**Supporting information for:**

# *A new environment-sensitive fluorescent amino acid building block for Fmoc-based solid phase peptide synthesis*

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## *GENERAL SYNTHETIC METHODS*

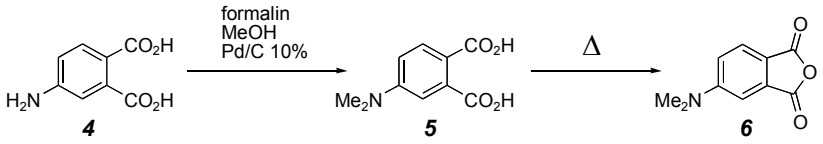
All starting amino acids are commercially available. Dichloromethane was distilled from calcium hydride under nitrogen, and tetrahydrofuran was distilled from sodium under argon. Analytical thin-layer chromatography (TLC) was carried out on F254 250-µm silica gel plates, and visualized by UV lamp.

$^{1}H$ and $^{13}C$ NMR spectra were acquired on a 400 MHz Bruker spectrometer. Chemical shifts are reported in ppm from a standard (tetramethyl silane for $^{1}H$), and *J* values are reported in Hz.

Electrospray Ionization Mass Spectrometry (ESIMS) was performed on a PerSeptive Biosystems Mariner Biospectrometry Workstation (Turbo Ion Source).

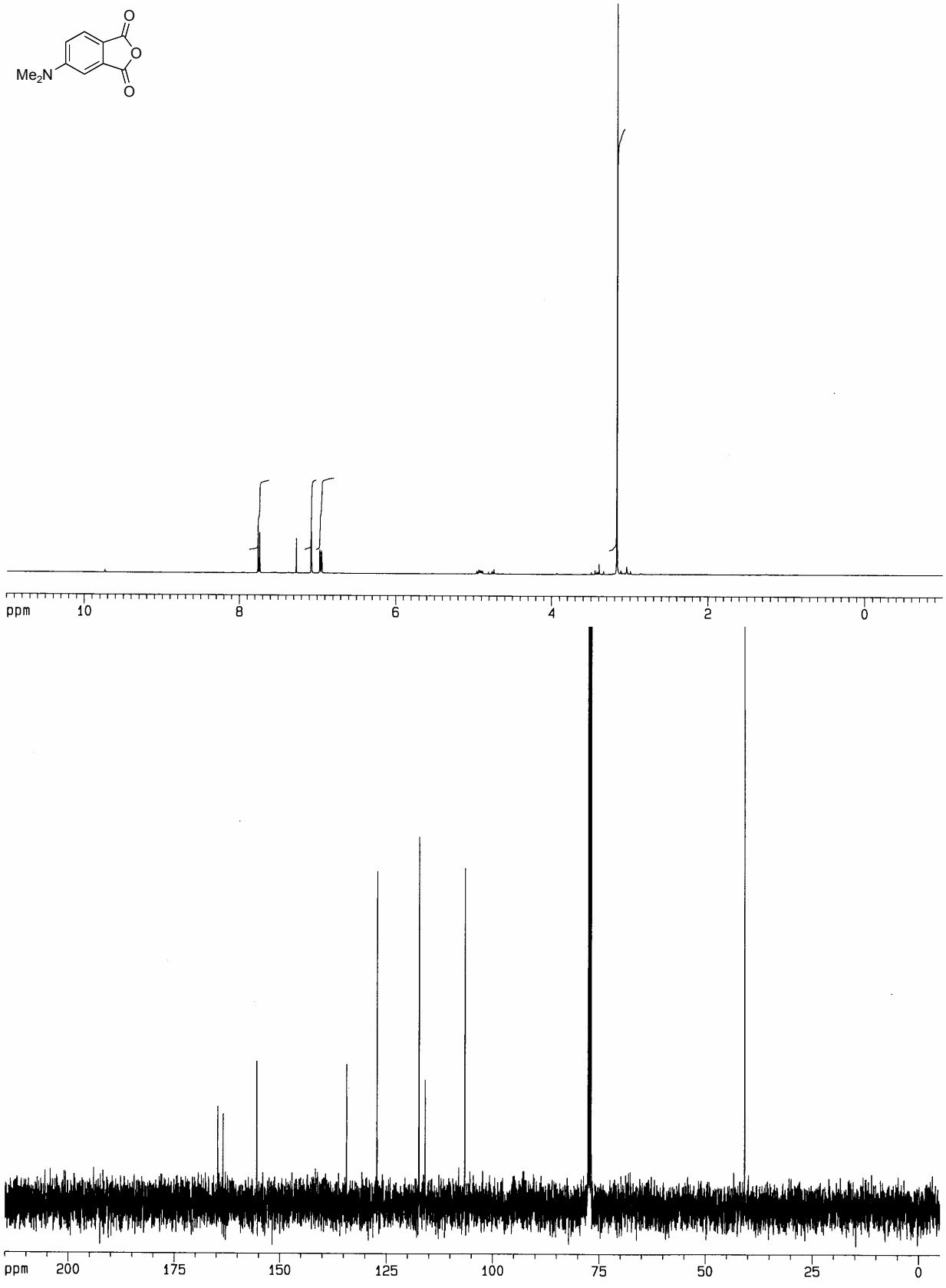
Fluorescence spectroscopy measurements were made using a Fluoromax-P spectrofluorimeter controlled by the DataMax 2.20 software, and coupled to a NesLab RTE-111 water bath for temperature control.

