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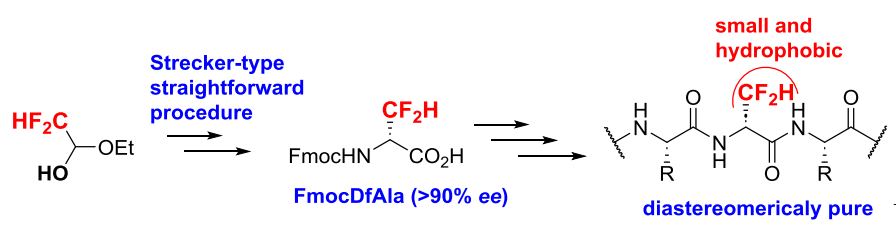
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Difluoroalanine: Synthesis, Incorporation in Peptides and Hydrophobic Contribution Assessment

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Supporting Information Placeholder



ABSTRACT: The straightforward synthesis of chiral (*R*)- and (*S*)-difluoroalanine (DfAla) is reported. The key step is a Strecker-type reaction on a (*R*)-difluorophenethylimine followed by hydrogenolysis, Fmoc protection and acidic hydrolysis. Peptide coupling reactions at its *N*- and *C*-terminal position provide diastereomerically pure tripeptides. Based on hydrophobicity index measurements, the hydrophobic contribution of difluoroalanine in a peptide chain was found to be quite similar to that of isoleucine for a smaller van der Waals volume of the side chain.

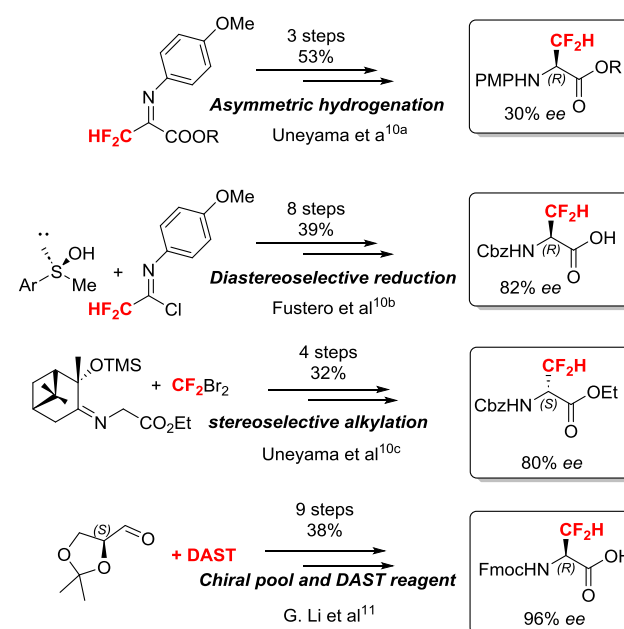
Fluorine containing compounds are attracting increasing attention because of the remarkable properties conferred by this element.¹ Among the latter, we can mention its pivotal role as bioisostere in medicinal chemistry.² Because of its specificities, the fluorine atom exerts a strong influence on the physicochemical properties of molecules. As the main effects, we can cite the modulation of the acidity or basicity of neighboring functions (pKa), lipophilicity, conformational constraints and metabolic stability.³ In this context, incorporating fluorinated amino acids in peptides constitutes a powerful strategy for the tuning of their biophysical and biological properties.⁴

The CF₂H group is recently emerging as a very attractive fluorinated group in medicinal chemistry because of its very specific properties. It is considered as a bioisostere of thiol and hydroxyl groups, able to act as a H-bond donor and also to modulate the lipophilicity of the compounds.⁵ Despite its interest, this group is very poorly represented in the chemistry of α -fluoroalkyl amino acids and their peptides. Although the γ -disubstituted difluoroethylglycine has been synthesized and incorporated in an enantiopure form in peptides,⁶ the challenging difluoroalanine (DfAla) has never been incorporated in peptides so far.

In the line of our previous report dedicated to the use of CF₂H containing pseudoproline for the control of peptide conformation,⁷ our attention is now focused on β,β -difluoroalanine (DfAla). Although this non-natural fluoro amino acid should be considered as the simplest typical

representative of the α -CF₂H amino acid, its enantioselective synthesis has been very scarcely reported in the literature and to our knowledge, it has never been incorporated in an enantiopure form in peptides.

