Targeting Lipopolyplexes Using Bifunctional Peptides Incorporating Hydrophobic Spacer Amino Acids: Synthesis, Transfection and Biophysical Studies

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11-Aminoundecanoic acid benzyl ester *p*-toluenesulfonate 19 (*14*). To 11-aminoundecanoic acid (10.05 g, 50.0 mmol) in benzene (500 mL) was added *p*-toluenesulfonic acid monohydrate (10.46 g, 55.0 mmol, 1.1 eq.) and benzyl alcohol (150 mL, large excess). The reaction was then heated to reflux under argon and the liberated water, being trapped by aid of a Dean and Stark receiver was removed azeotropically. The reaction was heated at reflux for 5 h after which time no more water was distilled off. The reaction was then allowed to cool to room temperature. Diethyl ether (500 mL) was slowly added to the reaction at room temperature and the resulting solution was left to stand for 24 h at 4 ^{o}C. The crystalline solid that had formed was filtered and washed with diethyl ether (3 × 100 mL). The crystalline product was then re-crystallised from methanol/diethyl ether to yield **19** (21.2 g, 91%) as hygroscopic crystals. ^{1}H NMR (300 MHz, DMSO) δ 1.25 (12H, br m, (C*H*$_2$)$_6$CH$_2$CH$_2$CO$_2$Bn), 1.55 (4H, m, C*H*$_2$(CH$_2$)$_6$C*H*$_2$CH$_2$CO$_2$Bn), 2.31 (3H, tosyl), 2.36 (2H, t, *J* = 7.3 Hz, C*H*$_2$CO$_2$Bn), 2.77 (2H, t, *J* = 7.4 Hz, Tos$^-$H$_3$N$^+$C*H*$_2$(CH$_2$)$_9$CO$_2$Bn), 5.11 (2H, s, CO$_2$C*H*$_2$C$_6$H$_5$), 7.15 (2H, d, *J* = 7.7 Hz, Tos), 7.34 (5H, m, benzyl), 7.55 (2H, d, *J* = 7.7 Hz, Tos), 7.70 (3H, br s, Tos$^-$*H*$_3$N$^+$(CH$_2$)$_{10}$CO$_2$Bn); ^{13}C NMR (75 MHz, DMSO) δ 21.7, 25.4, 26.7, 27.9, 29.36, 29.42, 29.6, 29.7, 34.4, 39.9, 66.2, 126.4, 128.8, 128.9, 129.1, 129.3, 137.3, 138.8, 146.3, 173.7; IR ν_{max}/cm^{-1} (KBr): 3065, 2921, 2849, 1735, 1610; $C_{18}H_{30}NO_2$ (ESI$^+$) *m/z* 486 (M+Na$^+$, 2%), 292 ([$C_{18}H_{30}O_2N$]$^+$, 100%); HRMS (ESI$^+$): $C_{18}H_{30}NO_2$ calcd for [MH]$^+$ 292.2271, found 292.2283.

