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Chemical and Enzymatic Synthesis of Fluorinated Dehydroalanine-Containing Peptides

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2-(2-Acetylamino-acetylamino)-3-(4-methoxy-phenylsulfanyl)-propionic Acid Benzhydryl Ester (14). The procedure described by Hoeg-jensen et al was modified.[1] Compound 6 (0.633 g, 1.03 mmol) was dissolved in 10 mL of $CHCl_3$. 4-Aminomethylpiperidine (4-AMP, 3 mL) was added and the mixture was stirred for 30 min. The solution was diluted with $CHCl_3$ (80 mL), and extracted with 10% aqueous phosphate buffer, pH=5.5 (5 × 25 mL). The organic layer was dried and concentrated. The peptide coupling was started by adding Ac-Gly-OH (0.120 g, 1.03 mmol) and PyBOP (0.535 g, 1.03 mmol) in $CHCl_3$ (10 mL), followed by DIEA (0.279 g, 2.16 mmol). After stirring for 30 min, the solution was diluted with $CHCl_3$ (120 mL), washed with 1 N HCl (30 mL), saturated aqueous $NaHCO_3$ and brine. The organic layer was dried and concentrated. Purification over silica gel (CH_2Cl_2: MeOH / 20:1) provided the product as a white powder (0.386 g, 76%). R_f: 0.25. mp 169-171 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 1.96 (s, 3H, CH_3), 3.22 (B of ABX, Jab = 14.04 Hz, Jbx = 5.8, 6.2 Hz, 1H, CH_2), 3.39 (A of ABX, Jab =14.04 Hz, Jax = 4.6 Hz, 1H, CH_2), 3.75 (s, 3H, OCH_3), 3.82 (qd, J = 16.9 Hz, 5.12 Hz, 2H, CH_2), 4.85 (X of ABX, 1H, CH), 6.40 (t, J = 4.98 Hz, 1H, NH), 6.75 (d, J = 8.82 Hz, 2H, Ph), 6.78 (s, 1H, $CH(Ph)_2$), 7.04 (d, J = 7.52 Hz, 1H, NH), 7.25-7.35 (m, 12H, Ph); ^{13}C NMR (100 MHz, $CDCl_3$): 22.7 (CH/CH_3), 37.7 (C/CH_2), 42.8 (C/CH_2), 52.5 (CH/CH_3), 55.2 (CH/CH_3), 78.4 (CH/CH_3), 114.6 (CH/CH_3), 124.5 (C/CH_2), 126.9 (CH/CH_3), 127.9 (CH/CH_3), 128.0 (CH/CH_3), 128.4 (CH/CH_3), 134.1 (CH/CH_3), 139.1 (C/CH_2), 139.3 (C/CH_2), 139.5 (C/CH_2), 159.3 (C/CH_2), 168.9 (C/CH_2), 169.3 (C/CH_2), 170.6 (C/CH_2). IR: 3288, 3064, 1744, 1653, 1534, 1494, 1286, 1246, 1175, 1030, 827, 743, 670 cm^{-1}. HRMS (FAB^+): calculated $C_{27}H_{28}N_2O_5S$ 492.1719, found $[M^+ + H]$ 493.1796.

Fmoc-Ala-ODpm. The same procedure was followed as given for compound **4** (48%). R_f: 0.18 (hexane/ethyl acetate: 5/1). ^1H NMR (400 MHz, $CDCl_3$): δ ppm 1.48 (d, J=7.2 Hz, 3H, CH_3), 4.22 (t, J=7.1 Hz, 1H, CH), 4.39 (m, 2H, CH_2), 4.55 (q, J=7.4 Hz, 1H, CH), 5.40 (d, J=8.8 Hz, 1H, NH), 6.91 (s, 1H, $CH(Ph)_2$), 7.51 (m, 18H, Ph). ^{13}C NMR (500 MHz, $CDCl_3$, APT): δ ppm 18.9 (CH/CH_3), 47.3 (CH/CH_3), 50.0 (CH/CH_3), 67.3 (C/CH_2), 78.3 (CH/CH_3), 120.2 (CH/CH_3), 125.4 (CH/CH_3), 127.3 (CH/CH_3), 128.1 (CH/CH_3), 128.4 (CH/CH_3), 128.9 (CH/CH_3), 135.8 (C/CH_2), 140.4 (C/CH_2), 141.5

(C/CH_2), 155.9 (C/CH_2), 172.3 (C/CH_2). IR: 3338, 3063, 3033, 1722, 1518, 1450, 1248, 1207, 1173, 1076, 759, 741, 699 cm^{-1}. HRMS (FAB^+): calculated $C_{31}H_{28}NO_4$ [M+H] 478.2018, found 478.2017.

