Solid Phase Synthesis of Chlorofusin Analogues

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SUPPORTING INFORMATION

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General. All solvents and reagents were obtained commercially and were used without further purification with the exception of DCM, which was freshly distilled from CaH_2 prior to use. 2-(9H-fluoren-9-ylmethoxycarbonylamino)-decanoic acid (**14**), which is not commercially available, was prepared according to the method described by Koppitz *et al.* (Supporting Information).[24] NMR spectra were obtained using a 400 MHz spectrometer.

rac-2-aminodecanoic acid (12).[24] Diethyl acetamidomalonate (30.0 g, 0.14 mol) and 1-bromooctane (37.4 g, 0.19 mol, 1.4 equiv) were added to a solution of dry sodium ethoxide in ethanol and refluxed for 24 h. The latter was formed by carefully dissolving sodium (3.2 g, 0.14 mol) in absolute EtOH (300 mL). After cooling to room temperature, the mixture was poured onto ice water and the precipitated diethyl (acetamido)octylmalonate was filtered, washed thoroughly with ice water to get rid of any unreacted 1-bromooctane and dried briefly. The crude waxy, white solid thus obtained (28.8 g, 63% yield) was dissolved in DMF (25 mL) and refluxed with conc. HCl (160 mL) for 48 h. The suspension was then cooled to room temperature and poured into 300 mL of EtOH/H_2O (3:1). Conc. NaOH (15 M) was slowly added until the mixture became slightly alkaline. The precipitate was then filtered, washed (H_2O, EtOH/H_2O (3:1), $CHCl_3$, Et_2O) and dried. This gave a racemic mixture of 2-aminodecanoic acid as white solid (9.7 g, 53% yield): ES+ MS m/z: 188.1 $[M+H]^+$ (calcd for $C_{10}H_{21}NO_2$, 187.1572).

D-2-aminodecanoic acid (13).[24] Chloroacetyl chloride (5.2 g, 49 mmol, 1.05 equiv) was added to a suspension of rac-2-aminodecanoic acid (9.7 g, 46 mmol) in anhydrous MeCN (60 mL) and the mixture was refluxed for 2 h. It was then cooled to room temperature, evaporated, dissolved in EtOAc and filtered. The filtrate was evaporated and

the resulting crude product purified by recrystallization from EtOAc/Hexane (1:5). This yield rac-2- (chloroacetylamino)decanoic acid as white crystal (10.6 g, 87% yield). The product was then dissolved in distilled water (1.2 L) at 80^{o}C and the pH of the solution adjusted to 7 with dropwise addition of aqueous LiOH (2 M). After cooling to room temperature, acylase I (from Aspergillus melleus) (1 g) was added and the mixture was stirred for 72 h. The cloudy solution formed was filtered and the filtrate acidified to pH 2 with conc. HCl. The precipitate was then filtered, washed with H2O, dissolved in EtOAc and dried ($MgSO_4$). Further filtration and evaporation gave D-2-(chloroacetylamino)decanoic acid as white solid (2.4 g, 45% yield). The latter was then suspended in aqueous HCl (3 M, 150 mL) and carefully refluxed for 8 h. Upon cooling, the mixture was neutralized with solid KOH and the precipitate was filtered, washed (H_2O, EtOAc, Et_2O) and dried. This gave D-2-aminodecanoic acid as white solid (1.5 g, 88% yield) ES+ MS m/z: 188.1 $[M+H]^+$ (calcd for $C_{10}H_{21}NO_2$, 187.1572).

2-(9H-fluoren-9-ylmethoxycarbonylamino)-decanoic acid (14).[24] Trimethylsilyl chloride (1.7 g, 16 mmol, 2 equiv) was added to a solution of D-2-aminodecanoic acid (1.5 g, 8 mmol) in anhydrous DCM (150 mL) and the mixture was reflux under N2 for 15 min. Triethylamine (1.6 g, 16 mmol, 2 equiv) was then added and the solution refluxed for another 1 h. Upon cooling to 0^{o}C, 9-fluorenylmethyl chloroformate (2.1 g, 8 mmol, 1 equiv) was added in one portion and the mixture was allowed to stir at room temperature for 24 h under N_2. The solvent was then evaporated and aqueous HCl (0.01 M, 200 mL) was added to the residue. After stirring for 30 min, the crude product was extracted with DCM, evaporated and recrystallized with DCM/Hexane (1:1). This gave Fmoc-D-Ade-OH as white crystal (2.3 g, 70% yield): mp 129-130^{o}C; ^{1}H NMR (400 MHz, d_6-DMSO) δ 0.81 (3H, t, J = 6.7 Hz, H_θ), 1.20 (12H, s, H_γ-H_η), 1.60 (2H, m, H_β), 3.88 (1H, m,

CHCH_2OCO), 4.23 (2H, m, CH*CH_2*OCO), 4.23 (1H, m, H_α), 7.29 (2H, t, *J* = 7.4 Hz, 2'H,7'H), 7.39 (2H, t, *J* = 7.4 Hz, 3'H,6'H), 7.60 (1H, d, *J* = 8.2 Hz, NH), 7.69 (2H, d, *J* = 7.7 Hz, 4'H,5'H), 7.86 (2H, d, *J* = 7.6 Hz, 1'H,8'H); ES+ MS m/z: 410.2 $[M+H]^+$ (calcd for $C_{25}H_{31}NO_4$, 409.2253).

