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The substrate specificity of the heat-stable stereospecific amidase from *Klebsiella oxytoca*

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Abstract—The substrate specificity of the heat-stable stereospecific amidase from *Klebsiella oxytoca* was investigated. In addition to the original substrate, 3,3,3-trifluoro-2-hydroxy-2-methylpropanamide, the amidase accepted 2-hydroxy-2-(trifluoromethyl)-butanamide and 3,3,3-trifluoro-2-amino-2-methylpropanamide as substrates. Compounds with larger side chains and compounds where the hydroxyl group was substituted with a methoxy group, or in which the CF_3 group was substituted by CCl_3, were not accepted. The biotransformation is a new synthetic route to (*R*)-(+)-3,3,3-trifluoro-2-amino-2-methylpropanoic acid, and its related (*S*)-(−)-amide, and to (*R*)-(+)-2-hydroxy-2-(trifluoromethyl)-butanoic acid and its related (*S*)-(−)-amide.

1. Introduction

(*R*)- and (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropanoic acid are intermediates for the synthesis of a number of potential pharmaceuticals, which include ATP sensitive potassium channel openers for the treatment of incontinence,[1] and inhibitors of pyruvate dehydrogenase kinase for the treatment of diabetes.[2] A novel heat-stable stereospecific amidase from *Klebsiella oxytoca* was selected, purified, characterised and cloned, and used to synthesise enantiomerically pure (*R*)-3,3,3-trifluoro-2-hydroxy-2-methylpropanoic acid, (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropanoic acid and (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropanamide.[3,4] We now report on the substrate specificity of the enzyme.

2. Results

The substrates listed in Table 1 were incubated with the heat-stable amidase from *K. oxytoca*. The enzyme used was partially purified by heat treatment of a cell-free extract from an *Escherichia coli* strain that contained the recombinant *K. oxytoca* gene.[3,4] The biotransformations were carried out at 40 °C in 100 mM potassium phosphate buffer, pH 8.0, with substrate (5.0 mg/mL) and enzyme extract (0.2 mg/mL). The reaction was followed by chiral GC analysis of the (*R*)- and (*S*)-amide substrates. The enzyme accepted substrates **2** and **7** (Table 1) and the chiral GC analyses showed that the reactions were enantiospecific. The initial rate with **2** as substrate was about a quarter of that with the original substrate **1**, and the time to complete the reaction was correspondingly longer. However, with substrate **7**, the initial rate was about 30 times faster than that with the original substrate **1**, and the time to complete the reaction was much faster (about 300 times).

To isolate the reaction products from substrates **2** and **7**, biotransformations with larger amounts of substrate were carried out (see Section 5). For substrate **2**, the biotransformation was carried out with whole cells of *E. coli* containing the recombinant *K. oxytoca* amidase, and for substrate **7**, the biotransformation was carried out with the amidase immobilised on the carrier Eupergit C, in the presence of only substrate and water. This experimental regime was to facilitate the isolation of the product (see Section 3). The reactions are shown in Scheme 1, and the detailed reaction results are shown in Table 2. The amide **8** was obtained with a yield of 34.4% and an ee value of >98%. The corresponding acid **9** was obtained with a yield of 39.1% and an ee value of 85.2%. The amide **10** was obtained with a yield of about 50% and an ee value of >99%, whereas the corresponding acid **11** was obtained with a yield of 42% and an ee value of 95.4%.