***4-N,N-dimethylaminophthalic anhydride (6):***




4-aminophthalic acid **4** (500 mg, 2.76 mmol) was dissolved in MeOH (150 mL), formalin (15 mL, 36% formaldehyde solution) and Pd/C 10% (200 mg) were added, and the resulting solution was stirred at room temperature under an atmosphere of hydrogen for three hours. The reaction mixture was filtered through celite and concentrated under reduced pressure to give the desired 4-*N*,*N*-dimethylamino phthalic acid **5** as a white solid, which was placed in a sublimator and heated at 120 °C for 1 h under vacuum to give the desired product **6** as a bright yellow solid (489 mg, 92%).

$^{1}$H NMR (400 MHz, $CDCl_3$, δ): 3.0 (s, 6H), 6.9 (dd, 1H, *J1* = 2.4 Hz, *J2* = 8.7 Hz), 7.07 (s, 1H, *J* = 2.4 Hz), 7.75 (d, 1H *J* = 8.6 Hz).
$^{13}$C NMR (400 MHz, $CDCl_3$, δ): 40.8, 106.6, 115.9, 117.4, 127.1, 134.3, 155.5, 163.4, 164.7.
HRMS-ESI (m/z): [M+H$^+$] calcd for $C_{10}H_9NO_3$ 192.0655, found, 192.0652.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6
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ppm
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125
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75
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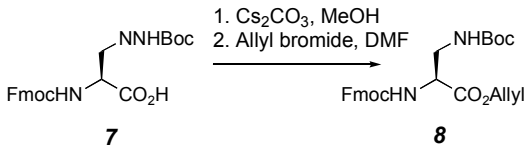
25
0

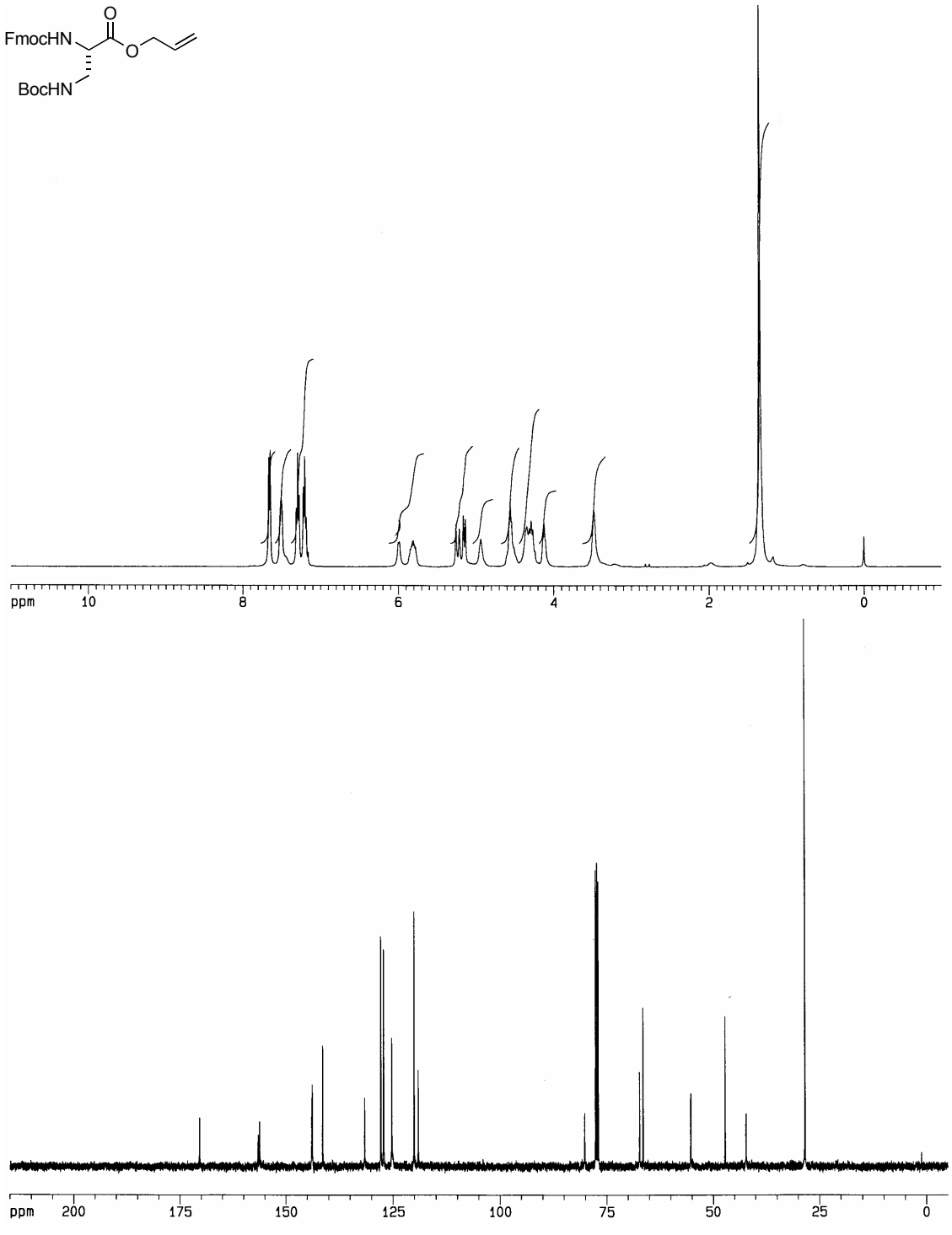
***allyl N-α-Fmoc-N-β-Boc-L-diaminopropionate (8):***