Scheme 1. Previous syntheses of chiral difluoroalanine.

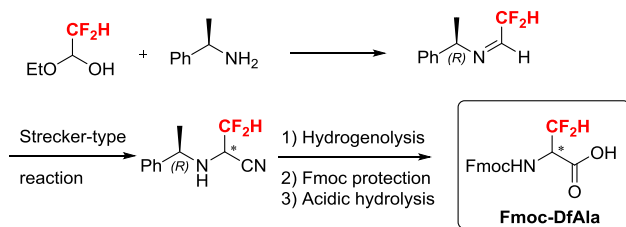


Conversely to the α,α -disubstituted (*R*)- and (*S*)-difluoromethylalanine (DfmAla)⁸ and trifluoromethylalanine (TfmAla),⁹ the main difficulty for obtaining enantiopure difluoroalanine lies in the easy epimerization of its stereocenter. In scheme 1 are presented the previous syntheses of chiral difluoroalanine reported in the literature. Three of them, based on stereoselective reduction or alkylation gave the β,β -difluoroalanine in low to moderate *ee* and yield.¹⁰ The last one, based on the chiral pool and DAST reagent, gave the (*R*)-difluoroalanine in poor yield after 9 steps but with 96% *ee*.¹¹ Nevertheless, using the DAST reagent is inadequate for multigram syntheses.

We present herein a straightforward and scalable synthesis of both enantiomer of β,β -difluoroalanine in good yield, few steps and high *ee*. The incorporation of β,β -difluoroalanine in a peptide chain has been investigated, as well as the assessment of the hydrophobicity provided by the CF₂H group.

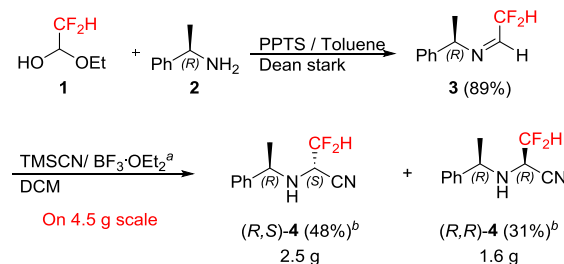
The straightforward scalable strategy envisioned for the synthesis of both (*R*)- and (*S*)- β,β -difluoroalanine involves as a key step a Strecker-type reaction on a chiral difluoroacetalimine (Scheme 2). After separating each diastereomer, hydrolysis of the nitrile function followed by removal of the chiral benzyl group by hydrogenolysis allows the formation of the (*R*)- and (*S*)-difluoroalanine. A Fmoc protection of the amino group is considered to provide an amino acid ready for use in peptide synthesis (Scheme 2). The main challenge of this synthesis is to avoid the epimerization of the highly sensitive stereogenic center in the α -position of the fluoroalkyl group during the nitrile acidic hydrolysis step (Scheme 2).

Scheme 2. Strategic Pathway for the synthesis of (*R*) and (*S*)-Fmoc-difluoroalanine (Fmoc-DfAla)



The condensation of the difluoroacetaldehyde ethylhemiacetal **1** with the (*R*)-phenethylamine **2** gave the imine **3** in good yield (89%).¹² The Strecker-type reaction of the imine **3** with TMSCN promoted by BF₃·OEt₂ proceeded in good yield and provide the amino nitrile (*R,S*)-**4** and (*R,R*)-**4** as a 60:40 diastereomeric mixture (Scheme 3). No improvement was observed with other catalysts such as for example Yb(OTf)₃, Sc(OTf)₃ and FeCl₃. Due to their large R_f difference, the separation of each diastereomer was easily performed by column chromatography to give (*R,S*)-**4** and (*R,R*)-**4** in 48% and 31% isolated yields, respectively. The diastereomeric purity (>99% *de*) was determined by HPLC (see supporting information). This reaction is very reproducible and can conveniently be performed on a multi-gram scale.