Boc-Cys(Ar)-OH (**18**). NaH (60 %) (0.258 g, 6.46 mmol) was suspended in DMF (15 mL) and 4-methoxybenzenethiol (0.905 g, 6.46 mmol) was added dropwise. The reaction mixture was stirred at ambient temperature for 30 min and then transferred to a 50-mL flask which contained the Vederas lactone[2] (1.007 g, 5.38 mmol) in 15 mL of DMF. The reaction mixture was then stirred overnight and quenched with 5% aq. $KHSO_4$ (40 mL). The aqueous layer was extracted with EtOAc (3 x 40 mL). The organic layer was collected, dried with $MgSO_4$ and concentrated to give colorless solid. Yield: 98%. ^1H NMR (400 MHz, $CDCl_3$): δ ppm 1.36 (s, 9H, Boc), 3.18-3.34 (ABX, Jab = 18.1 Hz, Jax = 10.6 Hz, Jbx =30.4 Hz, 2H, CH_2), 3.72 (s, 3H, OCH_3), 4.45 (q, J = 6.0 Hz, 1H, CH), 5.36 (d, J =7.6 Hz, 1H, NH), 6.83 (d, J = 8.3 Hz, 2H, Ph), 7.36 (d, J = 7.9 Hz, 2H, Ph). The ^1H NMR spectrum was consistent with literature values.[3]

Fmoc-Cys(Ar)-OH (**19**). Compound **18** (1.9 g, 5.80 mmol) was dissolved in 20 mL of CH_2Cl_2. TFA (10 mL) was added and the reaction mixture was stirred at ambient temperature for 35 min. The solvent and excess TFA were removed under reduced pressure. Water (8 mL) and Et_3N (1.62 mL, 11.6 mmol) were added and the mixture was cooled to 0 ^oC. Fmoc-OSu (1.95 g, 5.80 mmol) in CH_3CN (15 mL) was added into the solution. The reaction mixture was stirred at ambient temperature for 45 min and acidified to pH 2 with 1N HCl. The aqueous layer was extracted with EtOAc (3 x 40 mL), and the organic layer was collected, dried with $MgSO_4$ and concentrated. The residue was dissolved in sat. aq. $NaHCO_3$ (20 mL) and extracted with ether (2 x 40 mL). The aqueous layer was collected, acidified to pH 2 with 1N HCl and extracted with EtOAc (3 x 40 mL). The organic layer was combined, dried with $MgSO_4$ and concentrated to afford the product as a white solid. Yield: 67%. R_f: 0.18 (CH_2Cl_2/MeOH: 20/1). ^1H NMR (400 MHz, $CDCl_3$): δ ppm 3.28-3.37 (ABx, Jab = 14.3 Hz, Jax = 4.6 Hz, Jbx= 6.2 Hz, 2H, CH_2), 3.72 (s, 3H, OCH_3), 4.20 (t, J = 7.5 Hz, 1H, CH), 4.35 (dd, J = 7.6, 3.9 Hz, 2H, CH_2), 4.57 (q, J = 6.1 Hz, 1H, CH), 5.57 (d, J = 8.0 Hz, 1H, NH), 6.81 (d, J = 8.9 Hz, 2H, Ph), 7.32 (t, J = 7.1 Hz, 2H, Ph), 7.41 (t, J =7.7 Hz, 4H, Ph), 7.58 (t, J = 6.9 Hz, 2H, Ph), 7.77 (d, J = 7.7 Hz, 2H, Ph). ^{13}C NMR (400 MHz, $CDCl_3$): δ ppm 38.5, 47.3, 53.8, 55.5, 67.6, 115.0, 120.2, 125.4, 127.3, 128.0, 134.9, 141.5, 146.2, 159.9, 174.3. IR: 2984, 2939, 1737, 1495, 1374, 1240, 1046, 739 cm^{-1}. HRMS (FAB^+): calculated $C_{25}H_{24}NO_5S$ [M+H] 450.1375, found 450.1376.

Preparation of 28. A solution of D-[2-^2H]-3-fluoroalanine (fludalanine, 0.1 g, 0.8 mmol) in 2 N NaOH (1 mL) and 50% aq dioxane (1 mL) was cooled to 0 ^oC. Benzyl chloroformate (0.6 mL) and 4 N NaOH (1 mL) were added. The reaction mixture was stirred at 0 ^oC for an additional 2 h and extracted with ether (2 x 30 mL) and petroleum ether (30 mL). The aqueous layer was cooled to 0 ^oC, acidified to pH 1 with 1 N HCl, and extracted with EtOAc (60 mL). The organic layer was dried with $MgSO_4$ and concentrated to give 2-benzyloxycarbonylamino-3-fluoro-propionic acid **33**. Yield: 97%. m.p: 82-83 ^oC. ^1H NMR (400 MHz, $CDCl_3$): δ ppm 4.68 (dd, 1H, J = 47.7, 9.6 Hz, CH_2F), 4.87 (dd, 1H, J = 46.1, 9.5 Hz, CH_2F), 5.15 (s, 2H, CH_2Ph), 5.90 (s, 1H, NH),

