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Enzymatic hydrolysis of methyl 3,3-difluoro-2-amino esters. Synthesis of D- and L-3,3-difluoro-2-amino acids and their derivatives

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Abstract

The hydrolysis of methyl D,L-3,3-difluorophenyl alanate (**1a**) and methyl D,L-3,3-difluoro-2-aminobutanoate (**1b**) and their *N*-acetyl derivatives **2a** and **2b** by subtilisin has been studied. All derivatives examined were enzymatically resolved to separable mixtures of the corresponding 3,3-difluoro-L-amino acids (**3a** and **3b**) or *N*-acetylamino acids (**5a** and **5b**) and the unchanged 3,3-difluoro D-amino esters (**4a** and **4b**) or *N*-acetylamino esters (**6a** and **6b**). Acidic hydrolysis of methyl 3,3-difluoro-D-phenyl alanate (**4a**) or its *N*-acetyl derivative **6a** led to 3,3-difluoro-D-phenyl alanime (**8a**). In the same manner, L- and D-2-amino-3,3-difluorobutanoic acids **7b** and **8b** were prepared starting from **5b** and **6b**. Optical purity and enantiomeric excess were determined by GC analysis of the *N*-acetyl esters and by NMR analysis determined in the presence of $Eu(tfc)_3$. By these methods, unchanged methyl 3,3-difluoro-D-amino ester derivatives showed an *ee* of $\geq 90\%$ while L-amino acids were estimated to have $\geq 95\%$ *ee*.

Keywords: Enzymatic hydrolysis; Methyl difluoroamino esters; Difluoroamino acids; NMR spectroscopy; Subtilisin Carlsburg; Optical activity

1. Introduction

A number of synthetic biologically active peptides, including antibiotics, vaccines, enkephalines and other hormones, containing D-amino acid residues have been prepared and widely studied [1–5]. Fluorinated amino acids are important targets in the search for new drugs by application of the principle of isogeometric modification of metabolites with maximal shift of electron distribution in the design of anti-metabolites and drugs [6–14].

In our search for fluorinated amino acid derivatives with biological and pharmacological properties, we were interested in the preparation of enkephalines incorporating D- or L-3,3-difluoro-2-amino acids in their structures. The lack of chemical methods of synthesizing D- and L-3,3-difluoro-2-amino acids led us to examine the reactivity with enzymes of the now easily accessible 3,3-difluoro-2-amino acid alkyl esters. The potential of enzymes, namely proteases, as catalysts in synthetic organic chemistry has received much attention in recent years [15–17]. Because they can simultaneously display high chemical and stereochemical selectivity, their use may be suitable in the resolution of enantiomers by enantioselective hydrolysis.

Subtilisin Carlsberg is an enzyme which hydrolyses proteins and amino acid esters as well as their *N*-acetylated derivatives [18]. It is stable and active at room temperature or higher (e.g. 60 °C) and in pH range 6–12. Wang et al have found that, at pH 6–8, it resolved racemic amino acid esters to give *L*-amino acids and D-amino acid esters with high enantiomeric excess (*ee*). They also demonstrated that adding to the buffer solution of up to 30% (v/v) of several organic solvents including acetone, dioxan, acetonitrile or dimethyl sulphoxide in order to increase the solubility of the substrates did not alter the enzyme activity [19]. We have found that subtilisin Carlsberg catalyzes the stereoselective hydrolysis of the methyl ester of 3,3-difluorophenyl alanine and 3,3-difluoro-2-aminobutyric acids as well as their *N*-acetyl derivatives with good or satisfactory enantiomeric excess.

