Supporting Information

Effective gene-silencing of siRNAs that contain functionalized spacer linkages within the central region

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Experimental Procedures for the Synthesis of Novel Compounds and Characterizations:

General

Unless otherwise noted, all starting materials that were used were obtained from commercial sources and without any additional purification. Anhydrous CH$_2$Cl$_2$, THF and DMF were purchased from Sigma-Aldrich and degassed by stirring under a dry N$_2$ (g) atmosphere. Flash column chromatography was performed with Silicycle Siliaflash 60 (230-400 mesh) according to the procedure of Still, Kahn, and Mitra.$^1$ NMRs were performed on a Varian 400 MHz spectrometer. All $^1$H NMRs were recorded for either 32 or 64 transients at 400 MHz, all $^{13}$C NMRs were typically run overnight for 20k to 50k transients at 100 MHz and all $^{31}$P NMRs were recorded for 256 transients at 167 MHz. Spectra were processed and integrated using ACD/NMR Processor Academic Edition. $^1$H NMR peaks were referenced to 7.27 ppm for experiments performed in CDCl$_3$ or 2.50 ppm for experiments performed in $d_6$-DMSO. $^{13}$C NMR peaks were referenced to 77.00 for experiments performed in CDCl$_3$ or 39.51 for experiments performed in $d_6$-DMSO. ESI-HRMS were recorded on an Agilent Q-TOF and all novel compounds were analyzed through positive electrospray ionization using a mobile phase of acetonitrile/MeOH (95:5) with 0.1% formic acid.
**Synthesis of 2,2’-(prop-2-yn-1-ylazanediyl)bis(ethan-1-ol) – Compound (1)**

To a solution of diethanolamine (9.44 g, 89.8 mmol) in 150 ml of CH$_2$Cl$_2$ on ice was added anhydrous potassium carbonate (62.1 g, 449 mmol) with vigorous stirring. After 30 minutes of stirring on ice, dropwise addition of propargyl bromide (80% wt/wt, 10.0 ml, 89.8 mmol) was carried out over 5 minutes. The reaction was then left to stir for 60 hours at ambient temperature. The reaction mixture was then filtered using a sintered glass funnel to remove the potassium carbonate. The filtrate was concentrated in vacuo to produce a dark amber oil which was further purified using silica gel chromatography eluting with CH$_2$Cl$_2$ to 10% MeOH in CH$_2$Cl$_2$ to produce a clear amber oil (4.15 g, 32.2%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 2.20 (t, 1H, $J = 2.4$ Hz), 2.72 (t, 4H, $J = 5.2$ Hz), 3.47 (s, 2H), 3.62 (t, 4H, $J = 5.2$ Hz); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 42.16, 55.16, 58.11, 73.09, 78.36. ESI-HRMS (ES$^+$) m/z calculated for C$_7$H$_{13}$NO$_2$ = 143.0951, found = 143.0971 [M+H]$^+$. 

**Synthesis of 2-((2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethyl)(prop-2-yn-1-yl)amino)ethan-1-ol – Compound (2)**