7.37 (s, 5H, Ph), 10.46 (s, 1H, COOH). ^{13}C NMR (125 MHz, $CDCl_3$): δ ppm 54.6, 67.8, 83.3 (d, J = 173.3 Hz), 128.4, 128.6, 128.9, 136.0, 156.4, 173.7. ^{19}F NMR (376 MHz, $CDCl_3$): δ ppm –231.1 (t, J = 47.0 Hz). HRMS (FAB): calculated [M+H] $C_{11}H_{12}D_1F_1N_1O_4$ 243.0891, found 243.0892.

Compound **33** (0.763 g, 3.15 mmol) and *para*-nitrobenzyl bromide (0.681 g, 3.15 mmol) were dissolved in 8.8 mL of HMPA, and Et_3N (0.47 mL) was added. The reaction mixture was stirred at ambient temperature for 2 h, quenched with 20 mL of ice-cold water, and extracted with Et_2O/EtOAc (80 mL, 2:1). The organic layer was washed with 0.1 N HCl (20 mL) and water (20 mL), dried with $MgSO_4$ and concentrated to give the 4-nitrobenzyl ester of 2-benzyloxycarbonylamino-3-fluoro-propionic acid (**34**). Yield: 80%. R_f: 0.36 (hexane/ethyl acetate: 2/1). m.p: 127 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 4.69 (dd, 1H, J = 47.6, 9.5 Hz, CH_2F), 4.89 (dd, 1H, J = 46.1, 9.5 Hz, CH_2F), 5.14 (s, 2H, CH_2Ph), 5.33 (q, 2H, J = 9.2 Hz, CH_2Ph), 5.62 (s, 1H, NH), 7.36 (s, 5H, Ph), 7.49 (d, 2H, J = 8.80Hz, Ph), 8.23 (d, 2H, J = 8.7 Hz, Ph). ^{13}C NMR (125 MHz, $CDCl_3$, APT): δ ppm 66.4 (C/CH_2), 67.6 (C/CH_2), 83.4 (d, J = 137.2 Hz)(C/CH_2), 124.2 (CH/CH_3), 128.4 (CH/CH_3), 128.6 (CH/CH_3), 128.9 (CH/CH_3), 136.1 (C/CH_2), 142.2 (C/CH_2), 148.1 (C/CH_2), 156.1 (C/CH_2), 168.8 (C/CH_2). ^{19}F NMR (470 MHz, $CDCl_3$): δ ppm -124.2 (t, J = 47.9 Hz). HRMS (FAB): calculated [M+H] $C_{18}H_{17}D_1F_1N_2O_6$ 378.1212, found 378.1212. Compound **34** (0.047 g, 0.125 mmol) was stirred at ambient temperature for 30 min in 30% HBr/acetic acid (0.5 mL). Dry ether (5 mL) was added to precipitate the product, which was obtained by filtration and washed with dry ether to afford a colorless solid. Z-Ala-OH (0.022 g, 0.101 mmol) in DMF (0.3 mL) was cooled to –20 ^{o}C. Et_3N (0.014 mL, 0.101 mmol) and isobutyl chloroformate (0.014 g, 0.101 mmol) were added and the reaction mixture was stirred for an additional 20 min. Then the fluoroalanine solution in DMF (0.5 mL) and Et_3N (0.014 mL, 0.101 mmol) were added dropwise to the above mixture. The reaction mixture was warmed up to ambient temperature and stirred for 5 h. The mixture was added to ice-cold 0.05 M HCl (20 mL), and extracted with EtOAc (80 mL). The organic layer was washed with 0.05 M HCl (20 mL), diluted aqueous $NaHCO_3$ (30 mL), water (30 mL) and brine (30 mL). The organic layer was dried with $MgSO_4$ and concentrated. Purification by silica gel chromatography eluting with CH_2Cl_2/MeOH (40/1) gave 2-(2-benzyloxycarbonylamino-propionylamino)-3-fluoro-propionic acid 4-nitro-benzyl ester **35**. R_f: 0.42. Yield: (28 mg, 50%). m.p: 164 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 1.41 (d, 3H, J = 7.2 Hz, CH_3), 4.37 (q, 1H, J = 7.2 Hz, CH), 4.65 (dd, 1H, J = 48.0, 10.2 Hz, CH_2F), 4.88 (dd, 1H, J = 46.3, 9.2 Hz, CH_2F), 5.10 (ABq, 2H, Jab = 12.3 Hz, CH_2Ph), 5.28 (Abq, 2H, Jab = 13.3 Hz, CH_2Ph), 5.30 (d, 1H, J = 7.2 Hz, NH), 7.08 (s, 1H, NH), 7.33 (s, 5H, Ph), 7.48 (d, 2H, J = 8.9 Hz, Ph), 8.22 (d, 2H, J = 8.9 Hz, Ph). ^{13}C NMR (125 MHz, $CDCl_3$, APT): δ ppm 18.6 (CH/CH_3), 50.7 (CH/CH_3); 66.4 (C/CH_2), 67.5 (C/CH_2), 82.2 (C/CH_2), 84.0 (C/CH_2), 124.2 (CH/CH_3), 128.3 (CH/CH_3), 128.6 (CH/CH_3), 128.8 (CH/CH_3), 136.2 (C/CH_2), 142.2 (C/CH_2), 148.1 (C/CH_2), 168.5 (C/CH_2), 168.6 (C/CH_2), 172.7 (C/CH_2). ^{19}F NMR (376 MHz, $CDCl_3$): δ ppm -230.51 (t, J = 46.6 Hz). HRMS (FAB): calculated [M+H] $C_{21}H_{22}D_1F_1N_3O_7$ 449.1583, found 449.1584. Compound **35** (0.317 g, 0.71 mmol) and Pd/C (0.186 g, 5%) in warm acetic acid (56 mL), and water (5.6 mL) were placed in a 100-mL round-bottomed flask and flushed with N_2 for 5 min. The reaction mixture was stirred at ambient temperature with continuous bubbling of H_2 through the solution for 1.5 d. The catalyst was filtered off, the filtrate was concentrated to 2 mL, and

