 3
Acylase I (on Eupergit C)-catalyzed alcoholysis of methyl pyroglutamate **16a** in neat alcohols ($t = 4$ h)

Alcohol	Temperature (°C)	*c* (%)	*E*
EtOH	25	16	19 ± 8
PrOH	25	44	34 ± 9
BuOH	8	36	63 ± 5
BuOH	25	40	34 ± 4
BuOH	42	25	20 ± 2
Pentanol	25	33	40 ± 3
Hexanol	25	28	35 ± 7

Finally, the acylase I-catalyzed alcoholysis of methyl pyroglutamate **16a** was studied in neat alcohols (Table 3, Scheme 3). Compared to the open-chain structures of glutamic acid derivatives **6a** and **7a** the cyclic structure now makes the molecule less flexible. The present results clearly indicate that the butanolysis of **16a** proceeds smoothly, the time needed to obtain 50% conversion being about the same as in the case of **7a** (Tables 1 and 2). Clearly, reduced enantioselectivity is observed in the case of **16a** compared with **7a**. Efforts to enhance enzymatic enantioselectivity by performing the alcoholysis in different alcohols were unsuccessful (Table 3). However, a considerable temperature effect on E is evident in favor of low temperatures. The more reactive enantiomer of **16a** is the (*R*)-counterpart, i.e. an opposite enantiodiscrimination is observed compared to the open-chain derivatives **6a** and **7a**. This is in accordance with the expectation that the cyclic part of the molecule (–$NHCOCH_2CH_2$–) is bound to the subsite where the glutarate backbone of **6a** or **7a** is bound in the case of (*S*) stereoselectivity. Interestingly, when the *N*-acetylated pyroglutamic acid was previously subjected to traditional hydrolysis (Scheme 1) the substrate was practically unreactive.[3]

In the previous work, the gram-scale butanolysis of *N*-butanoyl dimethyl aspartate led to the separation of the two enantiomers in a single resolution step by acylase I from *Aspergillus melleus*.[6] The gram-scale resolution of **6a** has now been performed using acylase I on Eupergit C (see Section 4).

3. Conclusions

Butanolyses of α-substituted succinic and glutaric acid dimethyl esters **1a**–**15a** and that of methyl pyroglutamate **16a** in the presence of acylase I enzymes from *Aspergillus melleus*, *genus* and sp. on Eupergit C were studied (Tables 1–3; Schemes 2 and 3). The excellent regioselectivity of the enzymes makes them potential catalysts when the reactions at the sterically more hindered α-methyl ester group at the stereocenter of polyfunctional substrates are needed.

Enantioselectivity of the acylase I enzymes strongly depends on the structure of a substrate. Thus, acylase I on Eupergit C, in particular, is a fascinating resolution catalyst especially for the highly (*S*)-enantioselective butanolyses of *N*-substituted aspartic and glutamic acid esters **5a**–**7a**. Accordingly, the gram-scale resolution of *N*-butanoylated dimethyl glutamate **6a** with butanol allowed the simultaneous preparation of the unreacted (*R*)-**6a** (ee 91%) and the corresponding α-butyl β-methyl (*S*)-ester (*S*)-**6b** (ee 99%) at 48% conversion. Cyclic pyroglutamate **16a** also serves as a resolvable substrate, leading to the formation of the (*R*)-product. α-Substituted carboxylic acid esters are potential substrates for enantioselective alcoholyses by acylase I enzymes, but as is the case with many other hydrolytic enzymes,[13] enantioselectivity is strongly dependent on the structure of a chiral acid component.

4. Experimental

4.1. Materials

Acylase I from *Aspergillus melleus* was obtained from Sigma (Deisenhofen, Germany). Acylase I from *Aspergillus genus* and from *Aspergillus* on Eupergit C (388.4 U/g) were the products of Tokyo Kasei (Tokyo, Japan) and Fluka (Buchs, Switzerland), respectively. The origin of the *Aspergillus* enzyme immobilized on Eupergit C is not reported by the producer.