3. Discussion

There is considerable interest in the synthesis of chiral

Keywords: Biotransformation; Amidase; (*R*)-(+)-3,3,3-Trifluoro-2-amino-2-methylpropionic acid; (*S*)-(−)-3,3,3-Trifluoro-2-amino-2-methylpropanamide; (*R*)-(+)-2-Hydroxy-2-(trifluoromethyl)-butanoic acid; (*R*)-(+)-2-Hydroxy-2-(trifluoromethyl)-butanamide.
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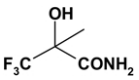
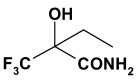
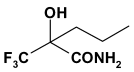
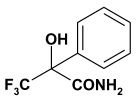
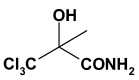
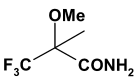
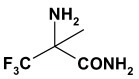
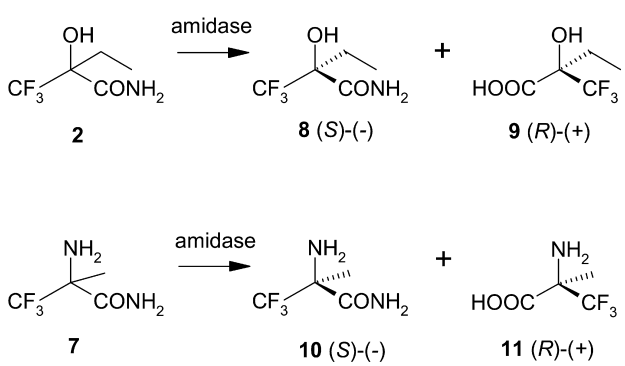
Table 1. Substrate specificity of the amidase

Substrate	Name	Structure	Initial rate (μmol/min/mg protein) (relative)	Time to complete conversion
1	3,3,3-Trifluoro-2-hydroxy-2-methylpropionamide		1.9 (1)	~5 h
2	2-Hydroxy-2-(trifluoromethyl)-butanamide		0.52 (0.27)	>80 h
3	2-Hydroxy-2-(trifluoromethyl)-pentanamide		0	
4	3,3,3-Trifluoro-2-hydroxy-2-phenyl-propionamide		0	
5	3,3,3-Trichloro-2-hydroxy-2-methylpropionamide		0	
6	3,3,3-Trifluoro-2-methoxy-2-methylpropionamide		0	
7	3,3,3-Trifluoro-2-amino-2-methylpropanamide		54.0 (28.41)	1.0 min

trifluoro-substituted acids as pharmaceutical intermediates[1,2] and fluorinated amino acids have been synthesised for use as mechanism based inhibitors of amino acid decarboxylases and transaminases.[5,6] Trifluoro-substituted acids have been synthesised chemically[1,7,8] and we[3,4,9] and another group[10] have used enzymatic methods for their synthesis. The fluorinated amino acid **11** (*R*)-(+)-3,3,3-trifluoro-2-amino-2-methylpropanoic acid (3,3,3-trifluoro-2-methyl-alanine) has been synthesised chemically,[5] and also by enzymatic resolution.[6]

Various derivatives of the original enzyme substrate with substitutions at each of the three positions other than the amide group were synthesised, and used to test the substrate specificity of the amidase. Cell-free extracts were used for these tests, to ensure that the permeability of the *E. coli* cell wall to the various substrates would not influence the results. When larger groups substituted the methyl group on the substrate, the enzyme was active with the ethyl compound (at 27% of the rate shown with **1** as substrate), but there was no measurable activity with the propyl and phenyl derivatives. It follows that the larger the substrate the more difficulty it has to fit in the active site. Substituting the CF_3 group with CCl_3 also resulted in no activity, again probably due to the larger size of the CCl_3 group and after a time the pH 8 buffer solution slowly began to hydrolyse the CCl_3 group. The influence of the hydroxyl group on the substrate was investigated by two further derivatives. Masking the hydroxyl group with a methoxy group resulted in no activity. Substituting the hydroxy group with an amino group resulted in a 28-fold increase in the rate of amide hydrolysis over substrate **1**. The amidase active site probably requires a direct or indirect proton interaction to function, either in binding or in a more direct chemical role.

The amides **8** and **10** rotated polarised light to the left (−) and the acids **9** and **11** rotated to the right (+). The absolute configurations of **9** and **11** have been reported as (*R*)-(+).[1,5,10]



2 **8** (*S*)-(-) **9** (*R*)-(+)

7 **10** (*S*)-(-) **11** (*R*)-(+)

Scheme 1. Amidase catalysed resolutions of **2** and **7**.