2. Results and discussion

Choosing racemic 3,3-difluorophenyl alanine methyl ester (**1a**), obtained by the ring-opening reaction of 2-carboxy-

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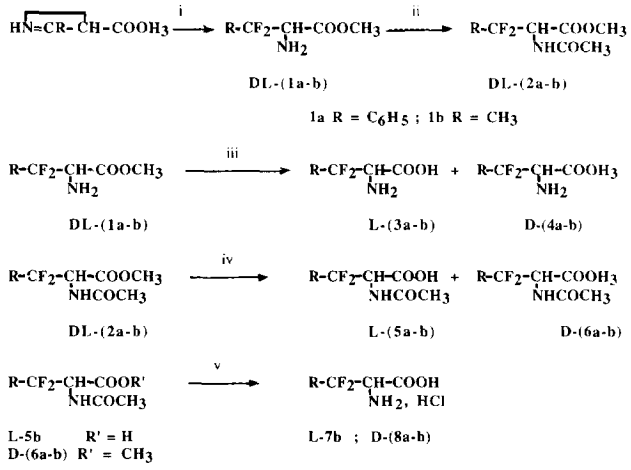

i: CH_2Cl_2/0 °C, HF/pyridine (70:30)
ii: $(CH_3CO)_2O$/pyridine/0 °C
iii: $NaHCO_3$ 0.2 M/subtilisin Carlsberg/20 °C
iv: H_2O/DMSO (80:20 v/v) subtilisin carlsberg/20 °C
v: H_2O/HCl 6 N/reflux 2.5–6 h

Scheme 1.

methyl-1-phenyl-1-azirine with hydrogen fluoride pyridine (70% w/w), as a model compound, a survey of the reactivity of subtilisin Carlsberg showed that it preferentially hydrolyzed the L-enantiomer with very good selectivity (Scheme 1). Likewise, D,L-*N*-acetyl-3,3-difluorophenyl alanine methyl ester (**2a**) was found to be a specific substrate for subtilisin capable of catalyzing its hydrolysis.

We have qualitatively investigated the influence of pH, temperature, co-solvents (acetone and DMSO) and protection of the NH_2 functional group with CH_3CO on the rate of the reaction. Fig. 1 shows the time course of the reaction of **2a**, the course of the hydrolysis having been monitored by measuring the decrease in the amino acid ester concentration by titration at pH 7.0 and 20 °C with 1.0 N ammonia solution.

Several tests showed that L-*N*-acetyl-3,3-difluorophenyl alanine methyl ester (**2a**) was hydrolyzed as fast as its unfluorinated analogues [19,20], since only the D-amino ester derivatives were recovered unchanged from the mixture after ca. 210 min of reaction, as indicated by ^{19}F NMR spectroscopic and gas chromatographic analyses.

The optical purities of the synthesized difluoroamino acid derivatives were established by NMR analysis. When the ^1H NMR spectra were recorded in the presence of the tris-[3-(trifluoromethyl-hydroxymethylene)-(+)-camphorato]-europium(III) derivative [Eu(tfc)$_3$], large chemical shift differences ($\Delta\Delta\delta$ up to 1.8 ppm) were observed for the methine proton for the enantiomers. Integration of these $CH_\alpha NH$ signals indicated that the *ee* was 95% for crude L-3,3-difluorophenyl alanine (**3a**) and 92% for methyl-*D*-3,3-difluorophenyl alanate (**4a**).

It is worthy of note that the best conditions were a pH value of 8 at 20–25 °C and that increasing the temperature at pH 8 resulted in a lower reaction rate, suggesting that at higher temperature some deactivation of the enzyme occurred.

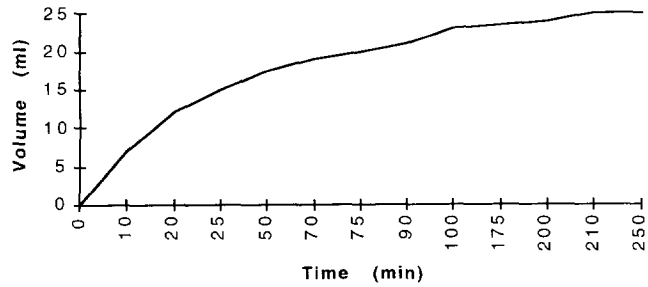

Fig. 1. Time course of the reaction of *N*-acetyl-D,L-3,3-difluorophenyl alanine methyl ester (**2a**) with subtilisin Carlsberg (**2a**, 10 mmol; subtilisin Carlsberg, 26 mg; DMSO, 20 ml; H_2O, 30 ml; pH, 6.8–7.0).