The esters of malic acid were products of Tokyo Kasei except for (*S*)-dimethyl malate which was obtained from Aldrich. The esters of aspartic and (*S*)-glutamic acid hydrochloride, *N*-benzyloxycarbonyl-(*S*)-glutamic acid and its α-methyl ester as well as (*S*)-bromo- and (*R*)-chlorosuccinic acids were purchased from Sigma. Glutamic acid monohydrate, chloro- and bromosuccinic acids and dimethyl methylsuccinate were products of Aldrich. (*R*)-Glutamic acid and (*S*)-glutamic acid γ-methyl ester were from Acros. (*R*)-3-Methylsuccinic acid was obtained from Tokyo Kasei. The solvents were of the highest analytical grade and obtained from Lab Scan Ltd or Aldrich.

4.2. Methods

The progress of the reactions was followed by taking samples (100 μl) at intervals, filtering off the enzyme and analyzing the samples by GLC on Chrompack CP-Chirasil-DEX CB (compounds **3a** and **8a**), Chrompack CP-Chirasil-L-Valine (compounds **4a**–**6a** and **16a**) and Astec Chiraldex G-TA capillary columns (compounds **1a**, **2a**, **9a**–**15a**). Compound **7a** was analyzed by HPLC on a Chirasil OD column. Conversion was calculated according to the equation $c = ee_S/(ee_S+ee_P)$ or by using dihexyl ether as an internal standard. The determination of E was based on the equation $E = \ln[(1-c)(1-ee_S)]/\ln[(1-c)(1+ee_S)]$.[14] Using linear regression E is achieved as the slope of a line. ^{1}H NMR spectra were measured in $CDCl_3$ on a Jeol Lambda 400 or Bruker 200 (compounds **6a**, **7a**, **10a**, **11a** and **13a**–**16a**) Spectrometer, tetramethylsilane being the 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

Racemates **1a**, **2a** and **8a** were commercially available. Dimethyl aspartate **3a** was prepared by bubbling ammonia through the hydrochloride solution in chloroform. Dimethyl glutamate was prepared from glutamic acid monohydrate and methanol with the aid of thionyl chloride. Compounds **4a**–**6a** and **11a**–**13a** were prepared by normal procedures from dimethyl malate, aspartate and glutamate and the corresponding acid anhydride (1.2 equiv.) or acid chloride (1.5–2.0 equiv.) in the presence of DMAP (*N*,*N*-dimethylaminopyridine; 0.05–0.1 equiv.) and triethyl amine (3–5 equiv.). The reaction between dimethyl glutamate (2.03 g, 11.6 mmol) and benzyl chloroformate [7.3 ml (50% solution in toluene), 22 mmol] in toluene (90 ml) in the presence of triethyl amine (4.0 ml, 28.9 mmol) resulted in the *N*-benzyloxycarbonyl (Cbz) protection in the formation of **7a** (2.41 g, 7.79 mmol). Methoxy substituted compound **9a** (0.54 g, 3.1 mmol after 42 h) was synthesized from dimethyl malate (1.00 g, 6.2 mmol) and iodomethane (1.16 ml, 18.5 mmol) in the presence of Ag_2O (1.43 g, 6.2 mmol) in diethyl ether.[15] Compound **10a** (1.11 g, 5.4 mmol after 150 h) was synthesized in a similar manner from dimethyl malate (1.00 g, 6.2 mmol), propyl iodide (2.41 ml, 24.7 mmol) and Ag_2O (4.29 g, 18.5 mmol). Halogenated compounds **14a** and **15a** (yields 71 and 88% after 48 and 72 h, respectively) were prepared from the corresponding acids (1 equiv.) by refluxing in methanol in the presence of *p*-toluenesulfonic acid (1 equiv.). The above compounds were purified by column chromatography using acetone/petroleum ether (1/9–3/7) as an eluent. As a modification of the known method,[16] methyl pyroglutamate **16a** was prepared by heating dimethyl glutamate in an oil bath

(3 h, 110°C) followed by chromatography with silica using elution with acetone/petroleum ether (1/1) for the purification of the product.

