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Enzymatic resolution of C$^{\alpha}$-fluoroalkyl substituted amino acids

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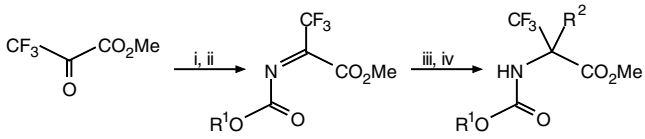
Abstract—A methodology for the enzymatic resolution of sterically constrained C$^{\alpha}$-fluoroalkyl substituted amino acids has been developed. Racemic *H*-(αTfm)Ala-NH_2, *H*-(αCF_2Cl)Ala-NH_2 and *H*-(αCF_2Br)Ala-NH_2 was separated with very high enantioselectivity (E >200) into their enantiomers using amidase from *Mycobacterium neoaurum*, yielding the corresponding (*R*)-acids. Furthermore, the first example of an enzymatic resolution of a C$^{\alpha}$-fluoroalkyl substituted Phe derivative has been established using amidase from *Ochrobactrum anthropi*.

1. Introduction

The incorporation of fluoride can have dramatic effects on peptide stability, protein–protein interactions and the physical properties of protein-based materials.[1] β-Fluorinated amino acids have gained prominence as mechanism-based inhibitors of amino acid decarboxylases and transaminases.[2] In particular, C$^{\alpha}$-fluoroalkyl substituted amino acids bearing a fluorinated substituent instead of the α-proton are known to be able to increase metabolic stability[3] as well as stabilize a peptide secondary structure.[4] The high lipophilicity of fluoroalkyl substituents has a positive effect on transport properties and in vivo absorption of peptides. Due to the high electron density, peptides containing a fluorinated alkyl substituent are capable of interacting with enzyme or receptor subsites in a manner, which is impossible for the fluorine-free pendants.[3] Furthermore, the ^{19}F atom as well as the C–F bond serve as a highly specific and powerful label for spectroscopic investigations of pathways, metabolisms and structure–activity relationships using NMR or Raman spectroscopy, respectively.[5] As a result, C$^{\alpha}$-fluoroalkyl substituted amino acids are valuable building blocks for the design of biologically active peptides.

Several routes towards racemic C$^{\alpha}$-fluoroalkyl substituted amino acids have been developed. Domino reactions starting from 5-fluoro-4-trifluoromethyloxazoles, including sequences such as nucleophilic substitution/Claisen rearrangement and nucleophilic substitution/benzyl group migration provide ready access to racemic C$^{\alpha}$-trifluoromethyl (αTfm) amino acids with side-chains of high structural diversity.[6] The most general approach is the amidoalkylation of carbon nucleophiles with alkyl 2-(alkoxycarbonylimino)-3,3,3-trifluoropropionates[7] (Scheme 1).



Scheme 1. Reagents and conditions (i) R^1OCONH_2; (ii) $(CF_3CO)_2O$/pyridine; (iii) R^2MgX; (iv) H_3O^+.

Considering the divergent biological activities of the enantiomers of C$^{\alpha}$-fluoroalkyl substituted amino acids and their diastereomeric peptide derivatives, respectively, the availability of these compounds in their enantiomerically pure form is highly desirable. Most synthetic routes to enantiomerically pure C$^{\alpha}$-fluoroalkyl substituted amino acids rely on chemical[8,9] and enzymatic resolution.[10] A promising strategy for the diastereoselective synthesis of αTfm amino acids proceeds via amidoalkylation of carbon nucleophiles with in situ formed homochiral cyclic acyl imines. The dioxopiperazines (DOP) obtained with good stereoselectivity can be transformed into homochiral dipeptide esters by

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regioselective acidolysis in methanol.[11] However, the majority of fluorinated $C^{\alpha,\alpha}$-dialkylated amino acids is prepared chemically followed by the enzymatic resolution of the enantiomers. The separation of the enantiomers of *H*-(αTfm)Ala by partial hydrolysis of the racemic *N*-trifluoroacetyl derivative with hog kidney aminoacylase has been reported.[8] Proteases like subtilisin, α-chymotrypsin or papain accept C^{α}-fluoroalkyl substituted amino acid esters as substrates but only to a very limited extent.[12] Therefore, the application of these proteases for the resolution of enantiomeric C^{α}-fluoroalkyl substituted amino acid derivatives is excluded with the exception of *Z*-(αTfm)Gly-OMe.[13]