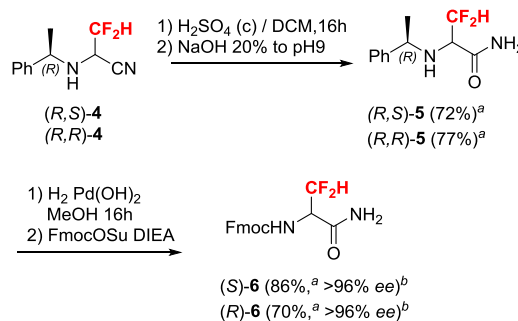
Scheme 3. Synthesis of the intermediate diastereomers (*R,S*)- and (*R,R*)-amino nitrile **4**.



^a 60:40 *dr*, ^b >99% *de* (HPLC)

Unfortunately, attempts of the Pinner reaction (HCl 37%/MeOH) or methanolysis in basic conditions (NaOH/MeOH) failed to achieve the transformation of the nitrile function of **4** into an ester function. In acidic conditions (conc HCl) difluoromethylene compounds and phenethylamine hydrochloride were detected among degradations products. This result suggests that the ammonium salt resulting from the protonation of the benzylic amine undergoes an elimination reaction favored by the acidic proton of the CF₂H group. This side reaction is specific to the CF₂H series because the acidic hydrolysis of the nitrile proceeds in good yield in the CF₃ series.¹³ On the other hand, in the presence of concentrated H₂SO₄, the elimination reaction was avoided, and the nitriles **4** were cleanly hydrolyzed into the corresponding amides **5** in good yields without any epimerization (Scheme 4). In order to proceed to the further hydrolysis of the amide function into the target carboxylic acid in acidic conditions and to avoid the ammonium elimination, we decided to protect the amino group with a Fmoc electron withdrawing group. The corresponding Fmoc-amino amides (*S*)- and (*R*)-**6** have been obtained in good yields and 96% *ee* through a sequence debenzylation and Fmoc protection (Scheme 4).

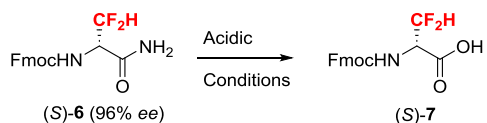
Scheme 4. Acidic hydrolysis of the nitrile function



^a Isolated yield, ^b Determined by chiral HPLC

The transformation of an amide function into a carboxylic acid function is known to require drastic reaction conditions. Many reactions in basic medium were tested on compound **5** (NaOH, H₂O₂/ Na₂CO₃), but none of them allowed to reach the expected product in a good yield. As basic conditions were unsuccessful, we turned to strong acid conditions. A methodological study for the acidic hydrolysis of the protected Fmoc amino amide (*S*)-**6** was then undertaken in order to find the best conditions (Table 1).

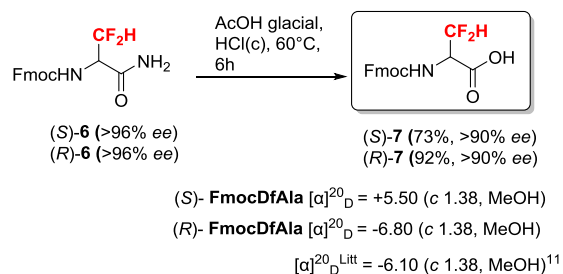
Table 1 : Optimization of the hydrolysis conditions of the amide function (S)-6 into the carboxylic acid (S)-7.



Entry	Acid	T (°C)	Time (h)	Solvent	Conversion (%) ^a	ee (%) ^a
1	HBr (48%)	rt	72	Dioxane	75	90
2	HBr (48%)	100	1.5	Dioxane ^b	100	88
3	HBr (48%)	rt	72	AcOH	45	95
4	HBr (48%)	60	8	AcOH	89	87
5	HCl (12N)	100	1.5	AcOH	100	79
6	HCl (12N)	60	6	AcOH	100	90

^a Determined by chiral HPLC ^b Dioxane degradation

Scheme 5. Optimized procedure for the acidic hydrolysis of the amide 6 into Fmoc-difluoroalanine 7