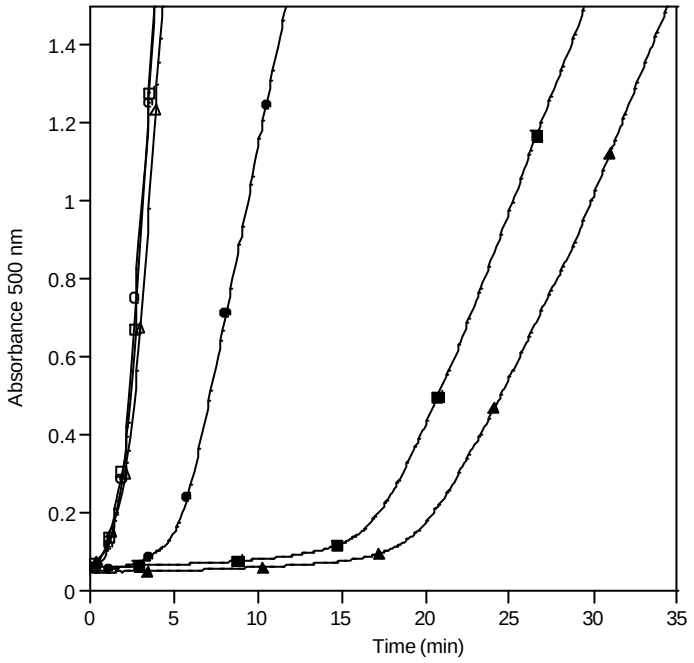
EtOH/acetone (1/2) was added to precipitate 2-(2-amino-propionylamino)-3-fluoro-propionic acid **28**. The precipitate was washed with acetone and dry ether. Yield: (96 mg, 75%). m.p: 219-220 °C (decomposed). ^{1}H NMR (400 MHz, D_2O): δ ppm 1.37 (d, 3H, J = 7.1 Hz, CH_3), 3.98 (q, 1H, J = 7.1 Hz, CH), 4.52 (dd, 1H, J = 51.1, 9.8 Hz, CH_2F), 4.63 (dd, 1H, J = 49.5, 9.8 Hz, CH_2F); ^{19}F NMR (376 MHz, D_2O): δ -228.2 (tt, J = 46.8, 4.18 Hz). HRMS (FAB): calculated [M+H] $C_6H_{11}D_1F_1N_2O_3$ 180.0895, found 180.0895.

