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Synthesis of 4,6-Difluoro-Tryptophan as a Probe for Protein ^{19}F NMR

Christina M. Monnie,[a] Iker Hernández,[b] Raixie Meléndez-Pacheco,[a] Fatema Bhinderwala,[a] Vadim A. Soloshonok,[b, c,*] Angela M. Gronenborn,[a,*] Aitor Landa,[b,*] and Mikel Oiarbide[b,*]

[a] Department of Structural Biology, University of Pittsburgh School of Medicine, 3501 Fifth Ave., Pittsburgh, PA 15261, USA
E-mail: amg100@pitt.edu

[b] Department of Organic Chemistry I, Faculty of Chemistry, University of the Basque Country UPV/EHU, Paseo Manuel Lardizabal 3, 20018 Donostia-San Sebastián, Spain
E-mail: vadym.soloshonok@ehu.eus; a.landa@ehu.eus; mikel.oiarbide@ehu.eus

[c] IKERBASQUE, Basque Foundation for Science, Bilbao 48011, Spain

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Abstract: A scalable procedure for the synthesis of 4,6-difluorotryptophan is reported based on a deaminative coupling of a 4,6-difluorogramine with 2-benzylthio-1,5-dihydro-4*H*-imidazolone as glycine equivalent. Thus prepared 4,6-difluorotryptophan was incorporated into the C-terminal domain of the HIV-1 capsid protein (CA-CTD), and ^{19}F spectra of the 4,6-difluoro Trp CA CTD were recorded and compared to the singly fluorinated counterparts.

Keywords: Fluorinated tryptophans; Amino acid synthesis; Gramines coupling; Protein ^{19}F NMR

Given the increasingly growing number of new pharmaceuticals containing fluorine atoms,[1] fluorine chemistry and applications of fluorinated compounds are becoming a flourishing areas of research. Fluorinated amino acids are in high demand,[2] and their synthesis[3] and incorporation into proteins are currently a very exciting and expanding subjects. Nowadays, application of fluorinated tailor-made amino acids to study protein dynamics, structure, folding and function is well beyond drug discovery campaigns, with fluorine NMR spectroscopy gaining much popularity as a valuable tool.[4] The 100% naturally abundant ^{19}F atom possesses favourable NMR properties: a spin 1/2 nucleus and the second highest gyromagnetic ratio after hydrogen, resulting in excellent sensitivity.[5] In addition, the resonance frequency of the fluorine nucleus is exquisitely responsive to its conformational and electronic environment, covering a range of >300 ppm.[6] Furthermore, since ^{19}F is virtually absent from all biological molecules, spectra of fluorinated proteins do not suffer from any background signals.

Incorporation of single fluorine atoms into proteins with as much chain position selectivity as possible, using fluorinated amino acid analogues, is an attractive approach to assess protein structure as well as ligand binding. One of the most used fluorinated amino acids in that regard is tryptophan (Trp). Monofluorinated tryptophans can be readily incorporated into proteins without the need of specialized strains.[7] Beyond monofluorinated tryptophans, here we aimed to enlarge this research space to difluoro-tryptophans, providing more than one probe to assess the behavior of the fluorinated protein. This necessitates convenient and facile access to such fluorinated amino acids. Here, we report an expedient and scalable synthesis of 4,6-difluoro-tryptophan, its incorporation into a protein and the characterization of this tailor-made amino acid as a probe in ^{19}F NMR (Scheme 1).