***7*** ***8***

Followed procedure from Ludolph *et. al*.[1] Fmoc-Dap(Boc)-OH (450 mg, 1.05 mmol) was dissolved in MeOH (15 mL), $Cs_2CO_3$ (172 mg, 0.53 mmol, 1.05 eq) was added and the resulting solution was stirred at room temperature for 10 minutes. The reaction mixture was concentrated under reduced pressure and redissolved in DMF (30 mL). Allyl bromide (280 µL, 3.3 mmol, 3.15 eq.) was added and the mixture was stirred for 1 h at room temperature. The reaction was added to 2% aqueous $NaHCO_3$ (150 mL) and extracted with EtOAc (4 x 50 mL), the combined organic layers were dried with $Na_2SO_4$ and concentrated under reduced pressure. The resulting brown oily residue was purified by flash column chromatography (1% $MeOH/CH_2Cl_2$) to give the desired product **8** as a white powder (516 mg, 78%). $R_f$ = 0.7 (1% $MeOH/CH_2Cl_2$).

$^1$H NMR (400 MHz, $CDCl_3$, δ): 1.34 (s, 9H), 3.48 (br s, 2H), 4.12 (br s, 1H), 4.2-4.4 (m, 2H), 4.55 (br s, 2H), 4.9 (br s, 1H), 5.14 (d, 1H, *J* = 10.3 Hz), 5.22 (d, 1H, *J* = 17.1 Hz), 5.8 (br s, 1H), 5.9 (d, 1H, 5.8 Hz), 7.18 (t, 2H, *J* = 7.3 Hz), 7.27 (t, 2H, *J* = 7.4 Hz), 7.5 (br s, 2H), 7.65 (d, 2H, *J* = 7.4 Hz).
$^{13}$C NMR (400 MHz, $CDCl_3$, δ): 28.39, 42.25, 47.21, 55.26, 66.48, 67.26, 80.10, 119.12, 120.87, 125.28, 127.18, 127.83, 131.58, 141.36, 143.83, 156.22, 156.57, 170.37.
HRMS-ESI (m/z): [$M+H^+$] calcd for $C_{26}H_{30}N_2O_6$ 467.2177, found, 467.2177.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BocHN
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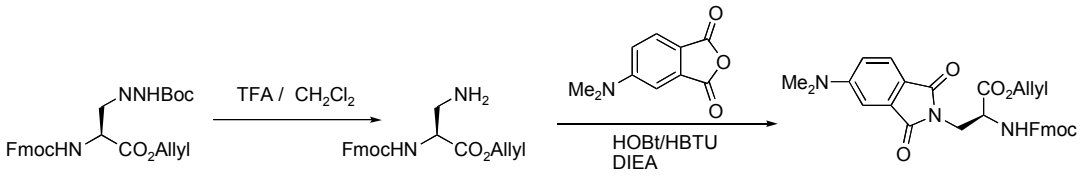
***allyl N-α-Fmoc-N-β-(4-N,N-dimethylaminophthalimidoyl)-L-diaminopropionate (10):***