4.4. Spectroscopic and analytical data

rac-(**4a**): ^{1}H NMR: δ (ppm) 2.04 (s, 3H, CH_3CONH), 2.95 (2×q, 2H, CH_2CO$_2$Me), 3.70 (s, 3H, CO$_2$CH_3), 3.77 (s, 3H, CO$_2$CH_3), 4.86 (m, 1H, CH), 6.54 (s, 1H, NH). ^{13}C NMR: δ (ppm) 23.0 (*C*H$_3$CO), 36.0 (*C*H$_2$CO$_2$Me), 48.4 (*C*H), 52.0 (CO$_2$*C*H$_3$), 52.7 (CO$_2$*C*H$_3$), 169.8 (*C*ONH), 171.2 (*C*O$_2$Me), 171.5 (*C*O$_2$Me). Elemental analysis: obs. C, 46.93%; H, 6.32%; N, 6.78%. Calcd C, 47.29%; H, 6.45%; N, 6.89% for the $C_8H_{13}NO_5$.

rac-(**6a**): ^{1}H NMR: δ (ppm) 0.95 (t, 3H, CH_3(CH$_2$)$_2$), 1.68 (m, 2H, CH_2CH$_3$), 2.01 (m, 2H, CH_2CH$_2$CO$_2$Me), 2.18 (m, 2H, CH_2CONH), 2.38 (m, 2H, CH_2CO$_2$Me), 3.68 (s, 3H, CO$_2$CH_3), 3.75 (s, 3H, CO$_2$CH_3), 4.65 (m, 1H, CH), 6.32 (d, 1H, NH). ^{13}C NMR: δ (ppm) 13.6 (*C*H$_3$), 18.9 (*C*H$_2$CH$_3$), 27.2 (*C*H$_2$CH$_2$CO$_2$Me), 30.0 (*C*H$_2$CO$_2$Me), 38.3 (*C*H$_2$CONH), 51.4 (CO$_2$*C*H$_3$), 51.8 (CO$_2$*C*H$_3$), 52.4 (*C*H), 172.4 (*C*O$_2$), 173.0 (*C*O$_2$), 173.3 (*C*ONH). Mass spectrum: M$^+$= 245.

rac-(**7a**): ^{1}H NMR: δ (ppm) 2.00 and 2.20 (2×m, 2H, CH_2CH$_2$CO$_2$Me), 2.42 (td, 2H, CH_2CO$_2$Me), 3.56 (s, 3H, CO$_2$CH_3), 3.74 (s, 3H, CO$_2$CH_3), 4.41 (m, 1H, CH), 5.10 (s, 2H, PhCH_2), 5.46 (d, 1H, NH), 7.35 (s, 5H, ArH). ^{13}C NMR: δ (ppm) 27.6 (*C*H$_2$CH$_2$CO$_2$Me), 29.9 (*C*H$_2$CO$_2$Me), 51.8 (CO$_2$*C*H$_3$), 52.5 (CO$_2$*C*H$_3$), 53.3 (*C*HNH), 67.0 (NHCO$_2$*C*H$_2$), 128.0–128.5 (5C, Ar–*C*), 136.1 (NHCO$_2$CH$_2$*C*), 155.9 (*C*ONH), 172.2 (*C*O$_2$), 173.0 (*C*O$_2$). Mass spectrum: M$^+$=309.