Preparation of 31. 2-Benzyloxycarbonylamino-3,3-difluoro-propionic acid was prepared as described in the literature[4] with some modifications as described in the Supporting Information. The compound was converted to its 4-nitrobenzyl ester using the same procedure as for compound **34** resulting in a 58% yield. R_f: 0.37 (hexane/ethyl acetate: 2/1). ^{1}H NMR (500 MHz, $CDCl_3$): δ ppm 4.86-4.94 (m, 1H, C<u>H</u>CHF_2), 5.14 (s, 2H, CH_2Ph), 5.32 (ABq, 2H, Jab = 14.0 Hz, CH_2Ph), 5.69 (d, 1H, J = 9.1 Hz, NH), 6.13 (td, 1H, J = 54.5, 1.7 Hz, CHF_2), 7.34 (s, 5H, Ph), 7.48 (d, 2H, J = 8.4 Hz, Ph), 8.21 (d, 2H, J = 8.5 Hz, Ph). ^{13}C NMR (100 MHz, $CDCl_3$): δ ppm 56.4 (t, J = 23.1 Hz), 66.8, 68.1, 113.1 (t, J = 247.4 Hz), 124.2, 128.5, 128.7, 128.8, 128.9, 135.8, 141.7, 148.2, 156.0, 166.3. ^{19}F NMR (376 MHz, $CDCl_3$): δ ppm –126.6 (dd, J = 54.0, 16.4 Hz, CHF_2), -126.7 (dd, J = 54.2, 13.2 Hz, CHF_2). HRMS (FAB): calculated [M+H] $C_{18}H_{17}F_2N_2O_6$ 395.1055, found 395.1056. 2-(2-Benzyloxycarbonylamino-propionylamino)-3,3-difluoro-propionic acid 4-nitro-benzyl ester was prepared using the same procedure as for compound **35** providing a yield of 67%. R_f: 0.44 (CH_2Cl_2/MeOH: 40/1). ^{1}H NMR (500 MHz, $CDCl_3$): δ ppm 1.41 (d, 3H, J = 7.1 Hz, CH_3), 4.40 (q, 1H, J = 6.2 Hz, 1H, C<u>H</u>CH_3), 5.07-5.17 (m, 3H, CH_2Ph + C<u>H</u>CHF_2), 5.31 (Abq, 2H, Jab = 13.0 Hz, CH_2Ph), 5.27-5.38 (m, 1H, NH), 6.12 (t, 1H, J = 54.1 Hz, CHF_2), 7.09 (d, 1H, J =7.3 Hz, NH), 7.32-7.35 (m, 5H, Ph), 7.48-7.51 (m, 2H, Ph), 8.21-8.25 (m, 2H, Ph). ^{13}C NMR (125 MHz, $CDCl_3$): δ ppm 18.3, 50.6, 54.5, 66.8, 67.5, 112.9 (t, J = 247.1 Hz), 124.2, 128.3, 128.6, 128.7, 128.8, 141.7, 164.7, 173.2. ^{19}F NMR (470 MHz, $CDCl_3$): δ ppm -127.1 - -125.3 (m, CHF_2). HRMS (FAB): calculated [M+H] $C_{21}H_{22}F_2N_3O_7$ 466.1426, found 466.1424. 2-(2-Amino-propionylamino)-3,3-difluoro-propionic acid **31** was prepared using the same procedure as for compound **28** providing the target in 52% yield. ^{1}H NMR (500 MHz, D_2O): Diastereomer A: δ 1.41 (d, 3H, J = 7.1 Hz, CH_3), 4.04 (q, 1H, J = 7.1 Hz, C<u>H</u>CH_3), 4.55-4.69 (m, 1H, C<u>H</u>CHF_2), 6.19 (td, 1H, J = 54.6, 2.0 Hz, CHF_2); Diastereomer B: δ 1.44 (d, 3H, J = 7.2 Hz, CH_3), 4.01 (q, 1H, J = 7.1 Hz, C<u>H</u>CH_3), 4.55-4.69 (m, 1H, C<u>H</u>CHF_2), 6.17 (td, 1H, J = 54.5, 2.1 Hz, CHF_2). ^{13}F NMR (400 MHz, D_2O): δ ppm -124.68 (ddd, J = 280.3, 54.7, 8.3 Hz, 1F), 125.0 (J = 279.8, 54.5, 7.0 Hz, 1F), -128.62 (ddd, J = 281.3, 54.5, 26.7 Hz, 1F), -129.22 (ddd, J = 279.5, 54.5, 26.0 Hz, 1F). HRMS (FAB): calculated [M+H] $C_6H_{11}F_2N_2O_3$ 197.0738, found 197.0737.