Organic solvents, including acetone and dimethyl sulphoxide, were useful for *N*-acetyl-3,3-difluorophenyl alanine methyl ester because they increased its solubility.

The fact that subtilisin Carlsberg hydrolyzed methyl 3,3-difluorophenyl alanate ester and its *N*-acetyl derivatives as fast as the racemic unfluorinated analogues [19] indicated that the introduction of two fluorine atoms at the α-position of the amino functional group, whilst lowering its pK_a value significantly [15], did not disturb the course of the reaction or alter the enzyme activity.

We have applied this resolution method to D,L-3,3-difluoro-2-aminobutyric acid methyl ester (**1b**) and its *N*-acetyl derivative **2b**. In these cases, although the same general conclusions could be drawn, the *ee* of the crude products was less and isolation by extraction of L-3,3-difluoro-2-aminobutyric acid (**3b**) from the reaction mixture with organic solvents such as diethyl ether, ethyl acetate or methylene dichloride was particularly difficult, probably because of its high solubility in water. In contrast, when *N*-acetyl-D,L-3,3-difluoro 2-aminobutyric acid methyl ester (**2b**) reacted with subtilisin, *N*-acetyl L-3,3-difluoro 2-aminobutyric acid (**5b**) and *N*-acetyl-D-3,3-difluoro-2-aminobutyric acid methyl ester (**6b**) were obtained from the reaction mixture in moderate yield (see Experimental details).

Isolation of D-methyl-3,3-difluorophenyl alanate ester and D-*N*-acetylmethyl-3,3-difluorophenyl alanate ester allowed their conversion to D-3,3-difluorophenyl alanine (**8a**) by reaction with aqueous 6 N HCl under reflux. In the same manner, when *N*-acetyl-L-2-amino-3,3-difluorobutyric acid and *N*-acetyl-D-2-amino-3,3-difluorobutyric acid methyl ester were heated under reflux with aqueous 6 N HCl, L- and D-3,3-difluoro-2-amino acids **3b** and **8b** were obtained.

This enantioselective enzymatic separation is convenient and provides a new route to bioactive fluorinated peptides because (i) the preparation of the 3,3-difluoro amino acid esters **1a,b** is simple, (ii) the enzyme is inexpensive, (iii) the products can be readily isolated, sometimes by extraction and (iv) the enatiomeric excesses are good or quite acceptable.

3. Experimental details

^1H and ^{19}F NMR spectra were recorded on Bruker spectrometers, models AC-200E (200 MHz), WP-90 (84.67

MHz) and/or WP-80 (80 MHz). Signal positions δ are given in ppm, with tetramethylsilane or trichlorofluoromethane as internal standards. Coupling constants values are given in Hz. Melting points were determined on a Totoli capillary model Büchi 510 melting point apparatus and are uncorrected. Optical rotations were measured as methanol or aqueous solutions on a Perkin-Elmer model 241 polarimeter or on a Roussel–Jouan automatic polarimeter. GC analysis was carried out on a HP 5890 gas chromatograph equipped with a flame ionization detector. Microanalyses were performed by the Service de Microanalyse de CNRS, Vernaison (France). Silica gel chromatography was performed on Grade 60, 70–230 mesh ASTM silica gel (Merck) and TLC on silica gel 60 F-254 glass plates (Merck).

Subtilisin Carlsberg (protease type VIII) was purchased from Sigma Chemical C. All analytical grade solvents and pyridinium polyhydrofluoride were purchased from Aldrich Chemical Co. and used without further purification.