To a solution of 1 (1.98 g, 13.8 mmol) in 25 ml of CH$_2$Cl$_2$ was added triethylamine (1.73 ml, 1.24 mmol) followed by the dropwise addition of 4,4’-dimethoxytrityl chloride (3.74 g, 11.0 mmol) in 5 ml of CH$_2$Cl$_2$. The reaction mixture was left to stir overnight at room temperature after which it was extracted with a saturated NaHCO$_3$ solution. The organic layer was collected and dried with Na$_2$SO$_4$ followed by the concentration in vacuo to produce a green-yellow oil which was further purified by silica gel chromatography eluting with a gradient of CH$_2$Cl$_2$ to 10% MeOH in CH$_2$Cl$_2$ to produce a clear yellow oil (2.83 g, 45.9%), $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 2.19 (t, 1H, $J = 6$ Hz), 2.73 (t, 2H, $J = 5.6$ Hz), 2.80 (t, 2H, $J = 5.6$ Hz), 3.19 (t, 2H, $J = $
6 Hz), 3.44 (d, 2H, J = 2.4 Hz), 3.59 (t, 2H, J = 5.2 Hz), 3.80 (s, 6H), 6.84 (dt, 4H, J = 8.8 Hz),
7.21 (m, 1H, J = 7.6 Hz), 7.30 (td, 2H, J = 7.6 Hz), 7.34 (dt, 4H, J = 8.8 Hz), 7.45 (d, 2H, J = 8
Hz); £ C NMR (125 MHz, CDCl 3 ) δ 42.77, 52.57, 55.20, 55.65, 56.57, 61.95, 72.82, 78.83,
86.21, 113.07, 126.71, 127.78, 128.14, 129.96, 136.29, 144.98, 156.41. ESI-HRMS (ES+) m/z
calculated for C 28 H 31 NO 4 = 445.225, found= 445.228 [M+H]+.

Synthesis of 2-((2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethyl)(prop-2-
yn-1-
yl)amino)ethyl (2-cyanoethyl) diisopropylphosphoramidite – Compound (3)

To a solution of 2 (204 mg, 0.458 mmol) in 5 ml of anhydrous CH 2 Cl 2 under an N 2 (g)
atmosphere was added freshly distilled triethylamine (0.32 ml, 2.29 mmol). After warming to
room temperature in a desiccator, 2-cyanoethyl-\textit{N,N}-diisopropylchlorophosphoramidite (0.31
mL, 1.38 mmol) was added to the reaction mixture which stirred for 2 hours. The reaction
mixture was then concentrated \textit{in vacuo} to produce a cloudy oil which was further purified using
silica gel chromatography eluting with a gradient of 20% EtOAc in hexanes to 80% EtOAc in
hexanes in 2% trimethylamine. The product was isolated as a clear oil (0.12 g, 41.2%) , \textit{1}H NMR
(400 MHz, CDCl 3 ) δ 1.17 (dd, 12H, J = 9.6 Hz), 2.19 (t, 1H, J = 2.4 Hz), 2.56 (m, 2H), 2.81 (dt,
4H, J = 14.2 Hz), 3.16 (t, 2H, J = 6.4 Hz), 3.46 (d, 2H, J = 1.2 Hz), 3.58 (m, 2H), 3.69 (m, 2H),
3.78 (m, 2H), 3.79 (s, 6H), 6.82 (dt, 4H, J = 9.2 Hz), 7.20 (tt, 1H, J = 7.2 Hz), 7.28 (t, 2H, J = 8
Hz), 7.34 (dt, 4H, J = 8.8 Hz), 7.46 (d, 2H, J = 7.2 Hz); \textit{13}C NMR (125 MHz, CDCl 3 ) δ 20.29,
24.51, 24.56, 42.94, 43.06, 43.60, 53.96, 56.15, 58.53, 61.81, 62.42, 72.89, 78.24, 86.01, 112.99,
117.83, 126.59, 127.89, 129.96, 136.38, 145.12, 158.32. ; ESI-HRMS (ES+) m/z calculated for
C 37 H 48 N 3 O 5 P: 645.3332, found 562.2233 [M+H]+ (hydrolyzed).
Synthesis of \((3S,8S,9S,10R,13R,14S,17R)-10,13\text{-dimethyl-17-}((R)-6\text{-methylheptan-2-yl})-2,3,4,7,8,9,10,11,12,13,14,15,16,17\text{-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl methanesulfonate} – \text{Compound (4)}