At DSM Research, several methods for the preparation of enantiomerically pure $C^{\alpha,\alpha}$-dialkylated amino acids via the enzymatic resolution of racemic amino acid amides have been developed. Amidases from *Mycobacterium neoaurum* (ATCC 25795) and *Ochrobactrum anthropi* (NCIMB 40321), both exhibiting a high L-stereoselectivity, have been applied for the large-scale preparation of many different optically active $C^{\alpha,\alpha}$-dialkylated amino acids.[14] Amidase from *M. neoaurum* (ATCC 25795) shows a high activity and enantioselectivity not only for α-*H*-amino acids but also for C^{α}-methyl substituted molecules. Although this amidase has proven to be quite efficient in the synthesis of various $C^{\alpha,\alpha}$-disubstituted amino acids containing several other alkyl substituents at the C^{α}-position, the system is not ideal for too sterically demanding groups; for instance, the attachment of an aromatic substituent to the stereogenic centre restricts enzymatic activity. In contrast, the substrate specificity of *O. anthropi* (NCIMB 40321) is remarkably broad and covers C^{α}-*H*-amino acids, $C^{\alpha,\alpha}$-dialkyl amino acids, C^{α}-hydroxy acid amides and *N*-hydroxy amino acid amides. The amidase also resolves a broad range of highly sterically demanding $C^{\alpha,\alpha}$-disubstituted amino acid amides such as α-phenyl-α-alkyl disubstituted amino acids while the enantioselectivity decreases in the cases of smaller substituents.

As part of a program aimed at the introduction of the potential of fluorinated amino acids into peptide and protein modification, we studied the applicability of the above mentioned amidases for the resolution of a variety of C^{α}-fluoroalkyl substituted amino acids. Although both of these enzymes have already been successfully applied for the resolution of many different $C^{\alpha,\alpha}$-dialkyl amino acids, nothing is known about the influence of the electronically modified substituents on individual enzyme–substrate interactions and, thus, the catalytic efficiency and enantioselectivity of the amidases. These effects are reported herein.

2. Results and discussion

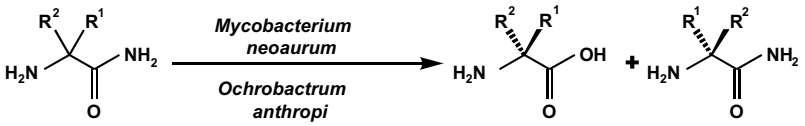
The results of the resolution experiments are summarized in Table 1.

2.1. Enzymatic resolution of (*RS*)-*H*-(αTfm)Ala-NH_2

At first, a small-scale resolution experiment was performed using amidase from *M. neoaurum* (with 1.47 mmol of substrate as 5 wt% solution in water at pH = 9 using 0.13 g amidase/g substrate) at 25 °C. After 5 h, the amide conversion was 46% and after 27 h only slightly higher (47%) with the ee values of the amide and acid being 98% and 99%, respectively. Evidently, the resolution is fast and almost completely stereospecific (E >200).