3.1. Synthesis of D,L-methyl-3,3-difluoro-2-amino esters 1a and 1b

Methyl-3,3-difluoro-2-amino acid esters were prepared by the ring-opening of 2-carboxymethyl-1-azirines with pyridinium polyhydrofluoride. In a typical experiment, 40 mmol (7 g) of 2-carboxymethyl-1-phenyl-1-azirine in 5 ml of dry methylene chloride was added dropwise to 50 ml of a cooled (0 °C) and stirred solution of hydrogen fluoride in pyridine (70% w/w). After complete addition, the resulting solution was stirred at 0 °C for 2 h, allowed to reach room temperature and carefully poured into cold water (100 ml). The mixture was extracted with diethyl ether (3×50 ml) and methylene chloride (2×50 ml). The organic layers were washed with water. The aqueous layers were combined, treated with dilute ammonia solution at 0 °C until the pH attained a value of 8.5 and extracted with diethyl ether (3×100 ml) and methylene chloride (2×50 ml). The organic layers were dried ($MgSO_4$) and evaporated under vacuum. For **1a**, excess pyridine was eliminated by azeotropic evaporation with toluene (2×25 ml). After precipitating the 3,3-difluoroamino acid ester as its hydrochloride salt in dry diethyl ether, it could be recrystallized ($EtOH/Et_2O$). For **1b**, the crude mixture obtained after evaporation of diethyl ether and dichloromethane in vacuo was separated by silica gel column chromatography (eluent: CH_2Cl_2/AcOEt, 8:2 v/v). Further purification was effected by recrystallization of the hydrochloride as above.

Methyl-3,3-difluoro-2-amino-3-phenylpropanoate (**1a**) (4.83 g, 48%): Hydrochloride, m.p. 167–168 °C. Analysis: Calc.: C, 47.71; H, 4.77; F, 15.10%. Found: C, 47.78; H, 4.83; F, 14.93%. ^{1}H NMR ($CDCl_3$) δ: 2.01 (2H, br s, NH_2); 3.61 (3H, s, OCH_3); 4.01 (1H, t, J=11.4 Hz, CH); 7.41 (5H, s, C_6H_5) ppm. ^{19}F NMR ($CDCl_3$) δ: −105.4 (dd, J=243.7 Hz, J=11.4 Hz); −104.5 (dd, J=243.7 Hz, J=11.4 Hz) ppm.

Methyl-3,3-difluoro-2-aminobutanoate (**1b**): Starting from 2-carboxymethyl-1-methyl-1-azirine (4.52 g, 40 mmol), 3.92 g (52%) of **1b** was obtained. Hydrochloride, m.p. 158–160 °C. Analysis: Calc.: C, 31.66; H, 5.27; F, 20.0%. Found: C, 31.72; H, 5.31; F, 19.89%. ^{1}H NMR ($CDCl_3$) δ: 1.68 (3H, t, J=18.3 Hz, CH_3); 2.50 (2H, br s, NH_2); 3.75 (1H, *t*, J=11.8 Hz. C*H*NH_2); 3.78 (3H, s, OCH_3) ppm. ^{19}F NMR δ: −100.6, −97.6 (2 dq, J=247 Hz, J=18.3 Hz, J'=11.8 Hz) ppm.

3.2. Synthesis of N-acetyl-D,L-3,3-difluoro-2-aminomethyl esters 2a and 2b

A stirred solution consisting of 11 mmol of **1a,b** or their hydrochloride salts in dry pyridine (12 ml), cooled at 0 °C, was treated with acetic anhydride (10 ml). The mixture was stirred at this temperature for 1 h, then evaporated to dryness under vacuum. The residue obtained was taken up in water (50 ml) and extracted with methylene chloride (3×50 ml). The organic layers were washed successively with dilute hydrochloric acid solution and water (2×20 ml). The organic layers were dried and the solvent evaporated under reduced pressure. The light yellow product obtained could be used for the next step without further purification. Analytical samples of **2a** could be obtained by recrystallization from CCl_4. Compound **2b** was purified by silica gel column chromatography (eluent: CH_2Cl_2/CH_3OH 98:2 v/v), followed by recrystallization from ether/hexane (white needles).

