**Protease catalyzed peptide synthesis for the site-specific incorporation of
 α -fluoroalkyl amino acids into peptides and proteins**

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Supporting Information

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Characterization of intermediate chemical products.

***N',N''*-Di-*tert.* butyloxycarbonyl-4-guanidinophenyl-*N*-(benzyloxycarbonyl)-**

aminoisobutyrate. Yield 91%. Eluent for Flash column chromatography, petroleum ether/ethyl acetate, 3/1 (v/v), R_f 0.28. $^1\text{H-NMR}$ (300.08 MHz, DMSO) δ (ppm) 1.40-1.48 (m, 24H), 5.07 (s, 2H), 6.93/7.51 (m/m, 4H), 7.34 (m, 5H), 8.02 (s, 1H), 9.98 (s, 1H), 11.40 (s, 1H). MS (m/z) 571 (M+H)⁺.

***N',N''*-Di-*tert.* butyloxycarbonyl-4-guanidinophenyl-*N*-(benzyloxycarbonyl)-(α -**

difluoromethyl)-alanineate. Yield 90%. Eluent for Flash column chromatography petroleum ether/ethyl acetate, 3/1 (v/v), R_f 0.37. $^1\text{H-NMR}$ (300.08 MHz, DMSO) δ (ppm) 1.41 (s, 9H), 1.52 (m, 12H), 5.10-5.17 (m, 2H), 6.40 (t, $J = 55.5$ Hz, 1H), 6.95/7.55 (m/m, 4H), 7.36 (m, 5H), 8.55 (s, 1H), 9.99 (s, 1H), 11.40 (s, 1H), $^{19}\text{F-NMR}$ (282.32 MHz, DMSO) δ (ppm) -52.79 (d/d, $J_{\text{FF}} = 279.4$ Hz, $J_{\text{FH}} = 55.4$ Hz, 1F), -49.57 (d/d, $J_{\text{FF}} = 279.4$ Hz, $J_{\text{FH}} = 55.4$ Hz, 1F). MS (m/z) 607 (M+H)⁺.

***N',N''*-Di-*tert.* butyloxycarbonyl-4-guanidinophenyl-*N*-(benzyloxycarbonyl)-(α -**

trifluoromethyl)-alanineate. Yield 93%. Eluent for Flash column chromatography, petroleum ether/ethyl acetate (3/1), v/v, R_f 0.38. $^1\text{H-NMR}$ (300.08 MHz, DMSO) δ (ppm) 1.40 (s, 9H), 1.52 (s, 9H), 1.71 (s, 3H), 5.12 (m, 2H), 7.00/7.56 (m/m, 4H), 7.35 (m, 5H), 8.79 (s, 1H), 9.99 (s, 1H), 11.39 (s, 1H), $^{19}\text{F-NMR}$ (282.32 MHz, DMSO) δ (ppm) 3.22 (s, 3F). MS (m/z) 625 (M+H)⁺.

***N',N''*-Di-*tert.* butyloxycarbonyl-4-guanidinophenyl-*N*-(benzyloxycarbonyl)-(α -methyl)-**

L-phenylalanineate. Yield 77%. Eluent for Flash column chromatography, petroleum ether/ethyl acetate, 6/1 (v/v), R_f 0.31. $^1\text{H-NMR}$ (300.08 MHz, DMSO) δ (ppm) 1.35 (s, 3H),

1.41 (s, 9H), 1.52 (s, 9H), 3.03-3.30 (m, 2H), 5.11-5.14 (m, 2H), 7.12/7.53 (m/m, 4H), 7.25-7.39 (m, 10H), 7.89 (s, 1H), 9.98 (s, 1H), 11.41 (s, 1H). MS (m/z) 647 (M+H)⁺.