To a solution of cholesterol (2.14 g, 5.81 mmol) in 15 ml of CH\(_2\)Cl\(_2\) was added triethylamine (0.88 ml, 8.71 mmol) followed by mesyl chloride (0.80 g, 6.97 mmol). The reaction was left stirring for 16 hours at which point the reaction was concentrated \textit{in vacuo} to produce a white solid which was purified further using silica gel chromatography eluting with a gradient of EtOAc/Hexanes (100\% Hexanes to 25\% EtOAc in Hexanes) to afford the title compound as a white crystalline powder (2.59 g, 96\%, \text{Rf} = 0.77 in 25\% EtOAc in hexanes). The NMR shifts match those reported by Neshchadin et. al.\(^3\) \(\text{H NMR (400 MHz, CDCl}_3\) \(\delta\) 0.64 (s, 6H), 0.82 (d, 3H), 0.87 (d, 3H), 0.98 (s, 6H), 1.03-1.15 (m, 5H), 1.22 (d, 2H), 1.30-1.34 (m, 3H), 1.41-1.53 (m, 9H), 1.75-1.81 (m, 1H), 1.85-1.88 (dd, 1H), 1.93-2.04 (m, 2H), 2.43-2.51, (m, 2H), 2.97 (s, 3H), 4.48 (s, 1H), 5.37 (s, 1H); \(\text{C NMR (125 MHz, CDCl}_3\) \(\delta\) 19.4, 20.7, 22.7, 23.2, 24.6, 27.3, 27.7, 28.1, 29.9, 30.0, 31.9, 35.8, 36.1, 36.76, 37.2, 37.7, 38.6, 39.9, 44.0, 50.8, 55.9, 56.49, 82.o, 123.69, 138.53. ESI-HRMS (ES\(^+\)) m/z calculated for \(\text{C}_{28}\text{H}_{48}\text{O}_3\text{S}\): 464.3324, found 464.3313 \([\text{M+H}]^+\).

Synthesis of \((3R,8S,9S,10R,13R,14S,17R)-3\text{-azido-10,13\text{-dimethyl-17-}((R)-6\text{-methylheptan-2-yl})-2,3,4,7,8,9,10,11,12,13,14,15,16,17\text{-tetradecahydro-1H-cyclopenta[a]phenanthrene} – \text{Compound (5)}

To a solution of 4 (1.04 g, 2.18 mmol) in 50 mL DMF was added sodium azide (0.71 g, 10.9 mmol). The round bottom flask was then equipped with a condenser column and the reaction was warmed to 75 °C where it proceeded for 24 hours. The reaction was cooled to room temperature
and subsequently concentrated \textit{in vacuo} which produced a white powder which was further purified using silica gel chromatography eluting with hexanes to afford the title compound as a white-yellow crystalline powder (0.56 g, 62%, Rf = 0.91 in 25% EtOAc in hexanes). The NMR shifts match those reported by Neshchadin et. al.\textsuperscript{3} \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) $\delta$ 0.60 (s, 6H), 0.79 (d, 3H), 0.84 (d, 3H), 0.93 (s, 6H), 0.97-1.04 (m, 5H), 1.18 (d, 2H), 1.25-1.27 (m, 3H), 1.36-1.46 (m, 9H), 1.5 (s, 1H), 1.76-1.78 (dd, 2H), 1.92-1.95 (m, 2H), 2.16-2.21 (m, 2H), 5.27 (s, 1H); $^{13}$C NMR (125 MHz, CDCl\textsubscript{3}) $\delta$ 19.4, 20.7, 22.7, 23.2, 24.6, 27.3, 27.7, 28.1, 29.9, 30.0, 31.9, 35.8, 36.1, 36.76, 37.2, 37.7, 39.9, 44.0, 50.8, 55.9, 56.49, 56.77, 121.76, 140.70. ESI-HRMS (ES$^+$) m/z calculated for C\textsubscript{27}H\textsubscript{45}N\textsubscript{3}: 411.3613, found 411.3622 [M+H]\textsuperscript{+}.