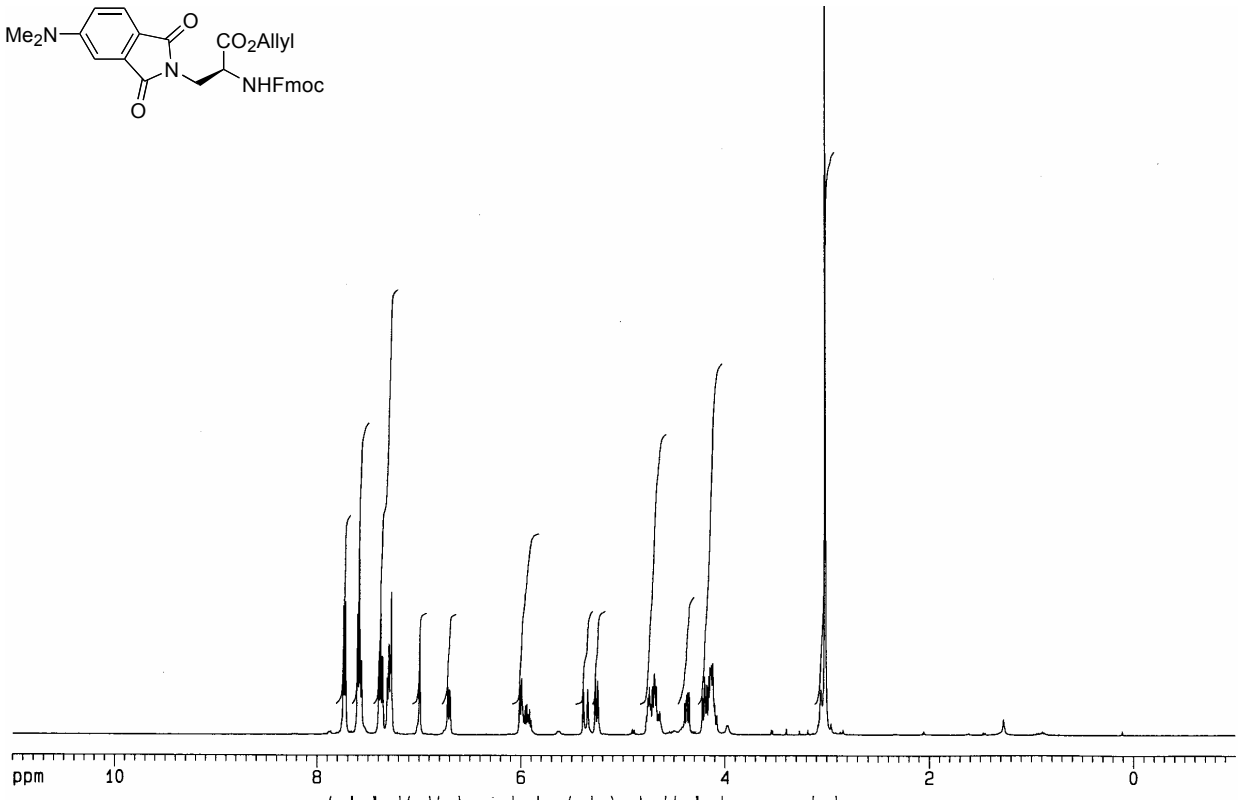



***8*** ***9*** ***10***

Fmoc-Dap(Boc)-OAll **8** (161 mg, 0.345 mmol) was dissolved in dry $CH_2Cl_2$ (6 mL) and the resulting solution cooled to 0 °C in an ice-water bath. TFA (6 mL) was added dropwise. The resulting mixture was allowed to stir at 0 °C for 15 minutes and at room temperature for another 45 minutes. The solution was concentrated under reduced pressure, redissolved and concentrated twice in dichloromethane. The brown oily residue was subjected to the next coupling step without further purification (118 mg, 93%).

The residue was dissolved in DMF (10 mL), DIEA (300 μL, 1.8 mmol, 5 eq) and 4-DMAP anhydride **6** (68 mg, 0.414 mmol, 1.2 eq.) were added, after 30 minutes HOBt/HBTU (2 mL of a 0.2M solution in DMF, 0.4 mmol) was added and the reaction mixture was stirred overnight. The reaction mixture was then poured over 1% $NaHCO_3$ (150 mL) and extracted with EtOAc (3 x 30 mL), the combined organic layers were dried with $Na_2SO_4$ and concentrated to give a bright yellow oil that was purified by flash column chromatography (30% EtOAc/hexanes) to give the desired product **10** as a yellow solid (112 mg, 63%). $R_f$ = 0.7 (60% EtOAc/hexanes).