N-Acetyl 3,3-difluoro-D,L-phenylalanine methyl ester (**2a**) (nc): m.p. 89–90 °C. Analysis: Calc.: C, 56.03; H, 5.06; F, 14.70%. Found: C, 56.00; H, 5.09; F, 14.41%. ^{1}H NMR ($CDCl_3$) δ: 1.97 (1H, s, $COCH_3$); 3.65 (3H, s, OCH_3); 5.41, 5.30 (1H, d of t, J=11.4 Hz, J'=10.5 Hz, C*H*NH); 7.1 (1H, br d, J=10.5 Hz, NH); 7.44 (5H, s, C_6H_5) ppm. ^{19}F NMR δ: −102.2 (dd, J=248 Hz, J'=10.5 Hz); −103.9 (dd, J=248 Hz, J'=11.4 Hz) ppm.

Methyl-*N*-acetyl D,L-3,3-difluoro-2-aminobutanoate (**2b**) (nc): m.p. 78–80 °C. Analysis: Calc.: C, 43.07; H, 5.64; F, 19.48%. Found: C, 43.01; H, 5.65; F, 19.60%. ^{1}H NMR ($CDCl_3$) δ: 1.70 (3H, t, J=11.9 Hz, CH_3); 2.07 (3H, s, $COCH_3$); 3.8 (3H, s, OCH_3); 5.01 (1H, d of t, J=19.1 Hz, J'=11 Hz, C*H*NH_2); 6.5 (1H, br d, J=11.8 Hz, NH) ppm. ^{19}F NMR δ: −97.6, −100.6 (2 d of q, J=247 Hz, J=19.1 Hz, J'=11.8 Hz) ppm.

3.3. Enzymatic hydrolysis of D,L-methyl-3,3-difluoro-2-amino esters 1a

The following procedure is representative. To a stirred solution of D,L-methyl-3,3-difluoro-2-amino-3-phenylpropanoate hydrochloride (**1a**) (2.51 g, 10 mmol) in 45 ml of sodium bicarbonate buffer (0.2 M, pH 8.1) at 20 °C, subtilisin Carlsberg (26 mg, 11.2 U mg^{-1}) was added and the pH maintained at a value of 8.1 using 1 N aqueous ammonia in conjunction with a pH-stat. At the end of the reaction (when the addition of small quantities of enzyme had no effect on

the pH), the reaction mixture was extracted with ether (3×50 ml) and dichloromethane (50 ml). The organic layers were washed with water, dried over $MgSO_4$ and the solvents evaporated to dryness under reduced pressure to give D-3,3-difluorophenyl alanine methyl ester (**4a**) as a white solid (1.19 g). $[\alpha]_D^{20} = +27.5°$ (*c* 0.2, CH_3OH).

^1H and ^{19}F NMR spectra were identical to those for **1a**. ^1H NMR spectrum recorded in the presence of Eu(tfc)$_3$ indicated that the *ee* of the product was ≥92%. ^{13}C NMR (CD_3OD) δ: −56.6 (s, OCH_3); −62 (t, CHNH, J=28.6 Hz); −122.4 (t, CF_2, J=250 Hz); −129.2 (t, $C_{\beta 6}H_5$, J=5.4 Hz); −132.5 (t, $C_{\gamma 6}H_5$, J=1.4 Hz); −135.7 (s, $C_{\delta 6}H_5$); 136.3 (t, $C_{\alpha 6}H_5$, J=24.5 Hz); −168.0 (s, CO) ppm.

Further purification by recrystallization of the hydrochloride salt from ethanol/ether yielded analytical pure samples, m.p. 158–159 °C. Analysis: Calc.: C, 47.71; H, 4.77; F, 15.10%. Found: C, 47.80; H, 4.85; F, 14.87%.