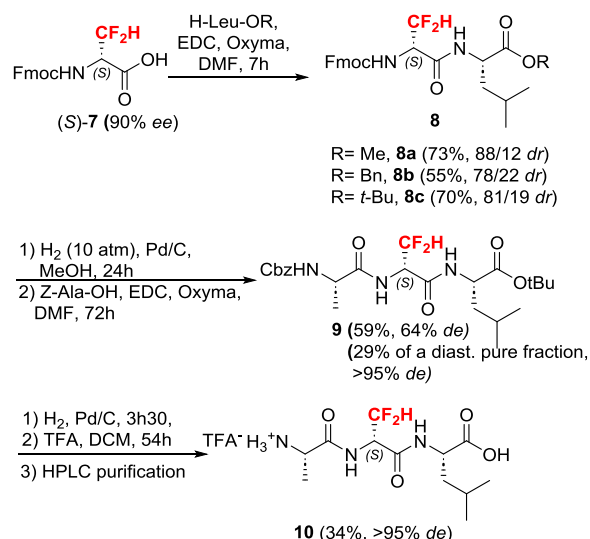


Having already successfully reported the transformation of an α -fluoroalkyl amide into a carboxylic acid in the presence of HBr,¹⁴ we carried out this reaction in dioxane at room temperature. After 72h, the conversion was moderate (75%), and the stereoselectivity was 90% *ee* (Entry 1). At 100°C, the conversion was complete, but the crude mixture presented side products resulting from the dioxane degradation (Entry 2). When the reactions were carried out in acetic acid as solvent, no improvement was observed either for the conversion or the enantioselectivity (Entries 3, 4). Using HCl 12N allowed the reaction temperature and time to be modulated for a better conversion (Entries 5, 6). The best conditions (60°C in AcOH during 6h, Entry 6) led to the expected amide **7** with a total conversion and a good *ee* (90%) (Entry 6, and Scheme 5). The absolute configuration of the stereogenic center of the Fmoc-DfAla (S)-**7** was assigned according to the work of Li et al.¹¹

With the enantioenriched Fmoc-DFAla-OH in hand, the challenge was then to find appropriate reactions conditions for its incorporation into a peptide chain while avoiding epimerization reaction due to the relative acidity of the proton in the α -position of the CF₂H group. The analogue trifluoroalanine has been included with difficulties in a peptide

chain for hydrophobicity measurement purposes but the easy epimerization of this amino acid due to the strong electron-withdrawing CF₃ group required several tedious chiral and reverse phase HPLC separations.¹⁵ In the CF₂H series, classical peptide coupling conditions (EDC, Oxyma) led to the peptide coupling of various protected Leucines at the C-terminal position of Fmoc-DFAla-OH with limited epimerization. The corresponding dipeptides **8a**, **8b** and **8c** were obtained in good yields (Scheme 6). The observed epimerization was probably promoted by the activation of the carboxylic function of the difluoroalanine. Unfortunately, removing the Fmoc protecting group in the presence of piperidine led to dipeptide degradation. This problem was circumvented by the removal of the Fmoc protecting group by hydrogenolysis in neutral conditions. Furthermore, in order to set up orthogonal protections avoiding basic medium, we turned to a *tert*-Butyl protection requiring acidic conditions for its removal. So, the Fmoc protecting group of the dipeptide **8c** has been removed by hydrogenolysis. Then the coupling reaction with the Z-Ala-OH gave the tripeptide **9** in 59% yield and 64% *de* (Scheme 6). A diastereomerically pure fraction of **9** could be isolated in 29% yield after silica gel chromatography separation. It should be noticed that in opposition to the α -trifluoromethylalanine series, the EDC/oxyma activation is appropriate to achieve this peptide coupling. Indeed, we reported that amino acid chlorides or mixed anhydride activation were mandatory to allow the peptide coupling on the more deactivated α -trifluoromethylalanine amino group.⁹ In order to obtain an enantiopure unprotected tripeptide, the protecting groups of **9c** were removed by hydrogenolysis and TFA treatment followed by an HPLC purification to give the peptide **10** in 34% yield and >95% *de* (Scheme 6).