$^1$H NMR (400 MHz, $CDCl_3$, δ): 2.93 (s, 6H), 4.1-4.2 (m, 3H), 4.35 (c, 1H, $J_1$ = 7.1 Hz, $J_2$ = 3.2 Hz), 4.6-4.8 (m, 3H), 5.25 (d, 1H $J$ = 10.3 Hz), 5.35 (d, 1H, $J$ = 16.3 Hz), 5.9-6.1 (m, 2H), 6.9 (d, 1H, $J$ = 2.0 Hz), 7.29 (m, 3H), 7.37 (t, 2H, $J$ = 7.4 Hz), 7.6 (t, 3H, $J$ = 7.7 Hz), 7.7 (d, 2H, $J$ = 7.4 Hz).
$^{13}$C NMR (400 MHz, $CDCl_3$, δ): 38.87, 40.45, 47.11, 53.74, 66.72, 67.47, 105.96, 114.84, 117.07, 119.22, 119.93, 125.49, 127.14, 127.69, 131.53, 134.42, 141.19, 143.79, 144.17, 154.37, 155.93, 168.63, 169.17, 169.82.
EIMS: 540.4 ($MH^+$), 562.4 ($MNa^+$), 1079.7 ($M_2H^+$), 1101.7 ($M_2Na^+$).
HRMS-ESI (m/z): [$M+H^+$] calcd for $C_{31}H_{29}N_3O_6$ 540.2129, found, 540.2133.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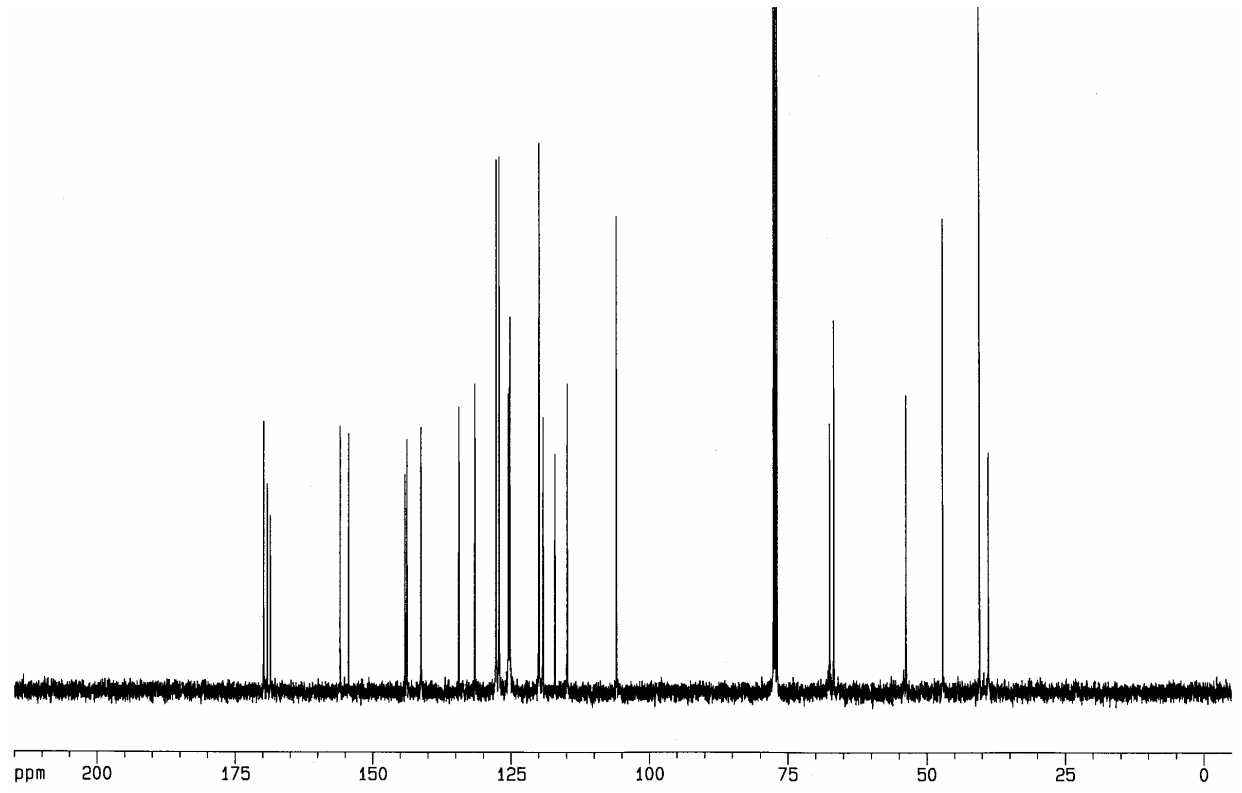
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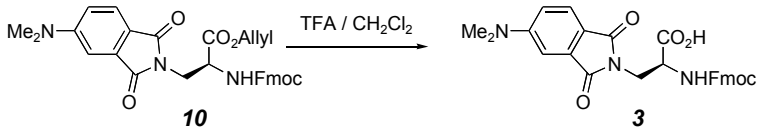
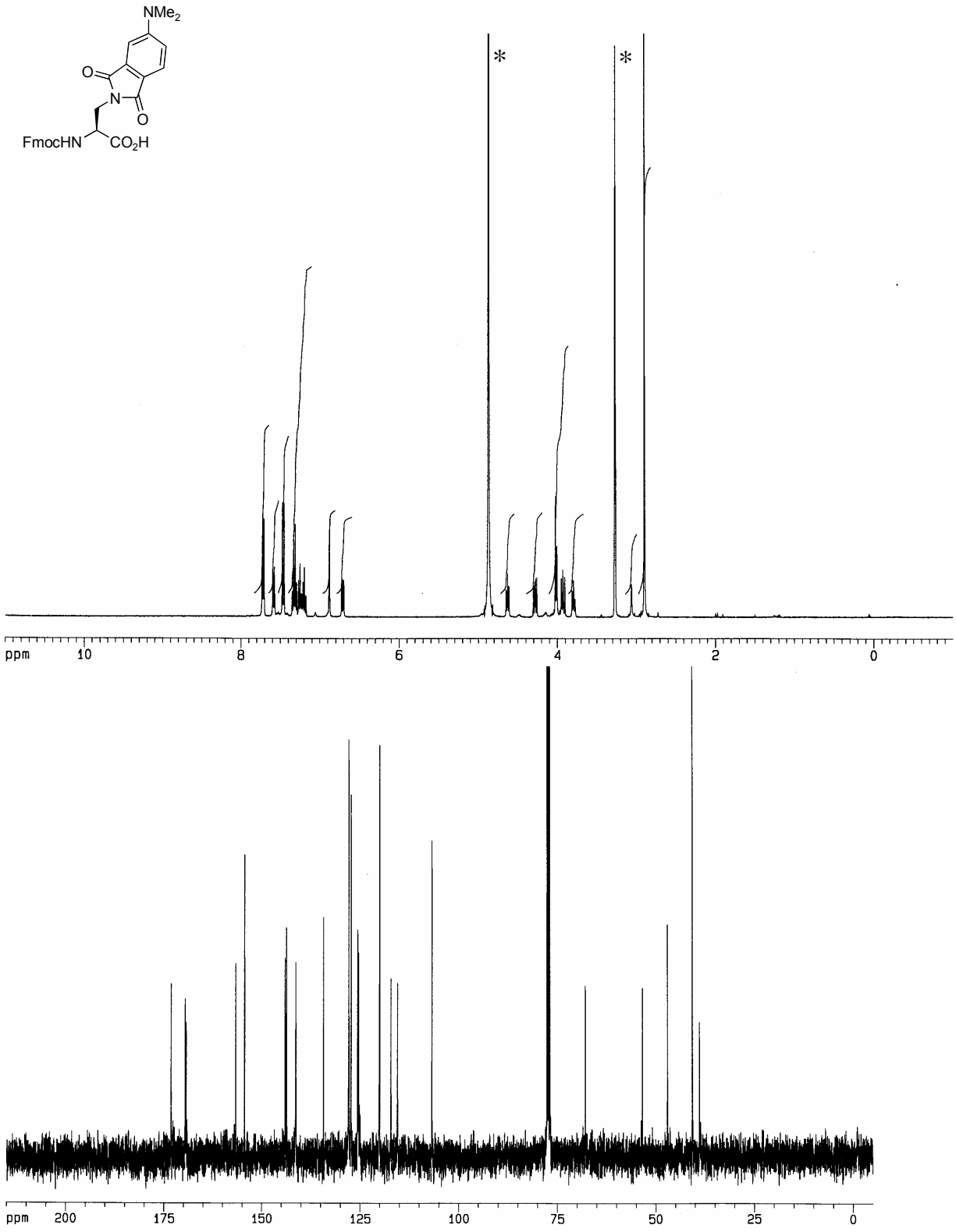