**Synthesis of 2-((2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethyl)((1-(((3R,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)methyl)-1H-1,2,3-triazol-4-yl)methyl)amino)ethan-1-ol – Compound (6)**

To a solution of 2-((2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethyl)(prop-2-yn-1-yl)amino)ethan-1-ol (0.62 g, 1.39 mmol) in a 1:1:1 mixture of THF, water and tert-butanol was added \textbf{5} (1.14 g, 2.77 mmol), copper sulfate pentahydrate (0.17 g, 0.69 mmol) and (+)-sodium ascorbate (0.55 g, 2.77 mmol). The reaction was stirred for 18 hours at room temperature at which the reaction was the concentrated \textit{in vacuo} and was subsequently extracted using water and CH\textsubscript{2}Cl\textsubscript{2}. The organic layer was collected and dried with Na\textsubscript{2}SO\textsubscript{4} which produced a green oil. Silica gel chromatography was then used to further purify the product using gradient eluting with hexanes/EtOAc (50% hexanes in EtOAc to 100% EtOAc) to afford the title compound as a thick clear oil (0.92 g, 77%); \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) $\delta$ 0.67 (s, 2H), 0.88 (m, 10H), 0.99-1.19 (m, 11H), 1.24-1.56 (m, 11H), 1.66 (d, 1H, $J$ = 8.7 Hz), 1.82 (s, 1H), 1.99 (m, 2H), 2.05-2.25 (m,
3H), 2.50 (d, 1H, J = 8.9 Hz), 2.73 (d, 4H, J = 5.6 Hz), 2.96 (m, 3H), 3.24 (s, 2H), 3.58 (s, 2H), 3.79 (s, 6H), 3.89 (s, 2H), 4.87 (s, 1H), 5.41 (s, 1H), 6.79-6.86 (m, 4H), 7.17-7.33 (m, 7H), 7.37 (m, 2H), 7.68 (s, 1H); 13C NMR (125 MHz, CDCl3) δ 11.51, 17.21, 18.37, 18.95, 20.29, 22.22, 22.48, 23.51, 23.84, 26.09, 27.67, 27.85, 31.31, 31.56, 32.48, 35.44, 35.67, 35.86, 36.72, 39.08, 39.18, 41.90, 49.62, 55.74, 56.14, 112.74, 124.09, 126.40, 127.45, 127.82, 129.66, 135.97, 137.59, 144.67, 158.06. ESI-HRMS (ES+) m/z calculated for C55H76N4O4: 856.5772 found 879.5783 [M + Na]+.

Synthesis of 2-((2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethyl)((1-(((3R,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)methyl)-1H-1,2,3-triazol-4-yl)methyl)amino)ethyl (2-cyanoethyl) diisopropylphosphoramidite – Compound (7)

To a solution of 6 (0.33 g, 0.38 mmol) in 10 ml of dry CH2Cl2 was prepared in flame dried glassware and put under a N2(g) atmosphere. Freshly distilled triethylamine (0.16 ml, 1.14mmol) and 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (0.26 ml, 1.15 mmol) were then added to the solution via an anhydrous transfer. The reaction was allowed to stir at room temperature for 1.5 hours at which point TLC analysis revealed the consumption of the starting material (Rf = 0.23 in EtOAc). The reaction mixture was the concentrated in vacuo which produced a yellow oil which was further purified using silica gel chromatography using gradient elution of hexanes/EtOAc (50% hexanes in EtOAc to 100% EtOAc) affording the title compound as a clear oil (0.27 g, 68%); 1H NMR (400 MHz, CDCl3) δ 0.67 (s, 2H), 0.86-1.56 (m, 44H), 1.66 (m, 2H), 1.79-2.12 (m, 6H), 2.22 (d, 1H, J = 8.9 Hz), 2.47 – 2.60 (m, 3H), 2.78 (m, 4H), 2.94 (d, 1H, J = 5.6 Hz), 3.18 (s, 2H), 3.62 (m, 4H), 3.70-3.81 (m, 9H), 3.88 (s, 2H), 4.14 (m, 1H), 5.39 (m, 1H),
6.77-6.87 (m, 4H), 7.16-7.35 (m, 7H), 7.37 (m, 2H), 7.71 (s, 1H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 11.81, 14.18, 18.68, 19.26, 20.32, 20.54, 22.54, 22.80, 23.83, 23.89, 23.96, 26.67, 27.99, 31.64, 31.98, 35.75, 36.16, 37.03, 39.49, 41.80, 42.92, 43.05, 49.94, 54.24, 55.16, 56.38, 56.42, 58.33, 58.51, 60.35, 62.48, 85.99, 112.99, 117.62, 121.91, 124.20, 126.61, 127.69, 129.98, 136.45, 136.44, 137.93, 144.27, 145.19, 158.32; $^{31}$P NMR (125 MHz, CDCl$_3$) $\delta$ 147.61 ; ESI-HRMS (ES$^+$) m/z calculated for C$_{64}$H$_{93}$N$_6$O$_5$P 1056.6002, found 1056.6063 [M+H]$^+$ (hydrolyzed).