Table 2. Amidase catalysed resolutions of **2** and **7**

#	Yield[a] (%)	Optical rotation	^{1}H NMR (DMSO)	^{13}C NMR (DMSO)	Elem. anal. or LC–MS	Mp (°C)	ee (%)
8	34.4	$[\alpha]_D=-13.2$ $c=3.75$ (MeOH)	7.60 (br s, 1H, $CONH_2$), 7.45 (br s, 1H, $CONH_2$), 6.46 (s, 1H, OH), 2.00 (m, 1H, CH_2), 1.70 (m, 1H, CH_2), 0.87 (t, 3H, J=6.7, CH_3)	169.3 (s, $CONH_2$), 124.9 (q, J_{CF}=287 Hz, CF_3), 77.2 (q, J_{CCF}=26.2, quaternary), 24.7 (t, CH_2), 6.7 (q, CH_3)	Calcd (found) for $C_5H_8F_3NO_2$ C 35.1 (35.4), H 4.7 (4.5), N 8.2 (8.1)	57–61	>98
9	39.1	$[\alpha]_D=+9.75$ c=3.31 (MeOH)[b]	1.92 (m, 1H, CH_2), 1.73 (m, 1H, CH_2), 0.88 (t, 3H, J=7.0 Hz, CH_3)	169.7 (s, COOH), 124.6 (q, J_{CF}=287 Hz, CF_3), 77.4 (q, J_{CCF}=27 Hz, quaternary), 25.6 (t, CH_2), 7.0 (q, CH_3)	Calcd (found) for $C_5H_7F_3O_3$ C 34.9 (35.2), H 4.1 (4.1)	107–111	85.2
10	53[c]	$Na_D=-5.4$ $c=0.6$ (water)	7.50 (s, br, 1H, $CONH_2$), 7.44 (s, br, 1H, $CONH_2$), 2.46 (s, br, 2H, NH_2), 1.35 (s, 3H, CH_3)	171.3 (s, $CONH_2$), 126.3 (sq, J_{CF}=285 Hz, CF_3), 60.2 (sq, quaternary C, J_{CCF}=26 Hz), 20.1 (qq, J_{CCCF}=2 Hz, CH_3)	Mass=156 Mass found=m/z 157 ($[M+1]^+$)	98–101[d]	>99[e]
11	42.0	$Na_D=+8.4$ c=0.8 (water)	5.60 (br, COOH, NH_2), 1.35 (s, 3H, CH_3)	170.4 (s, COOH), 126.9 (sq, J_{CCF}=284 Hz, CF_3), 60.1 (sq, quaternary C, J_{CCF}=25 Hz), 21.0 (q, CH_3)	Mass=157 Mass found=m/z 158 ($[M+1]^+$)		95.4[e]

[a] The theoretical maximum yield is 50%.
[b] Literature[10] (60% ee) $[\alpha]_D=+7.3°$ (c=5.33, MeOH).
[c] A yield of 53% is not theoretically possible. The higher figure is probably due to experimental error (trace moisture in the amino acid).
[d] This value was obtained from a separate larger biotransformation containing cell extracts. The sample passed elemental analysis, but the GC shows a trace impurity that may be (*S*)-**1**. $[\alpha]_D$ (MeOH, c=1.01) of this sample was −13.2 (23 °C). Anal. Calcd for $C_4H_7F_3N_2O$: C 30.8, H 4.5, N 17.9. Found C 31.1, H 4.7, N 17.7.
[e] Biotransformation with cell-free extract.

It follows, therefore, that the acids **9** and **11** that we isolated have the (*R*) configuration, and the amides **8** and **10** the (*S*) configuration.