For the synthesis of the target fluorinated tryptophan **1**, our plan envisioned the coupling of the corresponding fluorinated gramine with a nucleophilic

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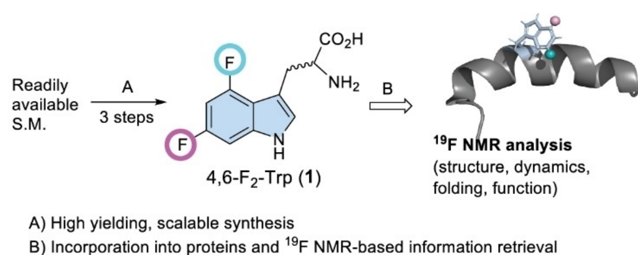
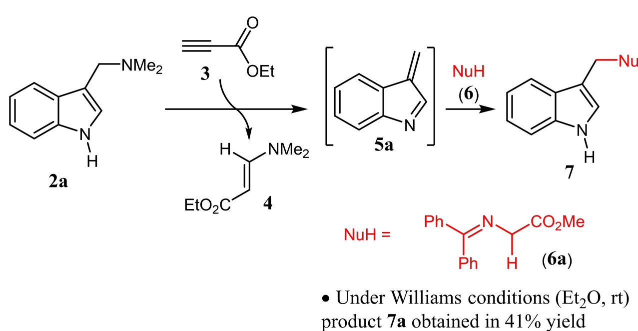


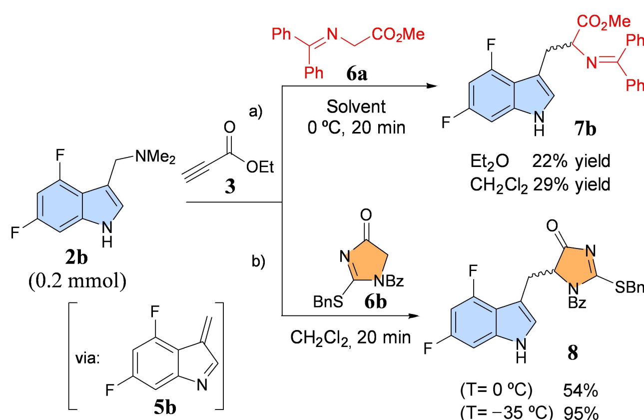
Scheme 1. Conceptualization of this work.

glycine equivalent. Gramines (3-aminomethylindoles), tend to lose the R^1R^2N unit attached at the benzylic position under various activation mechanisms, generating an electrophilic azafulvene intermediate which can be intercepted with a variety of nucleophiles *in situ.*[8] In particular, Sainsbury[9] reported that coupling of gramines with malononitrile or an α-cyanoester, respectively, could be promoted by dimethylacetylene dicarboxylate, which serves as the dimethylamino group scavenger, under smooth conditions. Further refinement of the method by Williams identified ethyl propiolate **3** as the optimal activating reagent[10] (Scheme 2). However, the coupling of gramine **2a** with *N*-(diphenylmethylene)glycine methyl ester **6a** under Williams conditions (Et_2O as solvent, 15 min at room temperature) was reported to provide the tryptophan derivative **7a** in a low 41% isolated yield. At the outset, and in view of these precedents, the suitability of the approach for the production of the desired fluorinated tryptophan was unclear.

For initial assessment of the approach, difluorinated gramine **2b** (synthesized through the Mannich reaction from the corresponding indole[11]) was subjected to reaction with iminoester **6a** in the presence of slight excess (1.1 equivalents) of ethyl propiolate **3** under the optimal conditions reported by Williams at 0 °C. The resulting adduct **7b** was indeed obtained, although, not surprisingly, in a low isolated yield (22%). Changing the solvent from Et_2O to CH_2Cl_2 improved the yield to a yet unsatisfactory 29% (Scheme 3a).



Scheme 2. Coupling of gramine **2a** with a glycine equivalent promoted by ethyl propiolate. (Williams *et al.* in ref [10]).



Scheme 3. Comparison of iminoester **6a** and 2-benzylthioimidazolone **6b** in the coupling with difluorogramine **2b**.