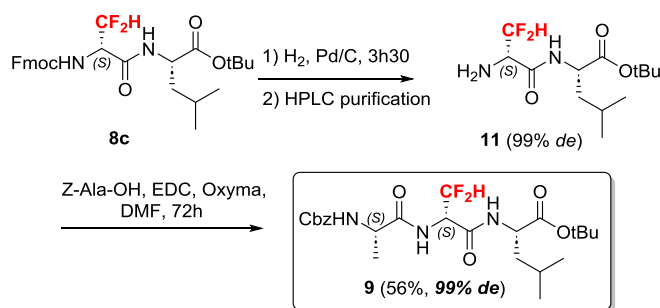
Scheme 6. Incorporation of Fmoc-DfAlaOH in peptide



In order to confirm that the *N*-terminal coupling can be achieved without epimerization, the diastereomerically pure dipeptide **11** (99% *de*) was prepared by hydrogenolysis of **8c** followed by a purification by preparative HPLC (Scheme 7). The *N*-coupling reaction of **11** with Z-Ala-OH under the same peptide-coupling conditions gave tripeptide **9** in a good chemical yield (56%) without any epimerization (99% *de*). It

is interesting to note that, once incorporated in a peptide chain, the enantiopure difluoroalanine is not prone to epimerization.

Scheme 7. Synthesis of enantiopure tripeptide **9**



The side chain hydrophobicity of amino acids is a key factor for the control of peptide and protein folding. The hydrophobicity modulation by incorporation of several fluorine containing amino acids has already been reported in the literature,^{6,16,17} but to our knowledge, this biophysical parameter has not yet been investigated for difluoroalanine. To achieve an accurate determination of the local hydrophobicity modulation imparted by the CF₂H group in a peptide context, we decided to measure the chromatographic hydrophobicity indexes (HI) of the tripeptide H₂N-Ala-DfAla-Leu-OH **10**. In this measurement, the nonpolar character of a reverse-phase HPLC column mimics a hydrophobic environment.¹⁸ The comparison of the HI of this peptide compared with those of the reference tripeptides H₂N-Ala-AA-Leu-OH with various amino acids in the central position^{17a} provides a precise assessment of the hydrophobic contribution of the CF₂H side chain (Figure 1 and supporting information). The hydrophobicity index shift dHI (HI_{DfAla} - HI_{Ala} = 9.9) constitutes a quantitative assessment of the increased hydrophobic contribution of the CF₂H substituent compared to the methyl group of alanine. It is very interesting to note that despite its smaller van der Waals volume, the CF₂H group provides nearly the same hydrophobic contribution as the side chain of isoleucine, which is known to be the most hydrophobic aliphatic amino acid (HI_{DfAla} - HI_{Ile} = 0.2). However, as expected difluoroalanine is less hydrophobic than trifluoromethylalanine (TfmAla), and the SCF₃ containing trifluoromethylcysteine (TfmCys) and trifluoromethionine (TFM) previously reported.^{17a}

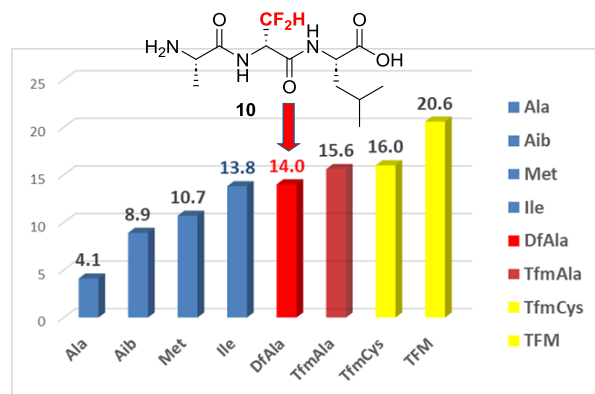


Figure 1. Chromatographic Hydrophobicity Index (HI) of tripeptide **10**.

In conclusion, we report a straightforward multigram synthesis of difluoroalanine in 5 steps, in good yield and with a good enantioselectivity (>90% *ee*). We demonstrated that, in opposition to the trifluoromethylated series, this unnatural amino acid can conveniently undergo coupling reactions at its C- and N-terminal positions to provide enantiopure tripeptides. The local hydrophobicity provided by the incorporation of difluoroalanine is almost equal to isoleucine for a smaller van der Waals volume of the side chains. The incorporation of difluoroalanine into a peptide chain should then constitute a valuable tool for the rational local hydrophobicity fine tuning of peptide sequences for improving peptide-protein interaction minimizing steric hindrance.

ASSOCIATED CONTENT

Supporting Information

The data underlying this study are available in the published article and its Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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