***N',N''*-Di-*tert.* butyloxycarbonyl-4-guanidinophenyl-*N*-(benzyloxycarbonyl)-(α -methyl)-*D*-phenylalanineate.** Yield 74%. Eluent for Flash column chromatography, petroleum ether/ethyl acetate, 6/1 (v/v), *R_f* 0.30. ¹H-NMR (300.08 MHz, DMSO) δ (ppm) 1.36 (s, 3H), 1.41 (s, 9H), 1.52 (s, 9H), 3.03-3.30 (m, 2H), 5.07-5.18 (m, 2H), 7.13/7.53 (m/m, 4H), 7.25-7.42 (m, 10H), 7.89 (s, 1H), 9.97 (s, 1H), 11.41 (s, 1H). MS (m/z) 647 (M+H)⁺.

***N',N''*-Di-*tert.* butyloxycarbonyl-4-guanidinophenyl-*N*-(benzyloxycarbonyl)-(α -difluoromethyl)-leucinate.** Yield 85%. Eluent for Flash column chromatography, petroleum ether/ethyl acetate (10/1), v/v, *R_f* 0.25. ¹H-NMR (300.08 MHz, DMSO) δ (ppm) 0.91-0.98 (m, 6H), 1.40 (s, 9H), 1.52 (s, 9H), 1.86 (m, 3H), 5.09 (s, 2H), 6.49 (t, *J* = 55.9 Hz, 1H), 7.00/7.56 (m/m, 4H), 7.36 (m, 5H), 8.27 (s, 1H), 9.99 (s, 1H), 11.40 (s, 1H), ¹⁹F-NMR (282.32 MHz, DMSO) δ (ppm) -51.54 (m, 1F), -51.35 (m, 1F). MS (m/z) 649 (M+H)⁺.

***N',N''*-Di-*tert.* butyloxycarbonyl-4-guanidinophenyl-*N*-(benzyloxycarbonyl)-(α -difluoromethyl)-phenylalanineate.** Yield 83%. Eluent for Flash column chromatography, petroleum ether/ethyl acetate, 10/1 (v/v), *R_f* 0.19. ¹H-NMR (300.08 MHz, DMSO) δ (ppm) 1.41 (s, 9H), 1.52 (s, 9H), 3.21-3.39 (m, 2H), 5.12 (s, 2H), 6.21 (t, *J* = 55.2 Hz, 1H), 6.88/7.53 (m/m, 4H), 7.24-7.39 (m, 10H), 8.49 (s, 1H), 9.97 (s, 1H), 11.39 (s, 1H), ¹⁹F-NMR (282.32 MHz, DMSO) δ (ppm) -52.08--51.75 (m, 2F). MS (m/z) 683 (M+H)⁺.

***N',N''*-Di-*tert.* butyloxycarbonyl-4-guanidinophenyl-*N*-(benzyloxycarbonyl)-(α -trifluoromethyl)-leucinate.** Yield 82%. Eluent for Flash column chromatography, petroleum

6H), 1.41 (s, 9H), 1.52 (s, 9H), 1.91-2.15 (m, 3H), 5.10 (m, 2H), 7.03/7.57 (m/m, 4H), 7.35 (m, 5H), 8.58 (s, 1H), 9.99 (s, 1H), 11.40 (s, 1H), ^{19}F -NMR (282.32 MHz, DMSO) δ (ppm) 5.92 (s, 3F). MS (m/z) 667 (M+H) $^{+}$.

***N',N''*-Di-*tert.* butyloxycarbonyl-4-guanidinophenyl-*N*-(benzyloxycarbonyl)-(α -trifluoromethyl)-phenylalanineate.** Yield 73%. Eluent for Flash column chromatography, petroleum ether/ethyl acetate, 10/1 (v/v), R_f 0.21. ^1H -NMR (300.08 MHz, DMSO) δ (ppm) 1.41 (s, 9H), 1.52 (s, 9H), 3.21-3.48 (m, 2H), 5.13 (s, 2H), 6.88/7.53 (m/m, 4H), 7.16-7.39 (m, 10H), 8.88 (s, 1H), 9.97 (s, 1H), 11.39 (s, 1H), ^{19}F -NMR (282.32 MHz, DMSO) δ (ppm) 7.05 (s, 3F). MS (m/z) 701 (M+H) $^{+}$.