Isolation of the product **11** from the reaction mixture was initially a problem. Although **10** could be easily extracted into ethyl acetate under basic conditions, **11** could not be isolated due to the presence of many buffer salts, free proteins and other cellular components. Extraction of amino acids such as **11** also requires precise knowledge of their iso-electric point in the mixture, and this point could not be determined. These problems were solved by using a reaction that contained only the substrate **7**, immobilised enzyme and water. The enzyme could easily be removed by filtration from the reaction after it was complete, **10** was extracted into ethyl acetate and **11** remained essentially alone in the water to be collected by evaporation of the water.

4. Conclusion

The amidase shows a marked preference for hydrolysis of only the (*R*) configured amide group, but it will accept only a limited range of substrates. However, the substrates that it did accept resulted in products that were unknown when this work was carried out (**8**, **9** and **10**) or have considerable synthetic interest (**11**). The biotransformation with the amidase therefore serves as a new synthetic route to these compounds.[11] Since this work was carried out, **9** has also been synthesised by enzymatic ester resolution,[10] and we were able to assign the configuration of the **9** that we synthesised based on the results in that paper.

5. Experimental

5.1. Analytical methods—general

The methods for NMR (Varian UNITY-400), chiral GC of the substrates to monitor the reactions, ammonia determination to monitor the reactions and chiral GC of the acid products have already been described.[3] For ^{1}H NMR the frequency was 400, 376 MHz for ^{19}F NMR and 100 MHz for ^{13}C NMR. For the ^{1}H and ^{13}C NMR the values reported are in ppm downfield from a reference of TMS, for ^{19}F NMR the values are reported upfield of the reference compound ($CFCl_3$). FTIR (Nicolet 20 SXB) was determined on KBr pellets. Only a sample of the wavenumbers of the major strong peaks is presented as a reference. Optical rotations were measured with a Perkin–Elmer PE 241 Polarimeter and LC–MS was carried out with a Finnigan LCQ Deca. Except where otherwise reported, MS refers to GC–MS.

5.2. Synthesis of racemic substrates for amide biotransformation

5.2.1. 3,3,3-Trifluoro-2-hydroxy-2-methylpropionamide 1. The title compound was synthesised from ethyl 4,4,4,-trifluoroacetoacetate by standard chemical methods[7,12] in three steps with an overall yield of 59.4%. Appearance: white powder. ^{1}H NMR (DMSO): 7.55 ppm (br s, 1H, NH_2); 7.53 ppm (br s, 1H, NH_2); 6.75 ppm (s, 1H, OH); 1.43 ppm (s, 3H, CH_3). ^{13}C NMR (DMSO): 170.7 ppm (s, $CONH_2$); 124.9 ppm (q, CF_3, J_{CF}=286.9 Hz); 74.1 ppm (q, quaternary C, J_{CCF}=27.5 Hz); 19.8 ppm (q, CH_3, J_{CCCF}=1.6 Hz). ^{19}F NMR (DMSO): −77.9 ppm (s, CF_3). Mp 143.6–145.0 °C (3× recrys. from AcOEt/Hex). IR: 3452 (s, OH); 3327 (s); 3271 (s); 1695 (sh); 1680 (s, $CONH_2$). Anal. Calcd for $C_4H_6F_3NO_2$: C 30.6, H 3.9, N 8.9. Found C 30.5, H 3.7, N 8.6.