**Synthesis of N,N’-bis(2-((tert-butyldimethylsilyl)oxy)ethyl)ethane-1,2-diamine – Compound (8)**

A solution of N,N’-bis(2-hydroxyethyl)ethandiamine (4.95 g, 33.4 mmol) in 175 mL of CH$_2$Cl$_2$ was prepared and allowed to stir for 10 minutes to dissolve the starting material. To this solution was added diisopropylethylamine (14.5 mL, 83.5 mmol) followed by the drop-wise addition of a solution of tert-butyldimethylsilylchloride (10.57 g, 70.1 mmol) in 50 mL of CH$_2$Cl$_2$ over three minutes. The reaction was left stirring at room temperature for 20 hours. A saturated NaHCO$_3$ solution extraction was performed and the organic layer was dried with Na$_2$SO$_4$. The organic layer was concentrated *in vacuo* to afford an oil which was purified by silica gel chromatography eluting with a gradient of MeOH/CH$_2$Cl$_2$ (100% CH$_2$Cl$_2$ to 5% MeOH in CH$_2$Cl$_2$) to afford the title compound as a clear yellow oil (8.39 g, 68%); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 0.04 (s, 12H), 0.87 (s, 18H), 2.40 (br. s, 2H), 2.72 (t, 4H, $J = 5.86$ Hz), 2.76 (s, 4H), 3.70 (t, 4H, $J = 5.5$ Hz); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ –5.36, 18.26, 25.90, 53.13, 57.54, 62.82, 77.85; ESI-HRMS (ES$^+$) m/z calculated for C$_{18}$H$_{44}$N$_2$O$_2$Si$_2$: 377.3014, found 377.3023 [M+H]$^+$.
Synthesis of N,N'-bis(2-((tert-butyldimethylsilyl)oxy)ethyl)-N,N'-di(prop-2-yn-1-yl)ethane-1,2-diamine – Compound (9)

A solution of 8 (5.07 g, 13.47 mmol) in 50 mL of dry CH₂Cl₂ was prepared in flame dried glassware sealed with a septum under a N₂ (g) atmosphere. To this was added diisopropylethylamine (7.1 mL, 40.4 mmol) that had been freshly distilled under N₂ (g). An anhydrous transfer of propargyl bromide (80% wt/mL solution in toluene) (3.1 mL, 33.7 mmol) to the reaction was accomplished and this reaction was stirred for 12 hours at room temperature under an N₂ (g) atmosphere. The reaction solution was extracted with a saturated NaHCO₃ solution. The organic layer was collected and dried with Na₂SO₄ and was subsequently dried in vacuo to afford an oil which was further purified using silica gel chromatography eluting with a gradient of hexanes/EtOAc (100% hexanes to 20% EtOAc in hexanes) to afford the title compound as a dark yellow oil (5.36 g, 88%); ¹H NMR (400 MHz, CDCl₃) δ 0.00 (s, 12H), 0.84 (s, 18H), 2.11 (s, 2H), 2.61 (m, 8H), 3.44 (t, 4H, J = 1.6 Hz), 3.61 (t, 4H, J = 6.3 Hz); ¹³C NMR (125 MHz, CDCl₃) δ -5.29, 18.32, 25.95, 52.73, 56.38, 58.58, 61.92, 117.13, 135.97; ESI-HRMS (ES⁺) m/z calculated for C₂₄H₄₈N₂O₂Si₂: 453.3327 found 453.3325 [M+H]⁺.