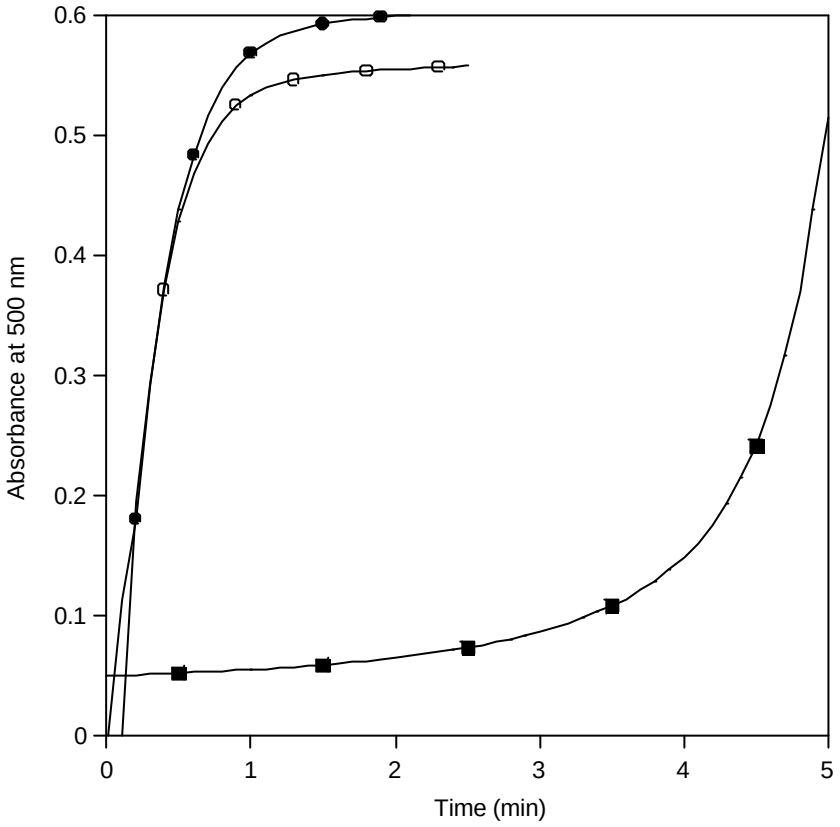
Investigation of the inhibition of the YcjG assay

As described in the text, the lag phase observed in the coupled enzyme assay after addition of a solution of compound **31** treated with YcjG is not due to inhibition of YcjG itself, but is likely caused by inhibition of another component of the assay. Investigation of the pH dependence of the length of the lag showed that at lower pH the lag time was significantly shorter (Supp. Figure 1). A logical candidate for the unstable species that inhibits one of the enzymes in the assay is the fluorinated dehydropeptide **32**. Therefore,

YcjG was separated from the dehydropeptide by ultrafiltration and the resulting solution of **32** was incubated with PepD, ADH, diaphorase, and saturating concentrations of NAD^+, INT and L-Ala-D-Glu. A distinct lag phase was observed indicating that **32** indeed causes temporary inhibition of one of these enzymes. When ADH, L-alanine, diaphorase, NAD^+ and INT were incubated with **32** in the absence of PepD, no lag phase was observed, whereas addition of PepD to this mixture induced the appearance of the lag period (Supporting Figure 2). Thus, cleavage of **32** by PepD produces a species that inhibits ADH or diaphorase. Analysis of the reaction of PepD with **32** showed the production of L-Ala as well as at least one other product, the exact structure of which we have not been able to determine. When the process was followed by ^{19}F NMR spectroscopy, rapid release of fluoride anion was observed and no organofluorine intermediates were observed. Although we were not able to determine the structure of the inhibitor generated by PepD, we did establish that the inhibitor is not fluoropyruvate as incubation of ADH, L-Ala, diaphorase and INT with this compound did not induce a lag phase.