Among the additional reaction parameters that might be susceptible for modification, we focused on plausible glycine equivalents other than α-iminoesters. In this respect, recently some of us introduced 2-benzylthioimidazolones as pronucleophilic hydantoin surrogates that add smoothly under (slightly basic) organocatalytic conditions to various Michael acceptors.[12] On this basis, we reasoned that pairing the C-4 pronucleophilic character of 2-benzylthioimidazolone **6b** with in situ formed electrophilic azafulvenes, like **5b**, might be favorable, providing a solution to the above problem since hydantoins may afterwards be transformed into the respective α-amino acid.[13] To that end, it was gratifying to observe that **6b** reacted with **2b**, in CH_2Cl_2 solvent, at 0 °C to afford the coupling adduct **8** in a promising 54% isolated yield (Scheme 3b). Further experimentation revealed that the temperature influenced the reaction outcome dramatically: running the reaction at −35 °C resulted in formation of compound **8** as analytically pure material in 95% isolated yield. Of practical importance, scaling up the reaction to the multigram scale was possible without any significant detriment on product yield or purity, allowing preparation of 2.54 g (5.34 mmol) of difluorinated adduct **8** starting from 1.25 g (5.94 mmol) of gramine **2b** (90% yield) in a single run.

Treatment of **8** with 6 *M* HCl in dioxane at room temperature for 16 h gave rise to the corresponding *N*-benzoyl hydantoin **9**. Then, straightforward hydantoin ring opening through basic hydrolysis,[14] protonation and purification under an ion-exchange column, yielded the target difluorinated amino acid **1**, with an overall 65% yield (three steps, Scheme 4).

All three fluorinated tryptophans, 4-fluoro tryptophan, 6-fluoro tryptophan and the synthesized 4,6-difluoro tryptophan were efficiently incorporated into protein, and yields of labeled protein were essentially identical. This indicates that the 4,6-difluoro trypto-

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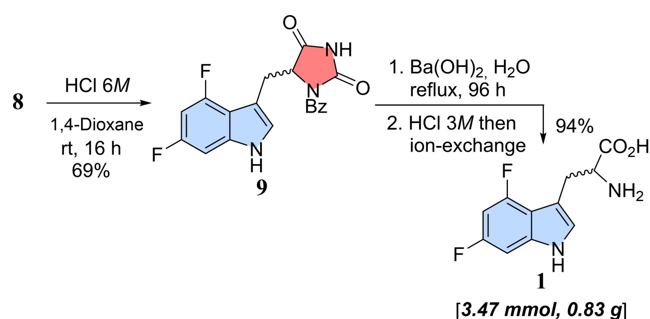

Scheme 4. Hydrolytic conversion of coupling adduct **8** to hydantoin **9** and target 4,6-F_2-Trp **1**.

phan is efficiently used by the biosynthetic protein synthesis machinery of E. coli. Note, in contrast to perfluorinated compounds, the two fluorine atoms on one amino acid in the CTD do not change the property of the protein significantly, although the dimerization constants seem to have been affected (see SI).

This domain dimerizes (Figure 1), and the dimer links two adjacent hexameric units in the overall capsid lattice via an antiparallel helix-helix interface. At the center of the interface, the single Trp residue in this domain plays a pivotal role in the stabilization of the extended lattice of the conical capsid shell.[15] As can be appreciated, both the 4- and 6-position of the six membered indole ring are separated by 5.2 Å (4F–4F), 6.1 Å (6F–6F) and 5.4 Å across the dimer interface in the structure, ideally suited to report on dimer

Figure 1. HIV1 CA-CTD (PDB ID; 2KOD) in ribbon representation with the Trp side chain at the dimer interface depicted in stick representation and the F atoms at the 4 and 6 positions as cyan and magenta spheres, respectively (top left). SDS PAGE of 4-F-, 6-F- and 4,6-F Trp CA-CTD with marker sizes indicated (bottom left). ^{19}F spectra at 25 °C of 4-F, 6-F and 4,6-F_2-Trp at 10 μM protein concentration. 4-F, 6-F and 4,6-F_2-Trp CA-CTD spectra are shown in cyan, magenta and blue, respectively. D, dimer; M, monomer.