11-(9-Fluorenylmethyloxycarbonylamido)-undecanoic acid benzyl ester 20. (*15*) To 11-aminoundecanoic acid benzyl ester *p*-toluenesulfonate **19** (11.59 g, 25.0 mmol) and sodium bicarbonate (3.31 g, 31.25 mmol, 1.25 eq.) in dioxane/water (2:1, 300 mL) at 0 ^{o}C was slowly added fluorenylmethyl chloroformate (9.70 g, 37.5 mmol, 1.5 eq.) in dioxane (100 mL). Once addition was complete the reaction was allowed to warm to room temperature and was left to stir for 7 h. The reaction was then concentrated *in vacuo* to remove the dioxane. The remaining solid/solution was partitioned twice with dichloromethane (2 × 200 mL). The organic fractions were combined and partitioned with saturatedaqueous NaCl (100 mL) adjusted to pH 5 with citric acid. The organic solution was then dried over anhydrous magnesium sulfate and concentrated *in vacuo* to give a yellow solid. The product was recovered from this solid by column chromatography eluting with chloroform/ethyl acetate (95:5) to yield **20** as a white solid (12.2 g, 95%), m.pt. 87-89 ^{o}C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.29 (12H, m, $(CH_2)_6CH_2CH_2CO_2Bn$), 1.51 (2H, m, $FmocHNCH_2CH_2$), 1.66 (2H, m, $CH_2CH_2CO_2Bn$), 2.37 (2H, t, *J* = 7.5 Hz, CH_2CO_2Bn), 3.19 (2H, m, $FmocHNCH_2$), 4.23 (1H, t, *J* = 6.8 Hz, $NHCOOCH_2CHC_{12}H_8$), 4.41 (2H, d, *J* = 6.8 Hz, $NHCOOCH_2C_{13}H_9$), 4.77 (1H, bs, NH), 7.32-7.58 (9H, m, benzyl & Fmoc), 7.59 (2H, d, *J* = 7.4 Hz, Fmoc), 7.76 (2H, d, *J* = 7.4 Hz, Fmoc); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.3, 27.0, 29.4, 29.5, 29.6, 29.7, 29.8, 30.3, 34.6, 41.4, 47.7, 66.4, 66.8, 120.3, 125.5, 127.3, 128.0, 128.5, 128.9, 136.5, 141.7, 144.4, 156.7, 174.0; IR ν_{max}/cm^{-1} (KBr): 3299, 2912, 2848, 1729, 1692; $C_{33}H_{39}NO_4$ (FAB^+) *m/z* 514 ($M+H^+$, 95%), 292 ($[C_{18}H_{30}O_2N]^+$, 71%); HRMS (FAB, NOBA matrix): $C_{33}H_{39}NO_4$ calcd for $[MH]^+$ 514.2957, found 514.2949.

11-(9-Fluorenylmethyloxycarbonylamido)-undecanoic acid 6. (*15*) To 11-(9-fluorenylmethyloxycarbonylamido)-undecanoic acid benzyl ester **20** (5.14 g, 10.0 mmol) in

tetrahydrofuran (80 mL) was added palladium on carbon (10 %, 100 mg). This reaction mixture was first de-gassed by stirring under vacuum and was then placed under a hydrogen atmosphere (standard atmospheric pressure). The reaction was left stirring under the hydrogen atmosphere until 241 cm^3 of hydrogen had been absorbed by the reaction. The reaction was then filtered through Celite™, washing with tetrahydrofuran (3 × 50 mL). The filtrate was concentrated *in vacuo* and a white solid was recovered. This white solid was re-crystallised from ethyl acetate to yield **6** as hygroscopic white crystals (3.70 g, 88%), m.pt. 120-122 ^oC (lit. m.pt. (*15*) 118-120 ^oC). ^1H NMR (300 MHz, DMSO) δ 1.25 (12H, m, $(CH_2)_6CH_2CH_2CO_2H$), 1.41-1.53 (4H, m, $CH_2(CH_2)_6CH_2CH_2CO_2H$), 2.20 (2H, t, *J* = 7.3 Hz, CH_2CO_2H), 2.99 (2H, m, FmocHNCH_2), 4.23 (1H, t, *J* = 6.7 Hz, $NHCOOCH_2CHC_{12}H_8$), 4.32 (2H, d, *J* = 6.7 Hz, NH_{CO}OCH2C13H9), 4.41 (1*H*, bs, NH), 7.34 (2H, dt, J = 7.4 Hz and 1.1 Hz, Fmoc), 7.43 (2^H, t, J = 7.4 Hz, Fmoc), 7.71 (2H, d, J = 7.4 Hz, Fmoc), 7.90 (2H, d, J = 7.4 Hz, Fmoc); ^{13}C NMR (75 MHz, DMSO) δ 25.6, 27.2, 29.56, 29.66, 29.72, 29.8, 29.9, 30.3, 34.9, 41.1, 47.8, 66.1, 121.0, 126.1, 127.9, 128.5, 141.7, 144.9, 157.0, 175.5; IR ν_{max}/cm^{-1} (KBr): 3346, 3018, 2925, 2850, 1949, 1913, 1688; $C_{26}H_{33}NO_4$ (FAB^+) *m/z* 446 ($M+Na^+$, 100%), 202 ($[C_{11}H_{24}O_2N]^+$, 10%); HRMS (FAB, NOBA matrix): $C_{26}H_{34}NO_4Na$ calcd for $[M+Ma]^+$ 446.23018, found 446.22930.