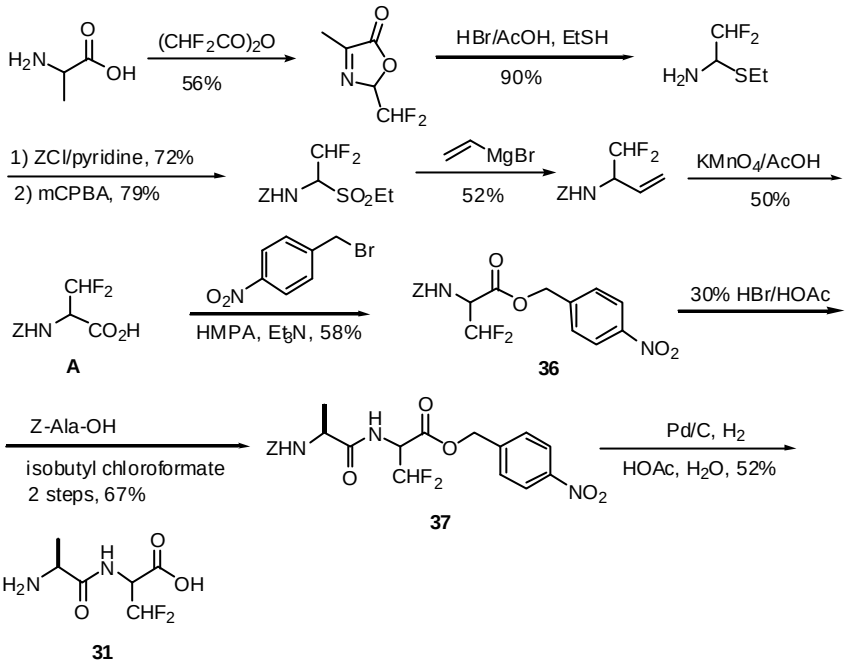


Supporting Figure 1. Activity assays contained L-Ala-D-Glu (250 μM), ADH (10 U), diaphorase (2 U), NAD^+ (1.5 mM), INT (1.5 mM), $CoCl_2$ (67 μM), PepD (200 μg), and YcjG (10 μg) at pH 6.5 (open circles), pH 8.5 (open squares), and pH 9.5 (open triangles). Addition of peptide **31** to YcjG for 5 min at pH 6.5 (closed circles), pH 8.5 (closed squares), and pH 9.5 (closed triangles) introduced a lag period of varying length with the assay conditions as above (all assays at pH 8.5).



Supporting Figure 2. Activity assay containing L-Ala (15 μM), ADH (7 U), diaphorase (2 U), NAD^+ (1.5 mM), INT (1.5 mM), $CoCl_2$ (67 μM) (closed circles). Addition of peptide **32** to the assay did not affect the production of reduced dye (open circles), but addition of PepD resulted in the appearance of a distinct lag period (closed squares).

Synthetic Scheme for the preparation of 31.



The conversion of alanine to benzyloxycarbonyl protected difluoroalanine was performed modifying the procedure described previously in the literature.[4]

2-Difluoromethyl-4-methyl-5(2H)-oxazolone. Alanine (0.5 g, 5.6 mmol) was added into difluoroacetic anhydride (2.44 g, 14 mmol) at 0 ^{o}C. The reaction mixture was stirred at ambient temperature for 1 h and then was heated at 90 ^{o}C for 4 h. The mixture was diluted with 100 mL of CH_2Cl_2 and washed with sat. $NaHCO_3$ (20 mL × 3), water (30 mL), and brine (30 mL). The organic layer was dried with $MgSO_4$ and concentrated to give the desired product (0.334 g). Yield: 40 %. ^{1}H NMR (500 MHz, $CDCl_3$): δ ppm 2.35 (d, 3H, J = 2.4 Hz, CH_3), 5.95 (td, 1H, J = 53.4, 2.5 Hz, CHF_2), 5.96-6.02 (m, 1H, C<u>H</u>CHF$_2$). The ^{1}H NMR spectrum is consistent with the literature.[4]

2,2-Difluoro-1-ethylthioethylamine, hydrobromide. A mixture of HBr in AcOH (30%, 0.44 mL) and EtSH (0.53 mL) was added to 2-difluoromethyl-4-methyl-5(2H)-oxazolone (0.222 g, 1.49 mmol) at 0 ^{o}C. The reaction mixture was stirred at 0 ^{o}C for 15 min and 3 h at ambient temperature. The reaction mixture was concentrated and solidified with ether/hexane to give the desired product. Yield: (0.314 g, 96%). ^{1}H NMR (400 MHz, D_2O): δ 1.15 (t, 3H, J = 7.4 Hz, CH_3), 3.41 (q, 2H, J = 7.1 Hz, CH_2), 4.69 (ddd, 1H, J = 19.8, 7.6, 2.4 Hz, C<u>H</u>CHF$_2$), 6.17 (td, 1H, J = 53.6, 2.3 Hz, CHF_2). The ^{1}H NMR spectrum is consistent with the literature.[4]

***N*-Benzyloxycarbonyl-2,2-difluoro-1-ethylthioethylamine**. 2,2-Difluoro-1-ethyl-thioethylamine (0.044 g, 0.198 mmol) in 0.5 mL of anhydrous CH_2Cl_2 was cooled to –15 ^{o}C. Pyridine (0.040 mL, 0.5 mmol) and benzylchloroformate (0.03 mL, 0.23 mmol) were added and the reaction mixture was stirred at –10 ^{o}C for 1 h, 0 ^{o}C for 5 h and at ambient temperature overnight. The mixture was diluted with CH_2Cl_2 (100 mL), washed with aq. 0.1 N HCl (30 mL), sat. aq. $NaHCO_3$ (30 mL), water (30 mL) and brine (30 mL). The organic layer was dried with $MgSO_4$ and concentrated. Purification over silica gel with hexane/EtOAc: 5/1 afforded the product as a solid (0.035 g, 89 %). R_f: 0.40. ^{1}H NMR (400 MHz, $CDCl_3$): δ ppm 1.30 (t, 3H, J = 7.3 Hz, CH_3), 2.70 (m, 2H, CH_2), 5.16 (s, 2H, CH_2Ph), 5.11-5.26 (m, 2H, C<u>H</u>CHF$_2$ + NH), 5.90