5.2.2. 2-Hydroxy-2-(trifluoromethyl)-butanamide 2. 2-Hydroxy-2-(trifluoromethyl)-butanenitrile (Fluorochem) 8 g was added slowly dropwise to conc. H_2SO_4 (15.3 g) the mixture was heated to 115 °C for 15 min, cooled to 8 °C and 21.8 g of water added. Diethyl ether (50 mL) was then added, and the organic phase was washed with water (25.0 mL), saturated aqueous $NaHCO_3$ (25.0 mL) and again with water (25.0 mL). The diethyl ether phase was dried over Na_2SO_4, filtered and evaporated. The resulting oil was treated with n-hexane, and the resulting crystals (4.27 g) were collected by filtration. Appearance: white powder. ^{1}H NMR ($CDCl_3$): 6.21 ppm (br s, 2H, NH_2); 4.09 ppm (s, OH); 2.01–1.91 ppm (m, 2H,CH_2); 0.98 ppm (t, 3H, J=7.1 Hz, CH_3). ^{13}C NMR ($CDCl_3$): 169.7 ppm (s, $CONH_2$); 124.2 ppm (q, CF_3, J_{CF}=284.9 Hz); 77.6 ppm (q, quaternary C, J_{CCF}=27.9 Hz); 25.8 ppm (t), 6.5 (q) (CH_2CH_3). ^{19}F NMR ($CDCl_3$): −79.1 ppm (s, CF_3). Mp 75.8–78.0 °C (cryst. from toluene). IR: 3472 (s, OH); 3286 (s); 1695 (s, $CONH_2$); 1593 (s). MS: *m/z* (rel. intensity) 172 (M+1, 1), 143 (10), 142 (17), 127 (4), 109 (6), 108 (100), 93 (20), 79 (6), 69 (7), 67 (6), 57 (30), 45 (18), 44 (57).

5.2.3. 2-Hydroxy-2-(trifluoromethyl)-pentanamide 3. 1,1,1-Trifluoropent-2-one was synthesised[13] and converted to the corresponding cyanohydrin in a similar way to that previously described.[4] The cyanohydrin was then converted to the amide (7.80 g, yield 42.7%) by reaction with H_2SO_4 similarly to the substrate above. Appearance: white powder. ^{1}H NMR ($CDCl_3$): 6.29 ppm (br s, 1H, NH_2); 6.24 ppm (br s, 1H, NH_2); 4.15 ppm (s, 1H, OH); 1.95–1.82 ppm (m, 2H); 1.61–1.48 ppm (m, 1H); 1.34–1.21 (m, 1H); 0.97 (t, 3H, J=7.4, CH_3). ^{13}C NMR (DMSO): 170.0 ppm (s, $CONH_2$); 124.1 ppm (q, CF_3, J_{CF}=285.7 Hz); 77.3 ppm (q, quaternary C, J_{CCF}=28.6 Hz); 34.7 ppm (t), 15.7 (t), 13.9 (q). ^{19}F NMR ($CDCl_3$): −79.2 ppm (s, CF_3). Mp 77.0–79.0 °C. IR: 3512 (s, OH); 3486 (s); 3400 (s); 3381 (s); 3253 (s); 1706 (s, $CONH_2$); 1687 (s); 1667 (s). MS: *m/z* (rel. intensity) 186 (M+1, 2), 148 (6), 143 (M+1−C_3H_7 or $CONH_2$, 26), 122 (64), 103 (7), 94 (56), 71 (24), 59 (11), 44 (100).

5.2.4. 3,3,3-Trifluoro-2-hydroxy-2-phenyl-propionamide 4. The title compound was prepared as described previously[14] and converted to the amide (7.89 g) by reaction with H_2SO_4 similarly to the substrate above. Appearance: pale orange powder. ^{1}H NMR (DMSO): 7.74–7.37 ppm (m, 7H, arom CH and NH_2); 3.35 ppm (s, 1H, OH). ^{13}C NMR (DMSO): 169.5 ppm (s, $CONH_2$); 124.1 ppm (q, CF_3, J_{CF}=286.5 Hz); 77.5 ppm (q, quaternary C, J_{CCF}=27.0 Hz); 135.1 ppm (s), 128.7 (d), 128.0 (d), 126.3 (d). ^{19}F NMR (DMSO): −73.4 ppm (s, CF_3). Mp 96.4–97.1 °C. IR: 3506 (s); 3390 (s); 3199 (s); 1700 (s); 1568 (s). Anal. Calcd for $C_9H_8F_3NO_2$ C 49.3, H 3.7, N 6.4. Found C 49.5, H 3.9, N 6.3.