***10*** ***3***

Allyl ester **10** (250 mg, 0.46 mmol) was dissolved in dry dichloromethane (20 mL) and $Pd(PPh_3)_4$ (53 mg, 0.1 eq.) and phenyl silane (1.4 mL, 11.6 mmol) were added. The resulting mixture was stirred at room temperature for 1 h, until the starting material was consumed. The crude reaction mixture was directly loaded onto a silica flash column (5% $MeOH/CH_2Cl_2$) and isolated as a yellow solid (230 mg, quantitative yield). $R_f$ = 0.3 (5% $MeOH/CH_2Cl_2$)

$^1$H NMR (400 MHz, $CDCl_3$, δ): 2.97 (s, 6H), 3.79 (t, 1H, $J$ = 7.1 Hz), 4.00 (d, 2H, $J$ = 1.7 Hz), 4.27 (t, 1H, $J$ = 4.0 Hz), 4.62 (t, 1H, $J$ = 6.3 Hz), 6.71 (dd, 1H, $J_1$ = 2.4 Hz , $J_2$ = 8.6 Hz ), 6.8 (d, 1H, $J$ = 2.3 Hz), 7.21 (td, 1H, $J_1$ = 0.9 Hz , $J_2$ = 7.5 Hz ), 7.26 (td, 1H $J_1$ = 1.0 Hz , $J_2$ = 8.6 Hz ), 7.32 (c, 2H, $J$ = 7.4 Hz), 7.46 (d, 2H, $J$ = 8.4 Hz), 7.58 (d, 1H, $J$ = 7.2 Hz), 7.70 (d, 2H, $J$ = 7.4 Hz). (Note * indicates residual methanol)
$^{13}$C NMR (400 MHz, $CDCl_3$, δ): 38.90, 40.25, 47.03, 53.59, 105.97, 114.64, 116.79, 119.81, 125.31, 125.51, 127.12, 127.60, 134.31, 141.16, 143.69, 144.17, 154.26, 156.23, 169.28, 172.45, 172.68.
EIMS: 500.1 $MH^+$, 522.1 ($MNa^+$), 999.2 ($M_2H^+$), 1021.2 ($M_2Na^+$).
HRMS-ESI (m/z): [$M+H^+$] calcd for $C_{28}H_{25}N_3O_6$ 500.1816, found, 500.1802.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CO2H
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10
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2
0
ppm
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175
150
125
100
75
50
25
0

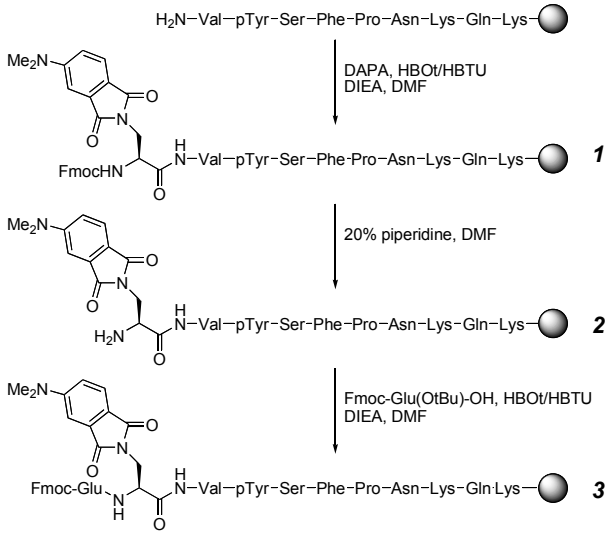
***PEPTIDE SYNTHESIS***

All peptide synthesis reagents and amino acid derivatives were purchased from Applied Biosystems or Novabiochem, and all other chemicals were purchased from Aldrich. High-performance liquid chromatography was performed using a Waters 600E HPLC fitted with a Waters 600 automated control module and a Waters 2487 dual wavelength absorbance detector recording at 228 and 280 nm. For analytical HPLC a Beckman Ultrasphere $C_{18}$, 5 μm, 4.6 x 150 mm reverse-phase column was used. For preparative separations a YMC-pack, $C_{18}$, 250 x 20 mm reversed phase column was used. The standard gradient for analytical and preparative HPLC used was 93:7 to 5:95 over 35 minutes (water:acetonitrile, 0.1% TFA).

Peptide synthesis was carried using standard Fmoc-based solid phase peptide synthesis (SPPS) protocols on a 0.05 to 0.1 mmol scale using a 0.21 mmol/g loading PAL-PEG-PS solid support. Amino acids were coupled in three-fold excess using a mixture of 0.2 M HBTU/0.2 M HOBt in DMF as activating agents. Each amino acid was activated for two minutes with the HBTU/HBOt mixture (1 eq.) and DIPEA 0.195 M in DMF (1.5 eq.) before being added onto the resin. Peptide coupling was monitored using TNBS test.[2]

Phosphopeptides were synthesized by introducing phosphoserine as Fmoc-Ser(PO(OBzl)OH)-OH and phosphotyrosine as Fmoc-Tyr(PO(OBzl)OH)-OH.