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formation. The WT CTD forms a dimer with a K_d value of ~10 μM. Interestingly, two different dimers were observed for WT CTD as well as 4-fluoro-Trp CTD, labeled D_1 and D_2 in Figure 1, whose relative amounts are both pH, temperature and concentration dependent.[16] Given the crucial positions of the fluorine atoms in 4-fluoro-Trp CTD, 6-fluoro-Trp CTD and 4,6-difluoro-Trp CTD, we used ^{19}F NMR to evaluate the protein. Spectra of both the free fluorinated amino acid and after incorporation into CA-CTD are displayed in Figure 1. Clearly, distinct ^{19}F chemical shifts are seen, with the 4-F and 6-F resonances of the isolated amino acids located at −46.4 ppm and −43.7 ppm, respectively. Interestingly, the equivalent resonances in 4,6-difluoro-Trp are located at −41.5 and −43.5 ppm, distinctly different from the resonance positions of the singly fluorinate Trp amino acids. As was noted previously, the isotropic ^{19}F chemical shift of a fluorine atom at the 6 position in Trp is downfield of that at the 4 position,[17] also seen here. In the 4-F-Trp CA-CTD protein spectra, both dimer forms (D_1 and D_2) are seen, as well as the monomer (M), while for 6-F-Trp CA-CTD only in the 10 μM sample the resonance for the monomer can be detected. Interestingly, however, the ^{19}F spectrum of the 4,6-F_2-Trp CTD shows four, not well-resolved resonances. Recording spectra at different concentrations (SI) did not permit unambiguous assignment of the resonances to the monomer and dimer species, different from the case of the monofluorinated 4F-Trp and 6-F-Trp CTD. Gratifyingly, though, the biosynthetic incorporation of 4,6-F_2-Trp into the C-terminal domain of the HIV-1 capsid protein (CA-CTD) was achieved, indicating that the E. coli protein synthesis machinery is capable of utilizing this di-fluorinated amino acid.

In conclusion, a scalable procedure for the synthesis of 4,6-difluorotryptophan **1** (4,6-F_2-Trp) is reported based on a deaminative coupling between a 4,6-difluorogramine **2b** and 2-benzylthio-1,5-dihydro-4*H*-imidazolone **6b**. Key for a clean reaction and efficient coupling is to keep the reaction temperature low (−35 °C) and to use **6b** as an advantageous glycine synthetic equivalent. The incorporation of prepared 4,6-F_2-Trp into the C-terminal domain of the HIV-1 capsid protein (CA-CTD) was realised with similar yield as with the individual mono fluorinated amino acids.