The combined aqueous solutions were concentrated, carefully acidified with concentrated HCl to pH 4.5–5, extracted with methylene dichloride (2×50 ml) and ethyl acetate (2×50 ml). The organic layers were combined, washed with cold water and dried. Evaporation under vacuum gave L-3,3-difluorophenyl alanine (**3a**) (nc) as a white solid (1.13 g), m.p. 176–179 °C. Analysis: Calc.: C, 53.73; H, 4.47%. Found: C, 54.19; H, 4.48%. ^1H NMR (CD_3OD) δ: 5.04 (1H, dd, J=5.1 Hz, J'=21.0 Hz, CH); 5.23 (s, br, exchangeable protons COOH and NH_2); 7.68 (5H, m, C_6H_5) ppm. ^{19}F NMR δ: −88.3 (dd, J=250 Hz, J=5.1 Hz); −107.2 (dd, J=250 Hz, J=21.0 Hz) ppm; $\alpha]_D^{20} = +14.5°$ (*c* 0.15, HCl 1.2 N).

3.4. Enzymatic hydrolysis of *N*-acetyl-D,L-3,3-difluoro-2-aminomethyl esters **2a** and **2b**

Method A

To a solution of **2a** (2.57 g, 10 mmol) in 20 ml of DMSO was added 30 ml of distilled water and the pH adjusted to pH 7.0 with 1 N aqueous ammonia solution. Subtilisin Carlsberg (26 mg) in 5 ml of water was added. The mixture was stirred at 20 °C for 2 h with the pH maintained at 6.8–7.0 with 1 N ammonia. The reaction mixture was diluted with water and the pH adjusted to 8.5 with 1 N ammonia. The mixture was then extracted with ethyl acetate (3×30 ml) and methylene chloride (2×30 ml). The organic layers were combined, washed with water, dried over magnesium sulphate and filtered. Evaporation of the solvents under vacuum gave 1.28 g of *N*-acetyl-3,3-difluoro-D-phenylalanine methyl ester (**6a**), m.p. 89–91 °C. The product was as 95% *ee* by a GC run on a 250 μm permethylated maltosyl cyclodextrin capillary column [21]. The ^1H and ^{19}F NMR spectra were identical to those of compound **2a**. ^{13}C NMR (CD_3OD) δ: −22 (s, CH_3); −53 (s, OCH_3); −58.9 (t, CHNH, J=30.1 Hz); −121 (t, CF_2, J=250 Hz); −126.6 (t, $C_{\beta 6}H_5$, J=5.4 Hz); −129.5 (t, $C_{\gamma 6}H_5$, J=20.4 Hz); −131.6 (t, $C_{\delta 6}H_5$, J=1.4 Hz); −135.2 (t, $C_{\alpha 6}H_5$, J=24.5 Hz); −168.3 (t, CO, J=2.5 Hz); −173 (s, CONH) ppm; $[\alpha]_D^{20} = -29°$ (*c* 0.25, CH_2Cl_2).

The combined aqueous solutions were evaporated under vacuo to dryness. The solid residue was dissolved in 20 ml of water, carefully acidified to pH 4.5–5 with concentrated HCl and extracted with ethyl acetate (3×25 ml) and with 50 ml of ether. The organic layers were combined, washed with cold water and dried ($MgSO_4$). Evaporation under vacuum gave *N*-acetyl-L-3,3-difluorophenyl alanine (**5a**) (nc) (1.25 g), m.p. 155–158 °C. Analysis: Calc.: C, 54.13; H, 4.54%. Found: C, 54.19; H, 4.49%; $[\alpha]_D^{20} = +21.6°$ (*c* 0.15, CH_3OH).