Peptide Synthesis. Peptide**7** was synthesised by Zinsser Analytic (Maidenhead, UK) at 80% purity.

All other peptides were synthesised as described below. The solid support used for SPPS was a NovaSyn-TGT resin pre-loaded with *N*-α-Fmoc-glycine. All SPPS reactions were carried out at room temperature.

Peptide **2** was assembled on the MilliGen 9050 using standard procedures (deblock: 30% piperidine in DMF, coupling: 0.6 M DIPCI in DMF/0.6 M HOBt in DMF, auxiliary wash: 0.3M NaI in DMF, wash DMF/DCM 3:2), **6**, and commercially available Fmoc-amino acids. Cleavage, deprotection, disulfide bond formation and purification were carried out according to the following general procedures:

Peptide-resin cleavage and peptide deprotection: general procedure: To the dry resin-bound peptide (0.110 mmol) in a Merrifield bubbler was added a solution (10 mL) consisting of trifluoroacetic acid (85%), thioanisole (5%), phenol (5%), water (2.5%) and triethylsilane (2.5%). The resin-bound peptide in the solution was left to agitate for 10 min by bubbling with a stream of nitrogen. After 10 min, the solution was drained into a flask. Another volume of the solution (10 mL) was then added to the resin-bound peptide and again bubbling with N_2 was re-commenced for 10 min. The solution was drained into the flask and the above procedure was repeated four more times. Once this was complete, the content of the flask was left to stir at room temperature, under nitrogen for 6 h. After 6 h, the solution was concentrated *in vacuo* and diethyl ether/hexane (1:1, 50 mL) was added to the residue that remained. The organic solution was carefully decanted to retain the solid precipitate that had formed, which was then dried under vacuum. The solid was then re-dissolved in water, degassed under vacuum and freeze-dried.

Disulfide bridge formation: general procedure: The peptide was dissolved in degassed water to make a final concentration of ~0.25 mg/mL. The solution was left to stir at room temperature exposed to the atmosphere for one week. After one week the reaction was concentrated *in vacuo*, the remaining residue was re-dissolved in degassed water and freeze-dried.

Synthesis of the fully-protected peptide-resin 21: The sequence Fmoc-Gly-Ala-Cys(Trt)-Arg(Pbf)-Arg(Pbf)-Glu(OtBu)-Thr(tBu)-Ala-Trp(Boc)-Ala-Cys(Trt)-Gly-OTGT was assembled on the MilliGen 9050 using standard procedures (deblock: 30% piperidine in DMF, coupling: 0.6 M DIPCI in DMF/0.6 M HOBt in DMF, auxiliary wash: 0.3M NaI in DMF, wash DMF/DCM 3:2). The resin-bound peptide was removed from the synthesiser and stored under Ar at 4 °C until required.

Peptide 3: Resin-bound peptide **21** (0.74 g, 0.110 mmol) was pre-swelled with DMF on a Merrifield bubbler under nitrogen. The DMF was removed, DBU/piperidine/DMF (1:1:48, 8 mL) was added to cover the resin-bound peptide and the reaction was agitated by nitrogen bubbling over 10 min. The deprotection solution was then removed and the resin-bound peptide was washed with DMF (2 x 10 mL). A further quantity of DBU/piperidine/DMF (1:1:48, 8 mL) was again added and bubbling was re-commenced for 10 minutes, followed by washing with DMF. This procedure was repeated a further three times. After the final deprotection, the resin-bound peptide was thoroughly washed with DMF (50 ml). (*Z*)-16-(9-fluorenylmethyloxycarbonylamido)-hexadeca-7-enoic acid **4** (0.108 g, 0.22 mmol), HATU (0.083 g, 0.22 mmol) and HOAt (0.029 g, 0.22 mmol) were dissolved in the minimum quantity of DMF/DCM (5 mL, 1:1). To this solution was added *N,N*-diisopropylethylamine (0.096 mL, 0.0711 g, 0.55 mmol) and the resulting solution was immediately added to the resin-bound

peptide. The reaction was agitated by bubbling with N_2 for 2 h. The reagent solution was then removed and the resin-bound peptide was washed with DMF, dichloromethane, and diethyl ether, then dried under vacuum. The remainder of the peptide sequence was then assembled on the MilliGen 9050 using standard procedures, coupling Fmoc-Lys(Boc) with 0.6M TBTU and 0.6 M HOBt in DMF/1.0 M DIPEA.