Initial peptide synthesis test were performed using a model sequence $H_2N$-Val-pTyr-Ser-Phe-Pro-Asn-Lys-Gln-Lys-PAL-PEG. Test cleavages were made with small aliquots in the steps following the coupling of DAPA to check the stability of the new amino acid side chain by analytical HPLC, mass spectrometry, fluorescence and absorption spectroscopy.




***Scheme.*** *Peptide synthesis of test peptides* ***1****,* ***2*** *and* ***3****.*

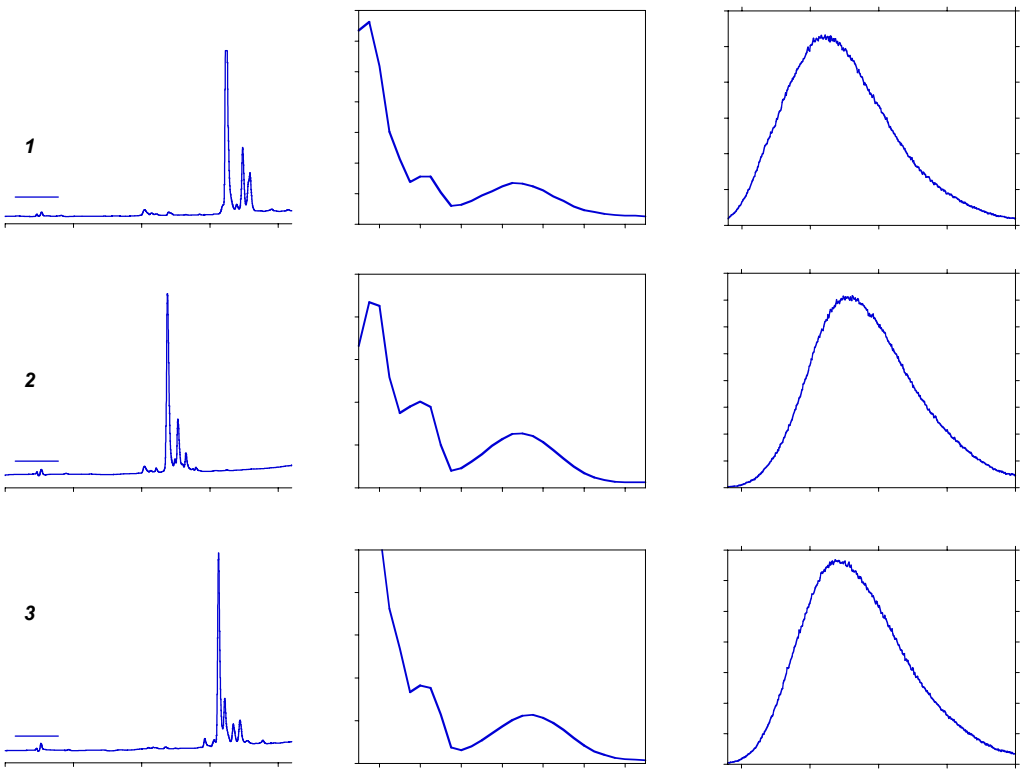



***Figure**. HPLC, absorbance and fluorescence spectra of crude samples of peptides **1**, **2** and **3** showing the stability of the side chain after the different treatments.*

Peptide **1**, calculated mass for $C_{80}H_{104}N_{17}O_{21}P$: 1669.7 ($MH^+$), 835.8 ($MH_2^{+2}$). Observerd 835.9 ($MH_2^{+2}$).
Peptide **2**, calculated mass for $C_{65}H_{94}N_{17}O_{19}P$: 724.8 ($MH_2^{+2}$), 483.5 ($MH_3^{+3}$). Observerd 724.9 ($MH_2^{+2}$), 483.6 ($MH_3^{+3}$).
Peptide **3**, calculated mass for $C_{85}H_{111}N_{18}O_{24}P$: 900.4 ($MH_2^{+2}$). Observerd 900.4 ($MH_2^{+2}$).

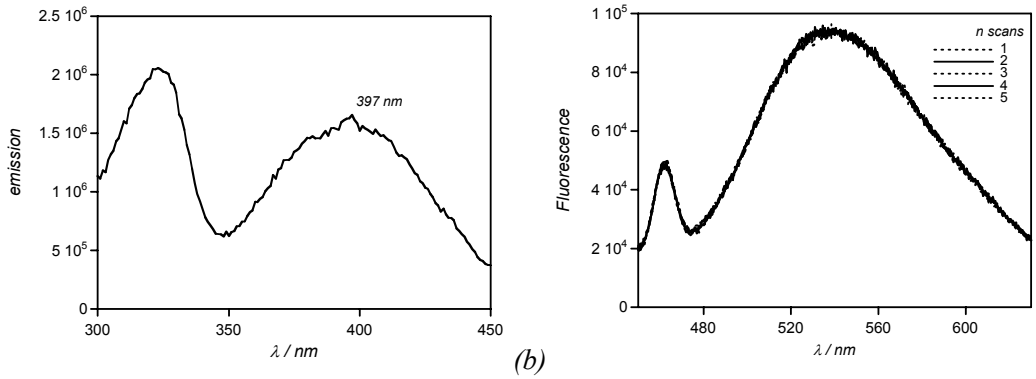
After checking that DAPA was stable to the coupling, deprotection and cleavage conditions, three other subsequent couplings were performed, introducing three glutamic acid residues. Final deprotection yielded the free N-terminal amino peptide that was acetylated using standard conditions (10% $Ac_2O$/0.195 M DIEA in DMF) to give the desired final test peptide. AcHN-$(Glu)_3$-DAPA-Val-pTyr-Ser-Phe-Pro-Asn-Lys-Gln-Lys-$CONH_2$; calculated mass for $C_{82}H_{117}N_{20}O_{29}P$: 1876.80 ($MH^+$), 939.4 ($MH_2^{+2}$), 950.4 ($MHNa^{+2}$), 961.4 ($MNa_2^{+2}$). Observed *m/z*: 939.8 ($MH_2^{+2}$), 950.8 ($MHNa^{+2}$), 961.8 ($MNa_2^{+2}$).