rac-(**9a**): ^{1}H NMR: δ (ppm) 2.78 (2×q, 2H, CH_2CO$_2$Me), 3.49 (s, 3H, CH_3O), 3.72 (s, 3H, CO$_2$CH_3), 3.79 (s, 3H, CO$_2$CH_3), 4.21 (dd, 1H, CH). ^{13}C NMR: δ (ppm) 37.5 (*C*H$_2$CO$_2$Me), 51.9 (CO$_2$*C*H$_3$), 52.1 (CO$_2$*C*H$_3$), 58.7 (*C*H$_3$O), 76.6 (*C*H), 170.4 (*C*O$_2$), 171.6 (*C*O$_2$). Elemental analysis: obs. C, 47.70%; H, 6.87%. Calcd C, 47.72%; H, 6.87% for the $C_7H_{12}O_5$.

rac-(**10a**): ^{1}H NMR: δ (ppm) 0.90 (t, 3H, CH_3CH$_2$), 1.59 (m, 2H, CH$_3$CH_2), 2.75 (2×q, 2H, CH_2CO$_2$Me), 3.50 (2×m, 2H, CH_2O), 3.71 (s, 3H, CO$_2$CH_3), 3.77 (s, 3H, CO$_2$CH_3), 4.21 (dd, 1H, CH). ^{13}C NMR: δ (ppm) 10.3 (*C*H$_3$CH$_2$), 22.8 (*C*H$_2$CH$_3$), 37.8 (*C*H$_2$CO$_2$Me), 51.9 (CO$_2$*C*H$_3$), 52.1 (CO$_2$*C*H$_3$), 73.1 (*C*H$_2$O), 75.3 (*C*H), 170.6 (*C*O$_2$), 172.1 (*C*O$_2$). M$^+$=204.

rac-(**11a**): ^{1}H NMR: δ 2.15 (s, 3H, CH_3CO$_2$CH), 2.89 (d, 2H, CH_2CO$_2$Me), 3.73 (s, 3H, CO$_2$CH_3), 3.78 (s, 3H, CO$_2$CH_3), 5.48 (t, 1H, CH). ^{13}C NMR: δ (ppm) 20.7 (*C*H$_3$CO), 36.0 (*C*H$_2$CO$_2$Me), 52.3 (CO$_2$*C*H$_3$), 52.9 (CO$_2$*C*H$_3$), 68.3 (*C*H), 169.5 (*C*O$_2$CH), 169.8 (*C*O$_2$Me), 170.1 (*C*O$_2$Me). Elemental analysis: obs. C, 47.09%; H, 6.03%. Calcd C, 47.06%; H, 5.92% for the $C_8H_{12}O_6$.

rac-(**13a**): ^{1}H NMR: δ (ppm) 0.88 (t, 3H, CH_3(CH$_2$)$_4$), 1.29 (m, 4×2H, CH_2), 1.64 (m, 2H, CH_2CH$_2$CO$_2$), 2.39 (t, 2H, CH$_2$CH_2CO$_2$), 2.89 (2×q, 2H, CH_2CO$_2$Me), 3.72 (s, 3H, CO$_2$CH_3), 3.77 (s, 3H, CO$_2$CH_3), 5.49 (t, 1H, CH). ^{13}C NMR: δ (ppm) 14.0 (*C*H$_3$CH$_2$), 22.5 (CH$_3$*C*H$_2$), 24.7 (*C*H$_2$CH$_2$CO$_2$), 28.8 (*C*H$_2$(CH$_2$)$_3$CO$_2$ and CH$_3$(CH$_2$)$_3$*C*H$_2$), 31.6 (CH$_3$CH$_2$*C*H$_2$), 33.8 (*C*H$_2$CO$_2$CH), 35.9 (*C*H$_2$CO$_2$Me), 52.0 (CO$_2$*C*H$_3$), 52.5 (CO$_2$*C*H$_3$), 68.0 (*C*H), 169.4 (*C*O$_2$CH) 169.5 (*C*O$_2$Me), 172.7 (*C*O$_2$Me). Elemental analysis: obs. C, 59.37%; H, 8.51%. Calcd C, 58.32%; H, 8.39% for the $C_{14}H_{24}O_6$.