The peptide was cleaved from the resin and deprotected as follows. To the dry resin-bound peptide (0.110 mmol) in a Merrifield bubbler was added a solution (10 mL) consisting of trifluoroacetic acid (85%), thioanisole (5%), phenol (5%), water (2.5%) and triethylsilane (2.5%). The resin-bound peptide in the solution was left to agitate for 10 min by bubbling with a stream of nitrogen. After 10 min, the solution was drained into a flask. Another volume of the solution (10 mL) was then added to the resin-bound peptide and again bubbling with N_2 was re-commenced for 10 min. The solution was drained into the flask and the above procedure was repeated four more times. Once this was complete, the content of the flask was left to stir at room temperature, under N_2 for 6 h. After 6 h, the solution was concentrated *in vacuo* and dried under vacuum. The solid was then re-dissolved in water, degassed under vacuum and freeze-dried. The residue was analysed by MS and was found to contain the desired peptide as well as large amount of the desired peptide but with one Boc group still remaining. The residue was therefore re-dissolved in a solution of TFA (37 mL), TES (1 mL) and water (2 mL). The solution was left to stir for 1.5 h under N_2, at room temperature. The solution was then concentrated *in vacuo* once again. MS analysis showed that there was no peptide with one Boc group remaining. The residue was then re-dissolved in water, de-gassed under vacuum and freeze-dried.

The residue was then purified by preparative reverse phase HPLC (water:acetonitrile, gradient 10-50% acetonitrile over 35 min: both water and acetonitrile contained 0.1 % trifluoroacetic acid). The retention time of the product was approximately 20 min (H_2O/MeCN, 75:25). MS analysis of the peptide recovered from HPLC purification however indicated that a peptide had been recovered that was two mass units greater than the desired peptide, indicating that the double bond had been reduced: MS m/z (+ve ES): Measured mass – 3585.74 (449.4 [M+8H$^+$]/8, 513.4 [M+7H$^+$]/7, 598.5 [M+6H$^+$]/6, 718.0 [M+5H$^+$]/5). Calculated mass (for original peptide) – 3582.69.

The resulting peptide was dissolved in de-gassed water/acetonitrile (2:1) to a final concentration of ~0.25 mg/mL. The solution was left to stir at room temperature exposed to the atmosphere for one week. After one week the reaction was concentrated *in vacuo*, the remaining residue was re-dissolved in de-gassed water and freeze-dried. The residue was then purified by preparative reverse phase HPLC, (water:acetonitrile, gradient 10-50% acetonitrile over 35 min: both water and acetonitrile contained 0.1% trifluoroacetic acid). The retention time of the product was approximately 22 min (H_2O/MeCN, 73:27). MS analysis of the peptide recovered from HPLC purification showed that the material had been successfully oxidised to afford peptide **3** as the TFA salt. MS m/z (+ve ES): Measured mass – 3584.00 (449.4 [M+8H$^+$]/8, 513.4 [M+7H$^+$]/7, 598.5 [M+6H$^+$]/6, 718.0 [M+5H$^+$]/5). Calculated mass (for original peptide) – 3581.67.

Peptide 5: Resin-bound peptide **21** (0.73 g, 0.110 mmol) was pre-swelled with DMF on a Merrifield bubbler under N_2. The DMF was removed, DBU/piperidine/DMF (1:1:48, 10 mL) was added to cover the resin-bound peptide and the reaction was agitated by nitrogen bubbling over 10 min. After 10 min, the deprotection solution was removed and the resin-bound peptide

was washed with DMF (2 x 10 mL). A further quantity of DBU/piperidine/DMF (1:1:48, 10 mL) was again added and bubbling was re-commenced for 10 minutes, followed by washing with DMF. This procedure was repeated a further three times. After the final deprotection, the resin-bound peptide was thoroughly washed with DMF (50 mL). (*Z*)-16-(9-fluorenylmethyloxycarbonylamido)-hexadeca-7-enoic acid **4** (0.216 g, 0.44 mmol), HATU (0.167 g, 0.44 mmol) and HOAt (0.06 g, 0.44 mmol) were dissolved in the minimum quantity of DMF/DCM (7 ml, 1:1). To this solution was added *N,N*-diisopropylethylamine (0.191 ml, 0.142 g, 1.10 mmol) and the resulting solution was immediately added to the resin-bound peptide. The reaction was agitated by bubbling with N_2 for 1.5 h. After 1.5 h, the reagent solution was removed and the resin-bound peptide was washed with DMF (2 x 20 mL). The procedure described above (Fmoc deprotection followed by coupling) was then repeated in order to attach the subsequent amino acid (LAA).