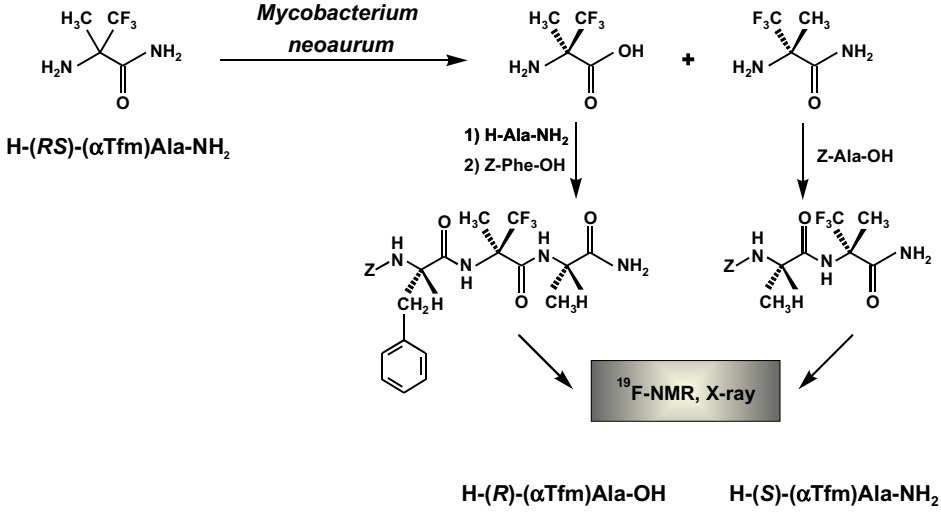
Secondly, the reaction was carried out on a preparative scale with both the amino acid and the unconverted amide being isolated. For more proof of the enantioselectivity of the amidase reaction, the amino acid was coupled to *H*-Ala-NH_2 via standard chemical peptide synthesis and the amino acid amide likewise coupled to *Z*-Ala-OH (see Scheme 2). The resulting dipeptide products were shown to be diastereoisomerically pure by ^{19}F NMR as well as ^{1}H NMR analysis. Furthermore, we determined the absolute configuration of the converted amide. *M. neoaurum* usually expresses a high enantioselectivity for the L-form of a racemic mixture. Therefore, *H*-(αTfm)Ala-Ala-NH_2 was coupled to *Z*-Phe-OH

Table 1. Results of the enzymatic resolutions of racemic C^{α}-fluoroalkyl amino acid amides



	R^1	R^2	Hydrolytic selectivity	Assignment	Enzyme	Conversion (%)	Amide ee (%)	Amino acid ee (%)	E value[a]
1	CF_3 (Tfm)	CH_3	*R*	Unambiguous	*M. neoaurum*	47	98	96	>200
2	CF_2Cl	CH_3	*R*	By analogy	*M. neoaurum*	48	99.5	94.7	>200
3	CF_2Br	CH_3	*R*	By analogy	*M. neoaurum*	49	99.5	94.8	>200
4	CH_2Ph	CF_3 (Tfm)	—	—	*O. anthropi*	No hydrolysis	—	—	—
5	CH_2Ph	CF_2Cl	—	—	*O. anthropi*	No hydrolysis	—	—	—
6	CH_2Ph	CF_2H (Dfm)	*S*	Tentative	*O. anthropi*	58	98.7	67.9	25

[a] E values were calculated on the basis of the experimentally determined ee values of the amide and the acid.



Scheme 2. Proof of the absolute configuration of the hydrolyzed enantiomer obtained by the resolution of racemic *H*-(αTfm)Ala-NH_2 by *M. neoaurum*.

to obtain the tripeptide *Z*-Phe-(αTfm)Ala-Ala-NH_2, which was analyzed with ^{19}F NMR (Scheme 2). The absolute configuration of (αTfm)Ala within this tripeptide sequence was assigned on the basis of an earlier published X-ray structural analysis of this peptide by comparison with the ^{19}F NMR spectra.[15]

This comparison showed that the amidase of *M. neoaurum* (ATCC 25795) hydrolyzes (*R*)-*H*-(αTfm)Ala-NH_2, which corresponds to D-Ala if comparing the positions of the methyl groups. Apparently, the Tfm group as the larger one of the two substituents at the C^{α}-atom, fits into the binding pocket for the methyl group of L-Ala.

2.2. Enzymatic resolution of (*RS*)-*H*-(αCF_2Cl)Ala-NH_2 and (*RS*)-*H*-(αCF_2Br)Ala-NH_2

After incubation of these racemates with *M. neoaurum* at 37 °C for 21 h the conversion of the substrates had reached 48% and 49%, respectively. The growing size of the substituents at the halogenated group did not seem to influence the enantioselectivity of the enzyme ($E > 200$), although the reaction rate with the αCF_2Cl and αCF_2Br substrates was significantly lower than with the αTfm congener. Analogously to the result with (*RS*)-*H*-(αTfm)Ala-NH_2, it is assumed that the amidase of *M. neoaurum* (ATCC 25795) also hydrolyzes the (*R*)-isomers of *H*-(αCF_2Cl)Ala-NH_2 and *H*-(αCF_2Br)Ala-NH_2.