rac-(**14a**): ^{1}H NMR: δ (ppm) 3.05 (2×q, 2H, CH_2CO$_2$Me), 3.73 (s, 3H, CO$_2$CH_3), 3.82 (s, 3H, CO$_2$CH_3), 4.66 (dd, 1H, CH). ^{13}C NMR: δ (ppm) 39.3 (*C*H$_2$CO$_2$Me), 51.1 (CO$_2$*C*H$_3$), 52.2 (CO$_2$*C*H$_3$), 53.2 (*C*H), 169.7 (*C*O$_2$), 173.1 (*C*O$_2$). Elemental analysis: obs. C, 39.90%; H, 5.38%. Calcd C, 39.91%; H, 5.02% for the $C_6H_9ClO_4$.

rac-(**15a**): ^{1}H NMR: δ (ppm) 3.10 (2×q, 2H, CH_2CO$_2$Me), 3.69 (s, 3H, CO$_2$CH_3), 3.78 (s, 3H, CO$_2$CH_3), 4.55 (dd, 1H, CH). ^{13}C NMR: δ (ppm) 37.7 (*C*H$_2$CO$_2$Me), 39.5 (*C*H), 52.1 (CO$_2$*C*H$_3$), 53.2 (CO$_2$*C*H$_3$), 169.5 (*C*O$_2$), 170.0 (*C*O$_2$). Elemental analysis: obs. C, 33.12%; H, 4.22%. Calcd C, 32.02%; H, 4.03% for the $C_6H_9BrO_4$.

rac-(**16a**): ^{1}H NMR: δ (ppm) 2.26 (m, 2H, CH_2CONH), 2.41 (m, 2H, CH_2CH), 3.78 (s, 3H, CO$_2$CH_3), 4.28 (t, 1H, CH), 6.61 (s, 1H, NH). Mass spectrum: $[M-H]^+ = 142$.

rac-(**5a**) and -(**12a**) were described in the previous paper.[6]

4.5. Enzymatic resolution

The resolutions were typically performed as small-scale experiments where one of the substrates **1a**–**16a** (0.1 M) was dissolved in butanol (2 ml). The enzyme preparation (75 mg/ml) was added in order to start the reaction and the reaction was shaken at room temperature (25°C) or at another given temperature.

For gram-scale resolution, acylase I on Eupergit C (4.6 g) was added on **6a** (1.51 g, 6.2 mmol) in butanol (62 ml). After 47 h the enzyme was filtered off at 48% conversion. Purification by column chromatography (acetone:petroleum ether 3:7) yielded the unreacted dimethyl ester (*R*)-**6a** (0.74 g, 2.6 mmol, ee 91%, $[\alpha]_D^{20}$ +26.1 ($c=1$, MeOH)) and the corresponding (*S*)-α-butyl γ-methyl ester (*S*)-**6b** (0.77 g, 2.7 mmol, ee 99%, $[\alpha]_D^{20}$ −23.6 ($c=1$, MeOH)). The spectroscopic data for (*R*)-**6a** are in accordance with those for racemic **6a**.

(*S*)-**6b**: ^{1}H NMR: δ (ppm) 0.94, 0.95 (m, 6H, 2×CH_3CH$_2$), 1.40 (m, 2H, CH_2CH$_3$), 1.65 (m, 4H, CH_2CH$_2$CH$_3$, CH_2CH$_3$), 2.00, 2.20 (2×m, 2H, CH_2CH$_2$CO$_2$), 2.23 (m, 2H, CH_2CONH), 2.38 (m, 2H, CH_2CO$_2$), 3.68 (s, 3H, CH_3), 4.14 (t, 2H, CO$_2$CH_2), 4.64 (m, 1H, CH), 6.32 (d, 1H, NH). ^{13}C NMR: δ (ppm) 13.6 (*C*H$_3$CH$_2$CH$_2$CONH), 13.8 (*C*H$_3$CH$_2$CH$_2$CH$_2$), 18.9 (*C*H$_2$CH$_2$CONH), 27.4 (CO$_2$CH$_2$CH$_2$*C*H$_2$), 30.0 (*C*H$_2$CH$_2$CO$_2$), 30.4 (*C*H$_2$CO$_2$), 34.8 (CO$_2$CH$_2$*C*H$_2$), 38.3 (*C*H$_2$CONH), 51.5 (CO$_2$*C*H$_3$), 62.5 (*C*H), 65.4 (CO$_2$*C*H$_2$), 172.0 (*C*O$_2$), 172.9 (*C*O$_2$), 173.3 (*C*ONH). $M^+ = 287$.