Experimental Section

Ethyl Propiolate-Promoted Addition of 2-Benzylthioimidazolone 6b to Gramine 2b

To a solution of gramine **2b** (42 mg, 0.2 mmol) in dry CH_2Cl_2 (2 mL) at room temperature, 2-benzylthioimidazolone **6b** (2 eq., 0.4 mmol, 124 mg) was added. After cooling the solution to −40 °C, ethyl propiolate **3** (1.1 eq., 0.22 mmol, 22 mg) was added and the reaction mixture was stirred at the same temperature for 10 minutes. Afterwards, the mixture was allowed to reach −35 °C slowly over 30 minutes. Then, the solvent was evaporated under reduced pressure and the crude compound was purified by column chromatography (eluent: hexane/ethyl acetate 5:1) to obtain **8** as a brown solid, m.p. = 215–217 °C. Yield 90.4 mg, 0.19 mmol, 95%. **^{1}H NMR (300 MHz, $CDCl_3$)** δ = 8.01 (s, 1H), 7.42–7.13 (m, 10H), 6.83 (d, J = 2.4 Hz, 1H), 6.74 (dd, J_{HF} = 8.9, J_{HH} = 2.1 Hz, 1H), 6.50–6.39 (m, 1H), 5.13–5.04 (m, 1H), 4.36 (d, J = 2.4 Hz, 2H), 3.34 (dd, J = 15.3, 4.8 Hz, 1H), 3.19 (dd, J = 15.1, 6.1 Hz, 1H). **^{13}C {^{1}H} NMR (126 MHz, $CDCl_3$)** δ = 185.8, 185.1, 167.5, 159.5 (dd, J = 239.8, 12.2 Hz), 156.2 (dd, J = 248.1, 15.1 Hz), 137.7 (t, J = 14.0 Hz), 135.2, 132.7, 132.5, 129.6, 128.9, 128.6, 128.0, 127.9, 124.1 (d, J = 3.2 Hz), 112.8 (d, J = 19.4 Hz), 107.2 (d, J = 3.1 Hz), 95.8 (dd, J = 28.7, 23.9 Hz), 93.9 (dd, J = 26.1, 4.4 Hz), 65.9, 38.3, 28.5 (d, J = 2.0 Hz). **^{19}F NMR (376 MHz, $CDCl_3$)** δ −118.14 (td, $J_{FH/FF}$ = 9.2, 4.6 Hz), −119.48 (dd, $J_{FH/FF}$ = 11.0, 4.8 Hz). **HRMS (ESI) *m/z*** $[M + H]^+$ Calcd for $C_{26}H_{20}F_2N_3O_2S$, 476.1239; found 476.1246. **IR** (cm^{-1}) 3354, 1717, 1674.

Large Scale Synthesis of 8

The same procedure as above was followed from 1.25 g (5.94 mmol) of starting gramine **2b** and using the following quantities of solvent and reagents: 50 mL dry CH_2Cl_2, 2-benzylthioimidazolone **6b** (2 eq., 11.88 mmol, 3.69 mg) and ethyl propiolate **3** (1.1 eq., 6.54 mmol, 0.64 g). Yield 2.54 g, 5.34 mmol, 90%.

Hydrolysis of 8 into Fluorinated Hydantoin 9

A solution of aq. HCl 6 M (11 eq. 58.74 mmol, 9.79 mL) was added dropwise to a solution of imidazolone **8** (5.34 mmol, 2.54 g) in 1,4-dioxane (80 mL) at 0 °C. Once the addition was complete, the reaction was stirred at room temperature overnight. Afterward, the mixture was cooled to 0 °C and a saturated solution of $NaHCO_3$ was added until basic pH was reached. The aqueous layer was extracted with CH_2Cl_2 threefold and the combined organic layers were dried over $MgSO_4$ and the solvent evaporated under reduced pressure. The crude product was purified by silica gel flash column chromatography (hexane/EtOAc 3:1 to 1:1) to obtain hydantoin **9** as a white foam. Yield: 1.37 g, 3.70 mmol, 69%. **^{1}H NMR (300 MHz, $CDCl_3$)** δ = 8.46 (s, 1H), 8.11 (s, 1H), 7.54–7.23 (m, 5H), 6.96 (d, J = 2.3 Hz, 1H), 6.83 (dd, J_{HF} = 8.9, J_{HH} = 2.0 Hz, 1H), 6.43 (ddd, J_{HF} = 11.0, 9.9, J_{HH} = 2.0 Hz, 1H), 5.24 (dd, J = 5.2, 3.3 Hz, 1H), 3.79 (dd, J = 15.1, 5.2 Hz, 1H), 3.56–3.43 (dd, J = 15.1, 3.3 Hz, 1H). **^{13}C NMR {^{1}H} (126 MHz, CD_3OD)** δ = 174.4, 170.1, 160.3 (dd, J_{CF} = 237.2, 12.0 Hz), 157.4 (dd, J_{CF} = 247.3, 15.0 Hz), 155.0, 139.4 (t, J_{CF} = 14.3 Hz), 135.5, 133.0, 130.2, 128.4, 126.2 (d, J_{CF} = 2.3 Hz), 114.6 (d, J_{CF} = 18.7 Hz), 107.0 (d, J_{CF} = 2.9 Hz), 95.5 (dd, J_{CF} = 29.2, 24.0 Hz), 94.8 (dd, J_{CF} = 26.0, 4.4 Hz), 62.9, 25.9 (d, J_{CF} = 2.5 Hz). **^{19}F NMR (376 MHz, $CDCl_3$)** δ −117.73 (td, $J_{FH/FF}$ = 9.5, 4.5 Hz), −118.41 (dd, $J_{FH/FF}$ = 11.1, 4.6 Hz). **HRMS (ESI) *m/z*** $[M + H]^+$ Calcd for $C_{19}H_{14}F_2N_3O_3$, 370.0998; found, 370.1005. **IR** (cm^{-1}): 3232, 1791, 1731.