(t, 1H, J = 55.5 Hz, CHF_2), 7.37 (s, 5H, Ph). The ^{1}H NMR spectrum is consistent with the literature.[4]

***N*-Benzyloxycarbonyl-2,2-difluoro-1-ethylsulfonylethylamine**. *N*-Benzyloxycarbonyl-2,2-difluoro-1-ethylthioethylamine was dissolved in CH_2Cl_2 (10 mL) and cooled to –20 ^{o}C. A solution of mCPBA (0.887 g, 60%, 3.08 mmol) in CH_3CN (12 mL) was added dropwise. The reaction mixture was stirred for an additional 1 h at –20 ^{o}C, then 1 h at 0 ^{o}C and finally overnight at ambient temperature. The reaction mixture was diluted with CH_2Cl_2 (100 mL) and washed with 10% $Na_2S_2O_3$ (30 mL), sat. aq $NaHCO_3$ (30 mL), water (30 mL) and brine (30 mL). The organic layer was dried with $MgSO_4$ and concentrated to give a colorless solid (79%). This compound was used in the next step without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.39 (t, 3H, J = 7.5 Hz, CH_3), 2.95 (m, 2H, CH_2CH_3), 5.19 (s, 2H, CH_2Ph), 5.28 (m, 1H, C<u>H</u>CHF_2), 6.19 (d, 1H, J = 10.5 Hz, NH), 6.43 (t, 1H, J = 54.6 Hz, CHF_2), 7.36 (s, 5H, Ph). The ^{1}H NMR spectrum is consistent with the literature.[4]

***N*-Benzyloxycarbonyl-2,2-difluoro-1-vinylethylamine**. A solution of *N*-benzyloxycarbonyl-2,2-difluoro-1-ethylsulfonylethylamine (0.203 g, 0.74 mmol) in anhydrous THF (3 mL) was added dropwise under N_2 to a stirred suspension of vinylmagnesium bromide in THF (1 M, 3.3 mL) at 0 ^{o}C. The reaction mixture was stirred at 0 ^{o}C for 1.5 h and quenched by addition of a AcOH-water mixture (2:1, 1.5 mL). The mixture was diluted with CH_2Cl_2 (100 mL) and washed with 0.1 N HCl (50 mL), sat. aq $NaHCO_3$ (30 mL), water (30 mL) and brine (30 mL). The organic layer was dried with $MgSO_4$ and concentrated. Purification by silica gel chromatography eluting with hexane/ethyl acetate (5/1) gave the desired product as a colorless solid. R_f: 0.24. Yield: 85%. ^{1}H NMR (400 MHz): δ 4.61 (b, 1H, C<u>H</u>CHF_2), 5.00 (b, 1H, NH), 5.14 (s, 2H, CH_2Ph), 5.39-5.44 (m, 2H, $=CH_2$), 5.84 (t, 1H, J = 55.6 Hz, CHF_2), 5.81-5.90 (m, 1H, -CH=), 7.37 (s, 5H, Ph). The ^{1}H NMR spectrum is consistent with the literature.[4]

***N*-Benzyloxycarboyl-3,3-difluoroalanine** (**A**). A solution of *N*-benzyloxycarbonyl-2,2-difluoro-1-vinylethylamine (0.131 g, 0.627 mmol) in AcOH (4 mL) was added dropwise to a $KMnO_4$ (0.272 g, 1.72 mmol) solution in water (19 mL) at 0 ^{o}C. The reaction mixture was stirred at 0 ^{o}C for 2.5 h and at ambient temperature overnight. After the addition of 10% $Na_2S_2O_3$, the reaction mixture was acidified to pH 2 with 1 N HCl and extracted with EtOAc (40 mL x 2). The organic layer was dried with $MgSO_4$ and concentrated to 20 mL. The EtOAc solution was extracted with sat. aq $NaHCO_3$. The aqueous layer was then acidified again to pH 2 with 1 N HCl and extracted with EtOAc (2 x 40 mL). The organic layer was dried with $MgSO_4$ and concentrated to give the desired product as a colorless solid. Yield: (0.097 g, 68%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.86-4.96 (m, 1H, C<u>H</u>CHF_2), 5.17 (s, 2H, CH_2Ph), 5.52 (d, 1H, J = 9.5 Hz, NH), 6.19 (t, 1H, J = 54.6 Hz, CHF_2), 7.37 (s, 5H, Ph). The ^{1}H NMR spectrum was consistent with the literature.[4]

For the conversion of **A** to **31** see the experimental section of the article.

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