4.6. Determination of absolute configurations

Absolute configurations of **3a**–**6a** and **8a**–**13a** were determined by synthesizing chemically the (*S*)-enantiomers from commercial (*S*)-dimethyl malate and (*S*)-dimethyl aspartate and glutamate hydrochlorides by the above-mentioned procedures. Compound (*S*)-**7a** was obtained from *N*-Cbz-(*S*)-glutamic acid and methanol by the normal DCC (*N*,*N*-dicyclohexylcarbodiimide) coupling procedure in the presence of DMAP. The enantiomers of **14a** and **15a** were prepared from (*R*)-chloro- and (*S*)-bromosuccinic acids. The absolute configuration of **1a** was determined simultaneously with regioselectivity by preparing α-butyl β-methyl (*R*)-2-methylsuccinate from the commercial β-methyl (*R*)-succinate by DCC and DMAP in butanol. The retention times of the enantiomeric peaks of racemic and enantiopure compounds in the chromatogram were then compared for recognizing the less reactive dimethyl ester enantiomer in the resolution mixtures. The commercial enantiomers gave a sign about the antibody helping in identifying the two peaks for a racemate.

4.7. Determination of enzymatic regioselectivity

The mixtures of dimethyl ester, methyl butyl regioisomers and dibutyl ester were obtained when the corresponding dimethyl ester was dissolved in butanol in the presence of sulfuric acid as a catalyst and the mixture was refluxed for 0.5–2 h. Analysis by GLC showed the retention times for the enantiomers of the four possible esters in the reaction mixture. Regioselectivity of the enzymatic reactions was then determined by preparing chemically the other one of the two possible regioisomers from the commercial half ester as shown below. The place of the peak in the chromatogram was then compared to that of the enzymatic reaction. In the case of **7a**, regioselectivity is based on the place of the $CO_2CH_2CH_2CH_2CH_3$ protons in ^{1}H NMR, giving the triplets at 4.14 ppm for the α-butyl protons and at 4.06 for the γ-butyl protons. The synthesis products were not usually isolated.

Mixed α-butyl esters were prepared from the respective commercial β- or γ-methyl ester of (*R*)-2-methyl succinic acid and (*S*)-aspartic, (*R*)-glutamic or (*S*)-glutamic acid hydrochlorides by the DCC coupling method in butanol followed by *N*-acylation with an acid chloride when necessary.[6] Mixed β- and γ-butyl esters of α-methyl (*S*)-malic and *N*-Cbz-(*S*)-glutamic acids were similarly prepared. For that purpose, α-methyl malic acid was synthesized by the known method.[17] β-Butyl α-methyl malate was transformed to the 2-methoxy analogue of **9a** by iodomethane using Ag_2O as a catalyst as above and to the 2-bromo analogue of **15a** with phosphorus tribromide in CH_2Cl_2.[15] For the reactions of **10a** and **14a** the same enzymatic regioselectivity was expected as for those of **9a** and **15a**, respectively. Regioselectivity in the case of **2a** was detected straight by the chiral GLC method. The two possible monobutyl regioisomers differ distinctively from each other. One is prochiral consisting of one peak in the chromatogram and the other one consists of two enantiomers that separate well in the column.

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