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Conversion of Hydantoin 9 into Fluorinated Tryptophan 1

To a solution of hydantoin **9** (1 eq., 3.70 mmol, 1.37 g) in 35 mL H_2O at 0 °C (ice bath), $Ba(OH)_2$ monohydrate (4 eq., 14.8 mmol, 2.80 g) was added. The resulting mixture was refluxed for 96 hours, with evolution of ammonia gas and formation of barium carbonate being observed. The mixture was then cooled down to room temperature and the precipitate was filtered and washed with water. The aqueous layer was acidified with HCl 3 *M* (6 eq., 22.2 mmol, 3.7 mL) and then water and the volatiles (HCl) were removed under reduced pressure in the rotary evaporator first and finally under vacuum pump. The resulting solid was dissolved in water and then loaded into a dowex® 50WX2 hydrogen form (50–100 mesh) ion exchange resin (previously washed with 1.0 M HCl). Afterwards, the resin was treated with water until the pH of the eluent was neutral. Then, the compound was liberated from the resin by eluting a 5% NH_3 aqueous solution. The obtained basic solution was concentrated under vacuum to yield the 4,6-difluoro-Trp **1** on its zwitterionic form as a pale brown solid, m.p. = 259–262 °C. Yield: 0.83 g, 3.47 mmol, 94%. **^{1}H NMR (300 MHz, D_2O)** δ = 7.22 (s, 1H), 7.08 (dd, J_{HF} = 9.6, J_{HH} = 2.1 Hz, 1H), 6.83–6.69 (m, 1H), 4.01 (dd, J = 8.9, 4.9 Hz, 1H), 3.53 (dd, J = 15.1, 4.8 Hz, 1H), 3.20 (dd, J = 15.0, 9.0 Hz, 1H). **^{13}C{^{1}H} NMR (126 MHz, CD_3OD)** δ = 173.9, 160.6 (dd, J_{CF} = 237.3, 12.3 Hz), 157.7 (dd, J_{CF} = 246.0, 15.3 Hz), 140.3 (t, J_{CF} = 14.7 Hz), 126.0 (d, J_{CF} = 2.9 Hz), 113.8 (d, J_{CF} = 19.4 Hz), 108.9, 95.4 (dd, J_{CF} = 29.3, 24.4 Hz), 94.9 (dd, J_{CF} = 26.0, 4.4 Hz), 57.3, 29.3. **^{19}F NMR (376 MHz, CD_3OD)** δ −121.54 (td, $J_{FH/FF}$ = 9.9, 4.1 Hz), −122.51 (dd, $J_{FH/FF}$ = 11.2, 4.3 Hz). **HRMS (ESI)** *m/z*: $[M+H]^+$ Calcd for $C_{11}H_{11}F_2N_2O_2$, 241.0783; found 241.0789. **IR** (cm^{-1}): 3457, 1646, 1557, 1540, 1507.