HPLC retention times and MS data for starting materials and products of enzymatic syntheses

Products were isolated after analytical HPLC; mass spectra were recorded using thermospray ionization.

HPLC data for starting compounds:

Z-Aib-OGp. HPLC-column: Vydac 214TP104, 10 μ m, 250 $\times$ 4 mm (The Separations Group, USA), gradient in 30 min from 5% acetonitril to 35% acetonitril. Retention time for acyl donor ester Z-Aib-OGp 22.1 min and hydrolysis product Z-Aib-OH 19.4 min.

Z-D,L-(α Dfm)Ala-OGp. HPLC-column: Capcell Pak SG 300 C8, 5 μ m, 250 $\times$ 4 mm (Shiseido Co, Ltd., Japan), gradient in 30 min from 5% acetonitril to 50% acetonitril. Retention time for acyl donor ester Z-D,L-(α Dfm)Ala-OGp 19.3 min and hydrolysis product Z-D,L-(α Dfm)Ala-OH 17.3 min.

Z-D,L-(α Tfm)Ala-OGp. HPLC-column: Luna C8 (2), 5 μ m, 250 $\times$ 4.6 mm (Phenomenex, USA), gradient in 30 min from 5% acetonitril to 70% acetonitril. Retention time for acyl donor ester Z-D,L-(α Tfm)Ala-OGp 20.1 min and hydrolysis product Z-D,L-(α Tfm)Ala-OH 22.4 min.

Z-L-(α Me)Phe-OGp and Z-D-(α Me)Phe-OGp. HPLC-column: Luna C8 (2), 5 μ m, 250 $\times$ 4.6 mm (Phenomenex, USA), gradient in 40 min from 5% acetonitril to 60% acetonitril. Retention time for acyl donor esters Z-L-(α Me)Phe-OGp and Z-D-(α Me)Phe-OGp 29.2 min and hydrolysis products Z-L-(α Me)Phe-OH and Z-D-(α Me)Phe-OH 33.6 min.

Z-D,L-(α Dfm)Phe-OGp. HPLC-column: Luna C8 (2), 5 μ m, 250 $\times$ 4.6 mm (Phenomenex,

donor ester Z-D,L-(α Dfm)Phe-OGp 26.8 min and hydrolysis product Z-D,L-(α Dfm)Phe-OH 32.6 min.

Z-D,L-(α Tfm)Phe-OGp. HPLC-column Capcell Pak SG 300 C8, 5 μ m, 250 $\times$ 4 mm (Shiseido Co, Ltd., Japan), gradient in 60 min from 5% acetonitril to 55% acetonitril. Retention time for acyl donor ester Z-D,L-(α Tfm)Phe-OGp 36.0 min and hydrolysis product Z-D,L-(α Tfm)Phe-OH 37.7 min.

Z-D,L-(α Dfm)Leu-OGp. HPLC-column: Luna C8 (2), 5 μ m, 250 $\times$ 4.6 mm (Phenomenex, USA), gradient in 30 min from 5% acetonitril to 60% acetonitril. Retention time for acyl donor ester Z-D,L-(α Dfm)Leu-OGp 24.5 min and hydrolysis product Z-D,L-(α Dfm)Leu-OH 31.6 min.

Z-D,L-(α Tfm)Leu-OGp. HPLC-column: Luna C8 (2), 5 μ m, 250 $\times$ 4.6 mm (Phenomenex, USA), gradient in 40 min from 5% acetonitril to 70% acetonitril. Retention time for acyl donor ester Z-D,L-(α Tfm)Leu-OGp 30.3 min and hydrolysis product Z-D,L-(α Tfm)Leu-OH 36.3 min.