Once the synthetic methodology had been assessed, the target peptide **14-3-3bp-DAPA** (AcHN-Arg-Leu-DAPA-Arg-pSer-Leu-Pro-Ala-$NH_2$) was synthesized using the same standard Fmoc-based SPPS procedures. Final peptide AcHN-Arg-Leu-DAPA-Arg-pSer-Leu-Pro-Ala-$NH_2$; calculated mass for $C_{50}H_{82}N_{17}O_{15}P$: 1191.59. Observed *m/z*: 1192.4, 1193.4 ($MH^+$) and 596.7 ($MH_2^+$).

The extinction coefficient for DAPA in the context of a peptide chain was derived from the concentration obtained by quantitative amino acid analysis of the peptide: UV (phosphate buffer pH 7.5, 100 mM NaCl) $\lambda_{max}$, nm ($\varepsilon$): 421, 6480 $M^{-1}$ $cm^{-1}$

***SPECTROSCOPIC MEASUREMENTS.***

Preliminary experiments were aimed at establishing the photophysical properties of the 4-DMAP side chain in the context of a peptidic sequence. The emission spectrum was collected obtaining an excitation wavelength of 397 nm, which is consistent with previously reported values. Consecutive spectra were also taken to test the photobleaching of the fluorescent reporter, which was found negligible under the conditions used in these experiments.

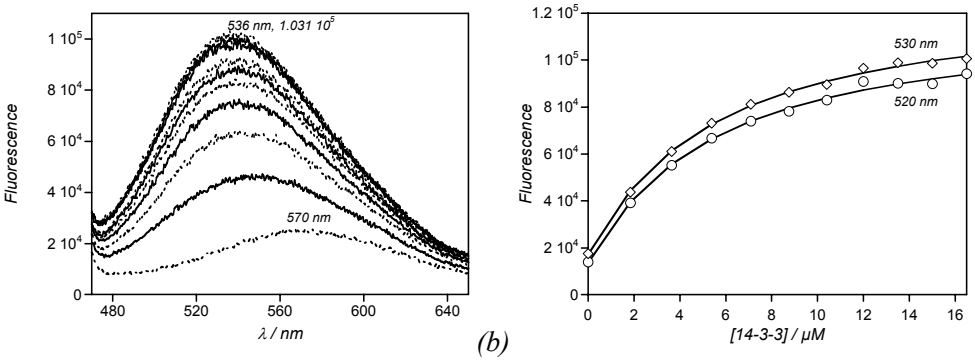



*(a)* *(b)*

***Figure**. a) Fluorescence excitation spectrum of peptide **14-3-3bp-DAPA** showing the maximum excitation wavelength. b) Consecutive emission spectra showing the stability of the side chain towards photobleaching in the conditions used in these experiments. (1 μM peptide, 100 μM phosphate buffer, pH 7.5, 100 mM NaCl. Excitation wavelength 395 nm, excitation slit width 2 nm, emission slit width 5 nm. Integration time 0.1 s. Increment 0.5 nm).*

***Fluorescence titration of 14-3-3bp-DAPA with 14-3-3 ζ.***

A typical titration of 14-3-3/**14-3-3bp-DAPA** binding is illustrated below. To 150 μL of a 1.3 μM solutions of peptide **14-3-3bp-DAPA** in phosphate buffer (10 mM phosphate pH 7.5, 100 mM NaCl), 2 μL aliquots of a stock solution (140 μM concentration in water measured by UV intensity) of the 14-3-3 ζ protein were successively added at 25 °C. The solutions were mixed with a micropipette, and the fluorescence spectra were recorded after each addition using the following parameters: *Temperature*: 25 °C. *Excitation wavelength*: 395 nm. *Slit width*: 5 nm (both excitation and emission). *Integration time*: 0.1 s. *Acquisition range*: 470-650 nm. The resulting spectra were analyzed with the SPECFIT program to obtain the corresponding dissociation constant for the peptide **14-3-3bp-DAPA**/14-3-3 ζ protein complex.




*(a)* *(b)*

***Figure**. a) Typical titration curve of peptide 14-3-3bp-DAPA with 14-3-3 showing the progressive increase in the fluorescence emission intensity and the blue shift in the maximum emission wavelength. b) Curves showing fitting of the titration curves at two different wavelengths.*

***REFERENCES***


[1] B. Ludolph, F. Eisele, and H. Waldmann, *ChemBioChem*, 2002, **9**, 901.

[2] W. S. Hancock and J. E. Battersby, *Analytical Biochemistry*, 1976, **71**, 260.