2.3. Enzymatic resolution of (*RS*)-*H*-(αTfm)Phe-NH_2 and (*RS*)-*H*-(αCF_2Cl)Phe-NH_2

The C^{α}-trifluoromethyl substituted Phe derivative was incubated with both *M. neoaurum* at 25 °C and *O. anthropi* at 37 °C, but (unlike earlier published results of a successful enantioselective hydrolysis of *H*-(αMe)Phe-NH_2[14] with *O. anthropi*) no conversion could be detected. Similarly, when the CF_2Cl-substituted analogue was reacted with the amidase from *O. anthropi*, no significant hydrolysis was observed. These results indicate that the steric constraint exhibited by the αTfm or αCF_2Cl group combined with the presence of an α-benzyl group is too high even for *O. anthropi* amidase.

2.4. Enzymatic resolution of (*RS*)-*H*-(αDfm)Phe-NH_2

The racemic C^{α}-difluoromethyl (Dfm) substituted Phe derivative was incubated with the amidase from *O. anthropi* at 37 °C. Surprisingly, after 21 h, the reaction reached 58% conversion with the ee values of the remaining amide and formed acid being 98.7% and 67.9%, respectively. Interestingly, the replacement of the αTfm by the αDfm group changed the steric situation at the C^{α}-atom to such an extent that the substrate can be accepted by the enzyme. The very high acidity of the proton in the CF_2H-group is another factor that has to be taken into account. While a CF_3-group can act as an electron pair donor and, therefore, as a hydrogen bond acceptor,[16] the CF_2H-group can be both a hydrogen bond donor and acceptor at the same time. This feature may be of importance for possible interactions between the substrate and active site of the enzyme and needs to be investigated further. In any case, this is the first example of an enzymatic resolution of a C^{α}-fluoroalkyl substituted Phe derivative. The enantioselectivity of this resolution ($E = 25$) was considerably lower when compared to the resolution of the fluorinated Ala derivatives with *M. neoaurum*, but high enough to obtain the amide in high enantiomeric excess (>99% ee). Moreover, it is likely that the *E* value can be significantly increased by decreasing the temperature (in combination with using more amidase and decreasing the pH to dissolve more of the substrate), so that the acid congener can also be

obtained in high enantiomeric excess. It should be noted that since also with *O. anthropi*, the hydrolyzed enantiomer has the more bulky substituent always at the R^1 position (see Table 1), we assume that in this case the (*S*)-enantiomer of *H*-(αDfm)Phe-NH_2 is hydrolyzed, although this assignment is tentative.

3. Conclusion

Several racemic C^α-fluoroalkyl amino acids have been synthesized from fluorinated pyruvates. Due to increasing interest in fluoroalkyl amino acids for peptide and protein modification, availability of these building blocks in their enantiomerically pure forms is highly desirable. Therefore, a method for the enzymatic resolution of racemic C^α-fluoroalkyl amino acid amides has been developed. Amidase from *M. neoaurum* (ATCC 25795) hydrolyzes (*RS*)-*H*-(αTfm)Ala-NH_2, (*RS*)-*H*-(αCF_2Cl)Ala-NH_2 and (*RS*)-*H*-(αCF_2Br)Ala-NH_2 with high enantioselectivity ($E > 200$) to give the pure (*R*)-amino acids. *O. anthropi* (NCIMB 40321) accepted (*RS*)-*H*-(αDfm)Phe-NH_2 as the substrate and allowed, for the first time, the successful enantioselective enzymatic hydrolysis of a C^α-fluoroalkyl substituted Phe derivative.

The technology described herein can now be applied for the preparation of a variety of enantiopure C^α-fluoroalkyl substituted amino acids. Currently we are exploring further the scope and limitations and incorporating the resulting enantiopure C^α-fluoroalkyl substituted derivatives into peptides with a view for application in structural analysis and medicinal chemistry.