5.2.5. 3,3,3-Trichloro-2-hydroxy-2-methylpropionamide 5. The title compound was synthesised from 1,1,1-trichloroacetone via the cyanohydrin (69% yield) and by reaction with H_2SO_4 (66% yield) similarly to the substrates above. Appearance: white powder. ^{1}H NMR (DMSO): 7.48 ppm (br d, 2H, $CONH_2$); 1.64 ppm (s, CH_3). ^{13}C NMR (DMSO): 170.8 ppm (s, $CONH_2$); 105.3 ppm (s, CCl_3); 82.5 ppm (s, quaternary C); 22.0 ppm (q, CH_3). Mp 172.5.0–175.2 °C (from toluene/hexane, lit.[15] 174–176 °C). IR: 3490 (s, OH); 3378 (s); 3196 (s); 3381 (s); 1689 (s, $CONH_2$); 1572 (s). MS *m/z* (rel. intensity): 208, 206 (M+1 Cl-isotopes, 0.6 and 0.4) 179 (2), 165 (6), 163 (16), 161 (17), 128 (62), 126 (100), 91 (13), 88 (20), 62 (15), 43 (73). Anal. Calcd for $C_4H_6Cl_3NO_2$ C 23.3, H 2.9, N 6.8. Found C 23.0, H 3.1, N 7.1.

5.2.6. 3,3,3-Trifluoro-2-methoxy-2-methylpropionamide 6. The title compound was synthesised by methylation of 3,3,3-trifluoro-2-hydroxy-2-methylpropionamide with dimethylsulfate resulting in a 38% yield of the methyl ether. Appearance: white powder. ^{1}H NMR (DMSO): 7.65 ppm (br s, 2H, $CONH_2$); 3.35 ppm (s, 3H,=OCH_3); 1.50 ppm (s, 3H, CH_3). ^{13}C NMR (DMSO): 168.5 ppm (s, $CONH_2$); 124.6 ppm (q, CF_3, J_{CF}=286.5 Hz); 80.0 ppm (q, quaternary C, J_{CCF}=27.0 Hz); 14.8 ppm (q, CH_3). Mp 82.3–86.8 °C (from Tol/Hex).

5.2.7. 3,3,3-Trifluoro-2-amino-2-methylpropanamide 7. The title compound was synthesised via low yielding (3% overall yield), but reliable, literature method[16] from trifluoroacetone. Appearance: white powder. ^{1}H NMR (DMSO): 7.50 ppm (br s, 1H, $CONH_2$); 7.43 ppm (br s, 1H $CONH_2$); 2.43 ppm (s, 2H, NH_2); 1.35 ppm (s, 3H, CH_3). ^{13}C NMR (DMSO): 171.3 ppm (s, $CONH_2$); 126.3 ppm (q, CF_3, J_{CF}=285.7 Hz); 60.2 ppm (q, quaternary C, J_{CCF}=26.3 Hz); 20.1 ppm (q, CH_3, J_{CCCF}=2.0 Hz). ^{19}F NMR (DMSO): −76.6 ppm (s, CF_3). Mp 81.3–84.8 °C (sublimation followed by EtOAc recrys, lit.[16] 84–85 °C). IR: 3452 (s, OH); 3327 (s); 3271 (s); 1695 (sh); 1680 (s, $CONH_2$). MS: *m/z* (rel. intensity) 157 (M+1, 1), 156 (M+, 1), 113 (4), 112 (100, M+−$CONH_2$), 94 (3), 93 (5), 92 (18), 69 (2), 62 (5), 42 (31).

5.3. Biotransformations

Biotransformations to test the substrate specificity of the enzyme were carried out with a cell-free extract from cells of *E. coli* XL-1 Blue MRF/pPRS7. Cells were cultivated in Nutrient Yeast Broth with ampicillin (100 μg/mL) to an OD_{650} of 3.6. They were then washed in 100 mM phosphate buffer, pH 8.0, and resuspended in the same buffer to an OD_{650} of 190. The cells were then broken open by 3 passes through a French press, after which the cell extract was heated to 75 °C for 5 min, and cell debris and precipitated protein removed by centrifugation at 20,000 *g*. The clear supernatant had a protein concentration of 9.75 mg/mL and was used in the biotransformations. To test the various substrates the conditions were: racemic substrate, 5.0 mg/mL; enzyme extract, 0.2 mg/mL; 100 mM potassium phosphate, pH 8.0; temperature, 40 °C. The reactions were followed by GC analysis and ammonia determination.[3] The same GC method was used for all the substrates. For substrates **4** and **5**, the enantiomers were not separated by the GC method, but it was still possible to determine if there was conversion of substrate by monitoring the size of the amide peak or by ammonia determination.