Method B

To a solution of **2a** (1.3 g, 5 mmol) in 20 ml of acetone was added 30 ml of water and the pH adjusted to 8.2 with 1 N aqueous ammonia solution. Subtilisin Carlsberg (13 mg) in 5 ml of water was added. The mixture was stirred at 20 °C with the pH maintained at 7.8–8 for 2 h with 1 N ammonia. During this time, small quantities of a solid precipitated. At the end of the reaction, the solvents were evaporated and the residue dissolved in water. Treatment as above and evaporation of the solvents under vacuum gave *N*-acetyl-D-3,3-difluorophenylalanine methyl ester (**6a**) (0.61 g) and *N*-acetyl-L-3,3-difluorophenyl alanine (**5a**) (0.58 g).

3.5. Enzymatic hydrolysis of *N*-acetyl-3,3-difluoro-D,L-2-aminobutyric acid methyl ester (**2b**)

Using method B above, 1.95 g (10 mmol) of **2b** were hydrolyzed with 25 mg of subtilisin Carlsberg at 20 °C. At the end of the reaction, the solvents were evaporated. The resulting residue was diluted with 10 ml of water and the pH adjusted to 8.5 with 1 N. The mixture was then extracted with ethyl acetate (3×20 ml) and methylene chloride (2×20 ml). The organic layers were combined, washed with water (10 ml), dried over magnesium sulphate and filtered. Evaporation of the solvents under vacuum provided D-*N*-acetyl-3,3-difluoro-2-aminobutyric acid methyl ester (**6b**) as a white solid (0.90 g, 92%), m.p. 78–80 °C. The ^1H and ^{19}F NMR spectra were identical to those of **2b**. GC indicated an *ee* value of 92%. ^{13}C NMR (CD_3OD) δ: −20.4 (t, CH_3); −20.5 (s, OCH_3); −57 (t, CHNH, J=30.1 Hz); −121 (t, CF_2, J=250 Hz); −168.3 (t, CO, J=2.5 Hz); −171.6 (s, CONH) ppm; $[\alpha]_D^{20} = +21°$ (*c* 0.2, CH_3OH).

The combined aqueous solutions were reduced under vacuum to 10 ml and carefully acidified to pH 4.5–5 with concentrated HCl. Most of the water was evaporated when the residue was extracted with ethyl acetate (3×20 ml) and ether (2×20 ml). The organic layers were combined, washed with aqueous saturated sodium chloride solution and dried ($MgSO_4$). After removal of the solvents under vacuum, the *N*-acetyl-L-3,3-difluoro-2-aminobutyric acid (**5b**) (nc) remained as a highly viscous oil (0.85 g, 87%) which solidified on standing at room temperature. Analysis: Calc.: C, 39.78; H, 4.97; F, 20.99%. Found: C, 39.81; H, 5.01; F,

19.89%. ^{1}H NMR ($CDCl_3$) δ: 1.5 (3H, t, J = 19.2 Hz, CH_3); 2.01 (3H, s, OCH_3); 5.0 (1H, d of t, J = 13 Hz, J' = 15.2 Hz, CHNH); 6.92 (1H, d, J = 13 Hz, NH); 9.2 (1H, s br, COOH) ppm. ^{19}F NMR showed two d of dq centred at δ −96.7 ppm (J = 247.6 Hz, J = 19.2 Hz, J' = 15.2 Hz) and at δ −98.4 ppm (J = 247.6 Hz, J = 19.2 Hz, J' = 14.4 Hz). Upon total decoupling of the hydrogens, the two pairs of multiplets collapsed to an AB pattern; $[\alpha]_D^{20}$ = +28° (c 0.4, CH_3OH).