4. Experimental

4.1. Synthesis of the racemic C^α-fluoroalkyl amino acid amides

The racemic C^α-fluoroalkyl amino acids (*RS*)-*Z*-(αTfm)Ala-OH, (*RS*)-*Z*-(αCF_2Cl)Ala-OH, (*RS*)-*Z*-(αCF_2Br)Ala-OH, (*RS*)-*Z*-(αTfm)Phe-OH, (*RS*)-*Z*-(αCF_2Cl)Phe-OH and (*RS*)-*Z*-(αDfm)Phe-OH and their unprotected amino acid counterparts were obtained via known procedures.[2,7] The six racemic unprotected amino acids (*RS*)-*H*-(αTfm)Ala-OH, (*RS*)-*H*-(αCF_2Cl)Ala-OH, (*RS*)-*H*-(αCF_2Br)Ala-OH, (*RS*)-*H*-(αTfm)Phe-OH, (*RS*)-*H*-(αCF_2Cl)Phe-OH and (*RS*)-*H*-(αDfm)Phe-OH, with the literature spectroscopic data[2,7] were used as reference compounds during the enzymatic resolution reactions. The racemic amides of the C^α-trifluoromethyl substituted derivatives were synthesized via reaction of the 1,3-oxazolidin-2,5-ones (αTfm)Ala-NCA and (αTfm)Phe-NCA[17] with ammonia at −40 °C in THF over night. The solvent was evaporated and the corresponding amides purified by chromatography on silica gel using MeOH/H_2O 1:1 (v/v) as eluent. The racemic amides of the C^α-difluoromethyl as well as chlorodifluoromethyl substituted derivatives (*RS*)-*Z*-(αCF_2Cl)Ala-OH, (*RS*)-*Z*-(αCF_2Cl)Phe-OH and (*RS*)-*Z*-(αDfm)Phe-OH were synthesized by the reaction of the N-*Z*-protected amino acids with DIC in CH_2Cl_2 followed by saturation with ammonia at −40 °C and reaction over night at 20 °C. The solvent was evaporated and the crude product purified by flash chromatography using ethyl acetate/petroleum ether/$CHCl_3$ 1:1:1 (v/v/v) as eluent. Deprotection of the amino function was easily realized by hydrogenation with Pd/C in methanol.[18] The racemic amide of the C^α-bromodifluoromethyl alanine was synthesized following the procedure described above for the C^α-difluoromethyl substituted amino acids but starting from the *N*-Boc-protected derivative due to the instability of the CF_2Br-group towards catalytic hydrogenation. Deprotection of the amino group could be readily carried out with TFA.[7b]

4.1.1. (*RS*)-*H*-(αTfm)Ala-NH_2. White solid. ^{1}H NMR (200 MHz, d_4-MeOH) δ: 1.48 (s, 3H).

4.1.2. (*RS*)-*H*-(αCF_2Cl)Ala-NH_2. White solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 1.56 (s, 3H), 1.81 (br s, 2H), 5.89 (br s, 1H), 7.07 (br s, 1H).

4.1.3. (*RS*)-*H*-(αCF_2Br)Ala-NH_2. White solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 1.56 (s, 3H), 1.89 (s, 2H), 5.80 (br s, 1H), 7.06 (br s, 1H).

4.1.4. (*RS*)-*H*-(αTfm)Phe-NH_2. White solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 1.67 (br s, 2H), 2.77–2.89 (m, 2H), 7.15–7.32 (m, 7H).

4.1.5. (*RS*)-*H*-(αCF_2Cl)Phe-NH_2. White solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 2.89 (d, $J = 13.6$ Hz, 1H), 3.58 (d, $J = 13.6$ Hz, 1H), 5.79 (s, 1H), 6.90 (s, 1H), 7.21–7.37 (m, 5H).

4.1.6. (*RS*)-*H*-(αDfm)Phe-NH_2. White solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ: 2.73 (d, $J = 13.6$ Hz, 1H), 3.23 (d, $J = 13.6$ Hz, 1H), 5.48 (br s, 1H), 6.92 (br s, 1H), 7.26–7.33 (m, 5H).

4.2. Enzymatic resolutions: materials and analysis

The amidases used for the enzymatic hydrolysis reactions were obtained from *M. neoaurum* (ATCC 25795) or *O. anthropi* (NCIMB 40321) as earlier described.[19] The progress of the amide hydrolysis reactions was estimated by electrochemical NH_3 determination. After reaching a conversion of approximately 50%, the reactions were stopped and the exact conversion and the ee values of the amide and acid were determined by HPLC analysis (with a chiral mobile phase) using a Gilson 302 pump (flow 1.0 mL/min), a Gilson 231 injector (injection volume 25 μL) and a UV detector (Spectra-Physics) operating at 280 nm. The HPLC column, consisting of

Nucleosil 120 C-18 (particle size 5 μm), had a size of 250 mm × 4.6 mm and was operated at 20 °C.