Biotransformations with **2** and **7** as substrates to produce product for isolation were carried out as follows. For substrate **2** cells of *E. coli* XL-1 Blue MRF/pPRS7 were cultivated in Nutrient Yeast Broth with ampicillin (100 μg/mL) to an OD_{650} of 4.7. They were then washed in 100 mM phosphate buffer, pH 8.0, and re-suspended in the same buffer to an OD_{650} of 140. The biotransformation conditions were: racemic substrate, 5.0 mg/mL; cells, OD_{650}=10; 100 mM potassium phosphate, pH 8.0; temperature, 37 °C; volume 1.0 L; stirring at 120 rpm. For substrate **7** the biotransformation conditions were: racemic substrate, 5.0 mg/mL; amidase from *E. coli* XL-1 Blue MRF/pPRS7, immobilised on Eupergit C, 3.0 g (118 mg protein extract immobilised/g Eupergit C); solvent, water; temperature, 20 °C; volume 200 mL; stirring at 120 rpm. For both substrates **2** and **7** the larger scale biotransformations were monitored by chiral GC analysis of the substrates, and the reactions were stopped when all of one of the two amide enantiomers had been converted to product.

5.3.1. (*S*)-(−)-2-Hydroxy-2-(trifluoromethyl)-butanamide 8 and (*R*)-(+)-2-hydroxy-2-(trifluoromethyl)-butanoic acid 9. Solution from the biotransformation (902 mL) was adjusted to pH 10.0 with NaOH, and then extracted 3 times with ethyl acetate (600 mL). The pH of the aqueous phase was re-adjusted to 10.0 between each extraction. The three portions of ethyl acetate were combined, dried over Na_2SO_4, and the ethyl acetate removed under reduced pressure at 40 °C. The resulting orange oil was dissolved in hexane and placed at −18 °C overnight. The product suspension was filtered, washed with cold hexane and dried. The product was then recrystallised from hot toluene and dried to give 1.72 g of **8** as an off-white solid.

The aqueous phase was adjusted to pH 1.0 with HCl and extracted 2 times with ethyl acetate. The combined ethyl acetate fractions were dried over Na_2SO_4, and the ethyl acetate removed under reduced pressure at 40 °C. Toluene (15 mL) was added to the residue and the mixture dried to give 2.18 g of brown solid. The product was then twice recrystallised from hot toluene and dried to give 1.97 g of **9** as an off-white solid (mp 104–112 °C). For characterisations of **8** and **9** see Table 2. Additional FT-IR data: compound **8**: 3471 (s, OH), 3295 (br s), 1696 (s, $CONH_2$), 1593 (s), 1277 (s), 1166 (s); compound **9**: 3422 (s, OH), 3035 (br s), 1750.4 (s, COOH), 1320 (s), 1268 (s), 1229 (s), 1200 (s), 1185 (s), 1161 (s).

5.3.2. (*S*)-(−)-3,3,3-Trifluoro-2-amino-2-methylpropanamide 10 and (*R*)-(+)-3,3,3-trifluoro-2-amino-2-methylpropanoic acid (3,3,3-trifluoro-2-methyl-alanine) 11. Removing the immobilised enzyme by filtration stopped the biotransformation. The aqueous reaction mixture was extracted 3 times with ethyl acetate. The combined ethyl acetate fractions were evaporated to give 0.53 g of **10** as a white powder. The aqueous phase was also evaporated to give 0.42 g of **11**, also as a white powder.

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