wt p53 (15-29) and biotinylated SGSG-p53 (17-27). Both linear peptides were assembled manually on Rink amide MBHA resin (250 mg, 0.165 mmol, substitution = 0.66 mmol/g) using standard procedures for Fmoc solid-phase peptide synthesis as described above. Coupling was achieved using 5 equiv of the following: protected amino acids, HBTU, HOBt and 10 equiv of DIPEA in DMF. Purification was carried out by semi-preparative reverse-phase HPLC performed on a Waters Prep LC C18 column (100 mm x 25 mm), eluting at a flow rate of 5 mL/min using the following gradient: 0% B to 60% B over 90 min.

wt p53 (15-29) (24 mg, 8% yield): MALDI-TOF MS *m/z*: 1807.4 $[M+H]^+$ (calcd for $C_{82}H_{123}N_{19}O_{27}$, 1805.8836); HPLC retention time = 16.267 min. Peptide sequence: H_2N-SQETFSDLWKLLPEN-$CONH_2$

biotinylated SGSG-p53 (17-27) (16 mg, 5% yield): MALDI-TOF MS *m/z*: 1990.3 $[M+H]^+$, 2012.2 $[M+Na]^+$ (calcd for $C_{90}H_{136}N_{22}O_{27}S$, 1988.9666); HPLC retention time = 22.500 min. Peptide sequence: Biotin-SGSGQETFSDLWKLLP-$CONH_2$

HDM2 (17-125). Competent *E. coli* JM109(DE3) cells were transformed with the plasmid encoding HDM2 (17-125) (a generous gift from Professor J. A. Robinson) *via* heat-shock method. The cells were then plated out in LB agar containing ampicillin (50 μg/mL) and were grown at 37ºC for 24 h. A single large colony was then introduced into 1 L of Terrific Broth and the culture was allowed to incubate at 37ºC, with shaking until

cell density reached OD_{600} = 0.7, at which point the temperature was adjusted to 27ºC and IPTG (1 mM) was added to induce protein production. After 5 h of incubation, the cells were harvested by centrifugation and were resuspended in buffer-A, which contained Tris HCl (20 mM), NaCl (300 mM) and imidazole (20 mM) at pH 7.9. They were then disrupted with lysozyme (0.2 mg/mL) and the debris removed *via* centrifugation. The supernatant thus obtained was purified using a Ni^{2+}-iminodiacetic acid (Ni-IDA) column where HDM2 (17-125) was eluted with buffer-A containing 150 mM of imidazole. It was then concentrated by centrifugal filtration into a storage buffer (0.66 mg/mL) in preparation for p53/HDM2 binding assay. The protein was analysed by 15% SDS-PAGE and MALDI-TOF MS *m/z*: 14683.0 $[M–Met+H]^+$ (calcd 14814.83).

Protein sequence:

MGSSHHHHHHSSGLVPRGSHMS$_{17}$QIPASEQETLVRPKPLLLKLLKSVGAQKDTY
TMKEVLFYLGQYIMTKRLYDEKQQHIVYCSNDLLGDLFGVPSFSVKEHRKIYTMI
YRNLVVVNQQESSDSGTSVSEN$_{125}$

p53/HDM2 Inhibition Assay. A streptavidin coated 96 microwell plate (Reacti-Bind™ Streptavidin High Binding Capacity coated plate, Pierce) was rinsed with wash buffer (PBS + 0.05% Tween-20) three times and incubated with 100 μL per well of biotinylated SGSG-p53 (17-27) peptide (2.5 μM in wash buffer) at 4ºC for 1 h. Any unbound peptide was then removed by rinsing with wash buffer three times. In a separate 96 microwell polypropylene plate (Pierce), 50 μL of HDM2 (17-125) (1 μM) was incubated with 50 μL of inhibitor (a range of concentrations) at 4ºC for 30 min in an incubation buffer containing PBS, 0.05% Tween-20, 2 mg bovine serum albumin, 5 mM DTT and 10% DMSO. 100 μL of the HDM2/inhibitors mixture was then transferred to each well in the streptavidin coated plate and was left to incubate at 4ºC for 1h. The wells

were then washed with wash buffer for five times, followed by the addition of 100 μL of anti-HDM2 [(Ab-5) mouse mAb(4B2C1.11)] (Calbiochem), diluted 500-fold with a dilution buffer (PBS + 0.05% Tween-20 + 2 mg bovine serum albumin). After 2 h of incubation at 4^{o}C, the wells were washed with wash buffer five times and a solution of anti-mouse IgG conjugated with horseradish peroxidase (AstraZeneca), diluted 10,000-fold in dilution buffer, was added. Again, the wells were incubated at 4^{o}C for 1 h and then washed with wash buffer five times. At this point, 200 μL of peroxidase substrate, 3,3',5',5-tetramethylbenzidine (Sigma-Aldrich), was added and the reaction was allowed to incubate at room temperature for 30 min to develop a blue product. Quenching the reaction with 100 μL of aqueous H_2SO_4 (0.5 N) produces a yellow colour which can be read at 450 nm. The percentage of p53/MDM2 binding was then calculated, from which the IC_{50} values for the analogues were determined. The assay was performed in triplicate using blank, which do not contain HDM2 (17-125), as a negative control and wt p53 (15-29) as a benchmark against which the activity of the test compounds was compared.