For the enzymatic resolution of (*RS*)-*H*-(αTfm)Ala-NH_2, (*RS*)-*H*-(αCF_2Cl)Ala-NH_2, (*RS*)-*H*-(αCF_2Br)Ala-NH_2 and (*RS*)-*H*-(αDfm)Phe-NH_2 the eluent consisted of triethyl amine (100 mg/L), copper(II) acetate·monohydrate (0.39 g/L) and *N*,*N*-di-*n*-propyl-L-alanine (0.68 g/L) in water adjusted to exactly pH = 5.15 with 1 M aq acetic acid solution. Under these conditions, both enantiomers of the amide and acid could be separated from each other, so that from one HPLC run both the conversion and the ee values of both the amide and the acid could be abstracted (Table 2).

4.3. Enzymatic resolution of (*RS*)-*H*-(αTfm)Ala-NH_2

4.3.1. Resolution on small scale. (*RS*)-*H*-(αTfm)Ala-NH_2 (0.23 g, 1.47 mmol) was dissolved in H_2O (4.7 mL). The amidase preparation (29 mg being 0.13 g/g substrate) from *M. neoaurum* (ATCC 25795) was added and the mixture shaken (190 rpm) at 25 °C with the pH ≈ 9.0 (not adjusted). NH_3 determination indicated that the hydrolysis reaction almost stopped at approximately 45%, conversion, reached after 5 h reaction time. Nevertheless, the reaction was continued and an additional sample was taken after 27 h. The samples of 5 and 27 h were analyzed by HPLC. After 5 h, the amide conversion appeared to be 46% with the ee values of the amide and acid being 97% and 96%, respectively. After 27 h, the amide conversion appeared to be 47% with the ee values of the amide and acid being 98% and 96%, respectively, corresponding to an *E* value of 226.

4.3.2. Resolution on preparative scale. (*RS*)-*H*-(αTfm)Ala-NH_2 (9.06 g, 58.1 mmol) was dissolved in H_2O (135 mL). The pH was adjusted to 8.6 with 5 M aq KOH solution. The amidase preparation (315 mg) from *M. neoaurum* (ATCC 25795) was added and the mixture shaken (190 rpm) at 25 °C. NH_3 determination indicated that after 23 h the conversion was approximately 50%. Subsequently, the enzyme was removed by centrifugation (20,000*g*, 20 min). The (*R*)-acid and the unconverted (*S*)-amide were separated by ion-exchange chromatography (DOWEX 1 × 8 Fluka, strongly alkaline, maximum load 1.5 equiv/g) and evaporation giving 3.7 g of (*S*)-*H*-(αTfm)Ala-NH_2 (41% yield based on racemate) with ee = 99% and 5.4 g of (*R*)-*H*-(αTfm)Ala-OH (59% yield based on racemate) with ee = 96.6%. Apparently, the actual conversion was approximately 59%.

4.3.3. (*R*)-*H*-(αTfm)Ala-NH_2. White solid. Ee = 99% (by HPLC). ^{1}H NMR (200 MHz, $CDCl_3$) δ: 1.53 (s, 3H), 1.82 (s, 2H), 6.0-7.1 (br s, 2H). $[\alpha]_D^{22} = -23$ (*c* 2.1, 1 M HCl).

4.3.4. (*R*)-*H*-(αTfm)Ala-OH. Yellowish solid. Ee = 96% (by HPLC). ^{1}H NMR (200 MHz, D_2O) δ: 1.68 (s, 3H), 2.04 (s, 1H). $[\alpha]_D^{22} = +6.1$ (*c* 2.0, 1 M HCl).

4.4. Enzymatic resolution of (*RS*)-*H*-(αCF_2Cl)Ala-NH_2 and (*RS*)-*H*-(αCF_2Br)Ala-NH_2

(*RS*)-*H*-(αCF_2Cl)Ala-NH_2 (0.111 g, 0.65 mmol) was dissolved in H_2O (1.03 mL). The amidase preparation (10.9 mg, being 0.1 g/g substrate) from *M. neoaurum* (ATCC 25795) was then added and the mixture shaken (160 rpm) at 37 °C with the pH ≈ 8.5. NH_3 determination indicated that the conversion was approximately 40% after 21 h. The enzyme was removed by filtration and the reaction mixture analyzed by HPLC. The conversion appeared to be 48% with the ee values of the amide and acid being 99.5% and 94.7%, respectively, corresponding to an *E* value of 218.