3.6. D-3,3-Difluorophenyl alanine hydrochloride (8a)

A sample of 3,3-difluoro-D-phenylalanine methyl ester (0.68 g, 2.25 mmol), prepared by the enzymatic hydrolysis of **1a**, was dissolved in 15 ml of 6 N HCl solution and the resultant mixture heated under reflux for 6 h. The reaction mixture was decolourized with charcoal and filtered. The water was evaporated and the residue was taken up in toluene and evaporated in vacuo to dryness. This procedure was repeated three times to eliminate excess HCl and water. In this manner, 3,3-difluoro-D-phenylalanine hydrochloride (**8a**) (nc) (0.45 g; a light yellow solid) was obtained, m.p. 152–156 °C. ^{1}H NMR (CD_3OD) δ: 5.03 (1H, dd, J = 5.1 Hz, J' = 21.0 Hz); 5.2 (br s, exchangeable protons COOH and NH_2); 7.62 (5H, m, C_6H_5) ppm. ^{19}F NMR δ: −87.8 (dd, J = 250 Hz, J' = 5.1 Hz); −106.2 (dd, J = 250 Hz, J' = 21 Hz) ppm; $[\alpha]_D^{20}$ = −17.8° (c 0.5, 1.2 N HCl). ^{19}F NMR analysis of the crude product indicated that it contained less than 5% of the L-isomer. The same compound could be obtained via the same reaction starting from *N*-acetyl-3,3-difluoro-D-phenylalanine methyl ester (**5a**).

3.7. Preparation of D- and L-3,3-difluoro-2-aminobutyric acids

A sample of *N*-acetyl-3,3-difluoro-D-2-aminobutyric acid methyl ester (**4b**) (0.605 g, 3.1 mmol), prepared by the enzymatic hydrolysis of **2b**, was dissolved in 10 ml of 6 N HCl solution and the mixture heated under reflux for 6 h. The reaction mixture was decolourized with charcoal and filtered. The water was evaporated and the residue then taken up in water and evaporated in vacuo to dryness. This procedure was repeated three times to eliminate excess HCl. D-2-Amino-3,3-difluorobutanoic acid hydrochloride (**8b**) (nc) was obtained as white solid (0.46 g, 94%), m.p. 258–261 °C (decomp.). ^{1}H NMR (D_2O/DMSO-d_6) δ: 1.5 (3H, t, J = 19.2 Hz, CH_3); 2.01 (3H, s, OCH_3); 5.0 (1H, d of t, J = 13 Hz, J' = 15.2 Hz, C*H*NH); 6.92 (1H, d, J = 13 Hz, NH); 9.2 (1H, s br, COOH)ppm. ^{19}F NMR spectroscopy showed two sets of signals which collapsed to an AB pattern upon total decoupling of the hydrogens. ^{19}F NMR δ: −90.3 (J = 243.5 Hz, J = 21 Hz, J' = 19.4 Hz); −98.8 (J = 243.5 Hz, J = 21 Hz, J' = 19.4 Hz) ppm; $[\alpha]_D^{20}$ = +1.5° (c 0.10, 6 N HCl). ^{19}F NMR analysis of the crude product indicated that it contained less than 10% of the L-isomer.

N-Acetyl-3,3-difluoro-L-2-aminobutyric acid (**5b**) (0.386 g, 2.13 mmol), prepared by the enzymatic hydrolysis of **2b**, was dissolved in 10 ml of 6 N HCl solution and the mixture heated under reflux for 2.5 h. The reaction mixture was decolourized with charcoal and filtered. The water was evaporated and the residue was taken up in water and evaporated in vacuo to dryness. This procedure was repeated three times to eliminate excess HCl. In this manner L-2-amino-3,3-difluorobutanoic acid hydrochloride (**7b**) (nc) (0.322 g, 95% as a white solid) was obtained. ^{19}F NMR ($CDCl_3$) showed two signals which collapsed to an AB pattern upon total decoupling of the hydrogens. ^{19}F NMR δ: −88.7 (J = 243.5 Hz, J = 19.2 Hz, J' = 15.2 Hz); −97.6 (J = 243.5 Hz, J = 19.2 Hz, J' = 14.4 Hz) ppm; $[\alpha]_D^{20}$ = −5.7° (c 0.15, 1 N HCl). ^{19}F NMR analysis of the crude product indicated that it contained less than 5% of the D-isomer.

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