Retention times and MS data for products of Trypsin-catalyzed peptide syntheses:

Z-Aib-Met-NH₂. Retention time: 21.1 min. MS (m/z) 368 (M+H)⁺.

Z-Aib-Ala-Ala-Lys-OH. Retention time: 14.9 min. MS (m/z) 508 (M+H)⁺.

Z-D,L-(α Dfm)Ala-Gly-NH₂. Retention time: 13.5 min. MS (m/z) 330 (M+H)⁺.

Z-D,L-(α Dfm)Ala-Ala-Arg-OH. Retention time: 13.3 min. MS (m/z) 501 (M+H)⁺.

Z-D,L-(α Tfm)Ala-Leu-NH₂. Retention time: 23.5 min. MS (m/z) 404 (M+H)⁺.

Z-D,L-(α Tfm)Ala-Ala-Arg-OH. Retention time: 16.3 min. MS (m/z) 519 (M+H)⁺.

Z-L-(α Me)Phe-Met-NH₂. Retention time: 32.2 min. MS (m/z) 444 (M+H)⁺.

Z-L-(α Me)Phe-Ala-Ala-Lys-OH. Retention time: 23.7 min. MS (m/z) 584 (M+H)⁺.

Z-D-(α Me)Phe-Met-NH₂. Retention time: 32.3 min. MS (m/z) 444 (M+H)⁺.

Z-D-(α Me)Phe-Ala-Ala-Lys-OH. Retention time: 23.2 min. MS (m/z) 584 (M+H)⁺.

Z-D,L-(α Dfm)Phe-Ala-Met-OH. Retention time: 31.4 min. MS (m/z) 552 (M+H)⁺.

Z-D,L-(α Dfm)Phe-Ala-Ala-Ala-OH. Retention time: 28.0 min. MS (m/z) 563 (M+H)⁺.

Z-D,L-(α Tfm)Phe-Ala-Arg-NH₂. Retention time: 28.6 min. MS (m/z) 595 (M+H)⁺.

Z-D,L-(α Tfm)Phe-Ala-Ala-Pro-OH. Retention time: 35.6 min. MS (m/z) 607 (M+H)⁺.

Z-D,L-(α Dfm)Leu-Gly-NH₂. Retention time: 24.6 min. MS (m/z) 428 (M+H)⁺.

Z-D,L-(α Dfm)Leu-Ala-Met-OH. Retention time: 30.9 min. MS (m/z) 518 (M+H)⁺.

Z-D,L-(α Tfm)Leu-Ala-Ala-OH. Retention time: 32.3 min. MS (m/z) 476 (M+H)⁺.

Z-D,L-(α Tfm)Leu-Ala-Ala-Pro-OH. Retention time: 31.2 min. MS (m/z) 573 (M+H)⁺.

Retention times and MS data for products of Trypsin-catalyzed peptide syntheses in frozen system:

Z-Aib-Leu-NH₂. Retention time: 24.4 min. MS (m/z) 350 (M+H)⁺.

Z-Aib-Ala-Met-OH. Retention time: 24.5 min. MS (m/z) 440 (M+H)⁺.

Z-D,L-(α Tfm)Ala-Gln-NH₂. Retention time: 17.1 min. MS (m/z) 419 (M+H)⁺.

Z-D,L-(α Tfm)Ala-Ala-Ser-OH. Retention time: 18.2 min. MS (m/z) 450 (M+H)⁺.

Retention times and MS data for products of α -Chymotrypsin-catalyzed peptide syntheses:

Z-Aib-Arg-Leu-OH. Retention time: 18.3 min. MS (m/z) 507 (M+H)⁺.

Z-D,L-(α Dfm)Ala-Arg-Ser-OH. Retention time: 12.4 min. MS (m/z) 517 (M+H)⁺.

Z-D,L-(α Tfm)Ala-Arg-NH₂. Retention time: 15.6 min. MS (m/z) 447 (M+H)⁺.