(*RS*)-*H*-(αCF_2Br)Ala-NH_2 (0.564 g, 2.60 mmol) was dissolved in H_2O (4.54 mL). The amidase preparation (57.3 mg being 0.1 g/g substrate) from *M. neoaurum* (ATCC 25795) was then added and the mixture shaken (160 rpm) at 37 °C with the pH ~8.5. NH_3 determination indicated that the conversion was approximately 40% after 21 h. The enzyme was removed by filtration and the reaction mixture analyzed by HPLC. The conversion appeared to be 49% with the ee values of the amide and acid being 99.5% and 94.8%, respectively, corresponding to an *E* value of 223.

4.5. Enzymatic resolution of (*RS*)-(αTfm)Phe-NH_2 and (*RS*)-(αCF_2Cl)Phe-NH_2

(*RS*)-H-(αTfm)Phe-NH_2 (0.20 g) was (partially) dissolved in H_2O (2.0 mL) and the amidase preparation (20.3 mg, being 0.1 g/g substrate) from *M. neoaurum* (ATCC 25795) then added and the mixture shaken (160 rpm) at 25 °C with the pH ≈ 8.5. NH_3 determination showed no significant conversion after 21 h. The reaction was repeated with 20.1 mg amidase preparation from *O. anthropi* (NCIMB 40321) but likewise no significant conversion was observed.

Table 2. Retention times in chiral HPLC of the amide and the acid enantiomers of the amino acid amides undergoing enzymatic resolution

Acid compound	Retention time of enantiomers (min)	Amide compound	Retention time of enantiomers (min)
(*RS*)-*H*-(αTfm)Ala-OH	10.4 and 13.8[a]	(*RS*)-*H*-(αTfm)Ala-NH_2	6.7 and 7.4[b]
(*RS*)-*H*-(αCF_2Cl)Ala-OH	14.1 and 21.3[a]	(*RS*)-*H*-(αCF_2Cl)Ala-NH_2	10.7 and 11.9[b]
(*RS*)-*H*-(αCF_2Br)Ala-OH	14.1 and 21.1[a]	(*RS*)-*H*-(αCF_2Br)Ala-NH_2	10.7 and 11.9[b]
(*RS*)-*H*-(αDfm)Phe-OH	16.0[a] and 17.6	(*RS*)-*H*-(αDfm)Phe-NH_2	20.5[b] and 21.7

[a] This enantiomer of the acid was predominantly formed during the resolution reaction.
[b] This enantiomer of the amide was predominantly hydrolyzed during the resolution reaction.

(*RS*)-*H*-(αCF$_2$Cl)Phe-NH$_2$ (0.212 g) was (partially) dissolved in H$_2$O (2.79 mL). The amidase preparation (29 mg being 0.13 g/g substrate) from *O. anthropi* (NCIMB 40321) was then added and the mixture shaken (160 rpm) at 37 °C with the pH ≈ 8.5. NH$_3$ determination indicated a conversion of less than 1% after 21 h and approximately 4% after 68 h.

4.6. Enzymatic resolution of (*RS*)-*H*-(αCF$_2$H)Phe-NH$_2$

(*RS*)-*H*-(αCF$_2$H)Phe-NH$_2$ (0.124 g, 0.58 mmol) was (partially) dissolved in H$_2$O (1.24 mL). The amidase preparation (15.8 mg, being 0.13 g/g substrate) from *O. anthropi* (NCIMB 40321) was then added and the mixture shaken (160 rpm) at 37 °C with the pH ≈ 8.5. NH$_3$ determination indicated that the conversion was approximately 51% after 21 h. The enzyme was removed by filtration and the reaction mixture analyzed by HPLC. The conversion appeared to be 58% with the ee values of the amide and acid being 98.7% and 67.9%, respectively, corresponding to an *E* value of 25.