Protein Preparation

BL21 (DE3) E. coli were transformed with a plasmid encoding a construct coding for the HIV capsid-CTD, as previously described.[15] Cultures were grown at 37 °C in M9 modified minimal medium containing 100 µg/L Carbenicillin, 1 g/L−$^{15}NH_4Cl$, and 2 g/L of glucose as the sole nitrogen and carbon sources, respectively. At OD_{600} of 0.6–0.7, the culture was supplemented with 1 g/L of glyphosate and 100 mg/L of the two aromatic amino acids, phenylalanine and tyrosine as well as 50 mg/L of the fluorinated amino acid, 4-fluoro tryptophan, 6-fluoro tryptophan or 4,6-difluoro tryptophan. After 45 minutes, protein expression was induced with 1 mM IPTG. The culture was then grown at 18 °C for an additional 16 h. Cells were harvested by centrifugation at 3,500 g for 30 min at 4 °C. Cell pellets were resuspended in 40 mL lysis buffer (25 mM sodium phosphate, pH 5.8, 5 mM DTT) and lysed by sonication. Cell debris was removed by centrifugation at 33,000 g for 40 min at 4 °C. The clarified lysate was loaded onto 5 mL ion-exchange column (HiTrap SP HP, 5 mL) and eluted using a gradient from 0–50% 1 mM NaCl in 25 mM sodium phosphate buffer, pH 5.8, 5 mM DTT. Protein eluted between 10–15% NaCl and protein containing fractions were collected, pooled, and concentrated in an Amicon with a 3000 Da cut-off. The concentrated protein was fractionated by size-exclusion over a Superdex 75 10/300 GL column (GE Healthcare Life Sciences) using phosphate buffered saline, pH 7.4, containing 2 mM DTT. Protein purity and F incorporation was assessed by PAGE and mass spectrometry.

^{19}F NMR Spectroscopy

All ^{19}F spectra of individual amino acids and fluorinated proteins in the same aqueous buffer were recorded at 298 K on a 14.1 T Bruker AVANCE spectrometer, equipped with a CP TXO F/C-H-D triple-resonance, z-axis gradient cryoprobe at 283 K. ^{19}F chemical shifts were referenced to trifluoracetic acid. ^{19}F spectra were collected with 16,000 data points and a spectral width of 50 ppm using a recycle delay of 5 s. The carrier frequency was set to −123 ppm. All spectra were processed using Bruker Topspin 3.2. ^{19}F spectra of compounds **1**, **2b**, **7b**, **8** and **9** were recorded at 298 K on a Bruker Avance Neo 400, equipped with Iprobe, at 376.5 MHz using monofluorobenzene as internal reference. These spectra were processed using Topspin 4.1.4 and Mestrenova software.

Acknowledgements

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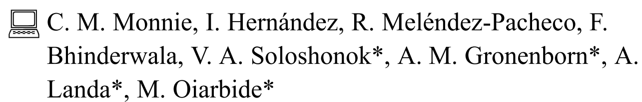
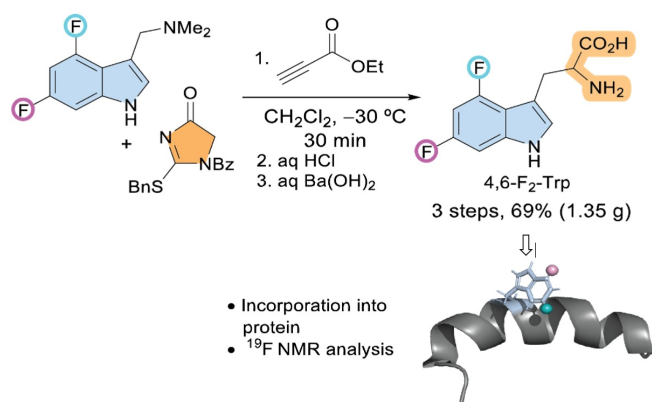


C. M. Monnie, I. Hernández, R. Meléndez-Pacheco, F. Bhinderwala, V. A. Soloshonok*, A. M. Gronenborn*, A. Landa*, M. Oiarbide*



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