After the next 1.5 h, the reagent solution was removed and the resin-bound peptide was washed with DMF (2 x 20 mL). The Fmoc deprotection procedure described above was again repeated. Fmoc-Lysine(Boc)-OH (0.206 g, 0.44 mmol), HATU (0.167 g, 0.44 mmol) and HOAt (0.06 g, 0.44 mmol) were dissolved in the minimum quantity of DMF (7 mL). To this solution was added *N,N*-diisopropylethylamine (0.191 mL, 0.142 g, 1.10 mmol) and the resulting solution was immediately added to the resin-bound peptide. The reaction was agitated by bubbling with N_2 for 1h. The reagent solution was removed and the resin-bound peptide was washed with DMF, dichloromethane, and diethyl ether, then dried under vacuum. The remainder of the peptide sequence was then assembled on the MilliGen 9050 using standard procedures, coupling Fmoc-Lys(Boc) with 0.6M TBTU & 0.6 M HOBt in DMF/1.0 M DIPEA.

For the first two Fmoc-Lys(Boc)-OH residues the deprotection times were extended by 5 min and the recycle times extended by 10 min.

The peptide was then cleaved from the resin and deprotected as follows. To the dry resin-bound peptide (0.110 mmol) in a Merrifield bubbler was added a solution (10 mL) consisting of trifluoroacetic acid (85%), thioanisole (5%), phenol (5%), water (2.5%) and 1,2-ethanedithiol (2.5%). The resin-bound peptide in the solution was left to agitate for 10 min by bubbling with a stream of nitrogen. After 10 min, the solution was drained into a flask. Another volume of the solution (10 mL) was then added to the resin-bound peptide and again bubbling with N_2 was re-commenced for 10 min. The solution was drained into the flask and the above procedure was repeated four more times. Once this was complete, the content of the flask was left to stir at room temperature, under N_2 for 4 h. The solution was concentrated *in vacuo* and dried under vacuum. The solid was then re-dissolved in water, degassed under vacuum and freeze-dried. This was then purified by preparative reverse phase HPLC, (water:acetonitrile, gradient 5-30% acetonitrile over 10 min, then 30-45% acetonitrile over 25 min: both water and acetonitrile contained 0.1% trifluoroacetic acid). The retention time of the product was approximately 23 minutes (H_2O/MeCN, 64:36). MS analysis of the products recovered from HPLC purification showed that the acyclic peptide had been purified and recovered. MS m/z (+ve ES): Measured mass – 3834.06 (480.2 $[M+8H^+]/8$, 548.7 $[M+7H^+]/7$, 639.9 $[M+6H^+]/6$, 767.7 $[M+5H^+]/5$). Calculated mass – 3834.13.

The resulting peptide was dissolved in de-gassed water/acetonitrile (2:1) to a final concentration of ~0.25 mg/mL. The solution was left to stir at room temperature exposed to the atmosphere for one week. After one week the reaction was concentrated *in vacuo*, the remaining residue was re-dissolved in de-gassed water and freeze-dried. The residue was then

purified by preparative reverse phase HPLC, (water:acetonitrile, gradient 5-30% acetonitrile over 10 min: both water and acetonitrile contained 0.1% trifluoroacetic acid). The retention time of the product was approximately 24 min (H_2O/MeCN, 63:37). MS analysis of the peptide recovered from HPLC purification showed that the material had been successfully oxidised to afford peptide **5** as the TFA salt: MS m/z (+ve ES): Measured mass – 3831.80 (480.1 $[M+8H^+]/8$, 548.4 $[M+7H^+]/7$, 639.6 $[M+6H^+]/6$, 767.4 $[M+5H^+]/5$). Calculated mass – 3832.10.