4.7. Peptide synthesis and proof of configuration

4.7.1. Synthesis of (*S*,*R*,*S*)-*Z*-Phe-(αTfm)Ala-Ala-NH$_2$. 1.0 mmol (157 mg) of the *H*-(αTfm)Ala-OH batch, which was obtained as a product of the preparative enzymatic resolution (see Section 4.3), was dissolved in 20 mL dry dichloromethane. 1.1 mmol DIC and 1.0 mmol (88 mg) *H*-Ala-NH$_2$ were added. The reaction mixture was stirred at room temperature for 12 h. After filtration of the precipitate, the mother liquor was evaporated in vacuo and the resulting product (mainly consisting of *H*-(αTfm)Ala-Ala-NH$_2$) used without further purification.

N-Methyl-morpholine (0.5 mmol) and isobutyl chloroformate (0.6 mmol) were added to a stirred solution of 0.5 mmol (150 mg) *Z*-Phe-OH in 20 mL absolute ethyl acetate at −15 °C. After 30 min 0.5 mmol (114 mg) *H*-(αTfm)Ala-Ala-NH$_2$ in 10 mL dry ethyl acetate were added. The reaction mixture was stirred at −15 °C for 1 h and at room temperature overnight. The reaction mixture was washed successively with H$_2$O, dilute citric acid, H$_2$O, satd NaHCO$_3$ and H$_2$O. The organic layer was dried over MgSO$_4$, evaporated in vacuo and purified by flash chromatography (ethyl acetate/*n*-hexane 4:1 (v/v); $R_f = 0.30$). Yield: 61%.

[M+H]: 509; ^{1}H NMR (360 MHz, d_4-methanol) δ: 1.32 (d, $J = 8.6$ Hz, 3H), 1.65 (s, 3H), 2.90 (dd, $J = 14.0$, 8.9 Hz, 1H), 3.10 (dd, $J = 14.0$, 6.1 Hz, 1H), 4.31 (m, 1H), 4.40 (m, 1H), 5.05 (s, 2H), 7.23–7.35 (m, 10H) ppm; ^{13}C NMR (360 MHz, d_4-methanol) δ: 17.69, 19.11, 38.15, 50.69, 58.01, 63.54 (q, $J = 27$ Hz), 67.75, 125.80 (q, $J = 285$ Hz), 127.84, 128.63, 129.00, 129.45, 129.51, 130.33, 137.94, 138.06, 158.66, 167.86, 174.45, 177.23 ppm; ^{19}F NMR (235 MHz, CDCl$_3$) δ: 0.85(s) ppm. This ^{19}F NMR shift corresponds to the (*S*,*R*,*S*)-diastereomer; the ^{19}F NMR shift of the (*S*,*S*,*S*)-diastereomer in CDCl$_3$ was determined earlier[3] to be 4.9 ppm.

4.7.2. Synthesis of (*S*,*S*)-*Z*-Ala-(αTfm)Ala-NH$_2$. *N*-Methyl-morpholine (1.0 mmol) and isobutyl chloroformate (1.2 mmol) were added to a stirred solution of 0.5 mmol (112 mg) *Z*-Ala-OH in 20 mL dry ethyl acetate at −15 °C. After 30 min a solution of 0.5 mmol (50 mg) of *H*-(αTfm)Ala-NH$_2$, which was recovered from the preparative enzymatic resolution experiment (see Section 4.3), in 20 mL dry ethyl acetate was added. The reaction mixture was stirred at −15 °C for 1 h and at room temperature over night. The reaction mixture was washed successively with H$_2$O, dilute citric acid, H$_2$O, satd NaHCO$_3$ and H$_2$O. The organic layer was dried over MgSO$_4$, evaporated in vacuo and purified by flash chromatography (ethyl acetate:*n*-hexane 3:1 (v/v); $R_f = 0.27$). Yield: 78%. ^{1}H NMR (360 MHz, CDCl$_3$) δ: 1.31 (d, 3H, $J = 7$ Hz), 1.46 (s, 3H), 4.21 (m, 1H), 4.38 (m, 1H), 7.21–7.29 (m, 5H), 5.93 (s, 1H), 7.42 (s, 1H) ppm; ^{13}C NMR (360 MHz, CDCl$_3$) δ: 16.90, 17.89, 62.53 (q, $J = 27$ Hz), 53.27, 67.44, 127.70 (q, $J = 286$ Hz), 127.32, 128.17, 128.55, 129.08, 135.69, 171.23, 172.51 ppm; ^{19}F NMR (235 MHz, CDCl$_3$) δ: −0.39(s) ppm; no signal for a second diastereomer was found.

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