Synthesis of N,N'-diallyl-N,N'-bis(2-((tert-butyldimethylsilyl)oxy)ethyl)ethane-1,2-diamine – Compound (10)

A solution of 8 (2.03 g, 5.395 mmol) in 50 mL of CH₂Cl₂ was prepared and allowed to cool in an ice bath while a mineral oil-sodium hydride dispersion (60% NaH) (0.68 g, 16.2 mmol) was washed three times with hexanes to remove as much mineral oil as possible. The sodium hydride was then slowly added to the solution of 8. Once the reaction mixture was no longer bubbling; allyl bromide (1.16 mL, 13.5 mmol) was added over the course of 3 minutes. The reaction was
gradually warmed to room temperature then left to stir for 6 hours and was then extracted with saturated NaHCO₃ solution. The organic layer was collected and dried with Na₂SO₄ then concentrated in vacuo to afford an oil which was purified using silica gel chromatography eluting with a gradient of hexanes/EtOAc (100% hexanes to 30% EtOAc in hexanes) to afford the title compound as a yellow oil (2.46 g, 74%); ¹H NMR (400 MHz, CDCl₃) δ 0.05 (s, 12H), 0.89 (s, 18H), 2.59 (s, 4H), 2.61 (t, 4H, J = 7.03 Hz), 3.15 (d, 4H, J = 6.3 Hz), 3.67 (t, 4H, J = 6.6 Hz), 5.13 (dd, 4H, J = 9.8 Hz), 5.84 (m, 2H): ¹³C NMR (125 MHz, CDCl₃) δ -5.29, 18.32, 25.95, 52.73, 56.38, 58.58, 61.90, 117.13, 135.97; ESI-HRMS (ES⁺) m/z calculated for C₂₄H₅₂N₂O₂Si₂ 468.3567, found 468.3498 [M+H]⁺.

Synthesis of N,N’-(ethane-1,2-diyl)bis(N-(2-((tert butyldimethylsilyl) oxy)ethyl)acetamide) – Compound (11)

To a solution of 8 (0.48 g, 1.28 mmol) in 15 ml of CH₂Cl₂ was added 4,4-dimethylaminopyridine (DMAP) (0.08 g, 0.67 mmol) and this mixture was stirred until both reagents fully dissolved. To this solution was added acetic anhydride (0.38 ml, 3.99 mmol) and the resulting mixture was left to stir for 20 hours at which point the reaction was extracted with a saturated NaHCO₃ solution. The organic layer was collected and dried with Na₂SO₄ which was concentrated in vacuo to afford a yellow oil which was further purified using silica gel chromatography eluting with a gradient of MeOH/CH₂Cl₂ (2% MeOH in CH₂Cl₂ to 5% MeOH in CH₂Cl₂) to afford the title compound as a white-yellow powder. (0.56 g, 93%); ¹H NMR (400 MHz, CDCl₃) δ 0.03 (s, 12H), 0.87 (s, 18H), 2.12 (s, 6H), 3.46 (m, 8H), 3.73 (t, 4H, J = 5.5 Hz); ¹³C NMR (125 MHz, CDCl₃) δ -5.55, 18.16, 21.72, 25.80, 43.22, 51.23, 60.77, 171.72: ESI-HRMS (ES⁺) m/z calculated for C₂₄H₄₈N₂O₄Si₂: 461.3225 found 461.3242 [M+H]⁺.
Synthesis of 2,2′-(ethane-1,2-diylbis(prop-2-yn-1-ylazanediyl))bis(ethan-1-ol) – Compound (12)

To a solution of 9 (1.79 g, 3.95 mmol) in 10 mL THF was added 1.0 M tetrabutylammonium fluoride (13.8 mL, 13.8 mmol) drop-wise over the course of 3 minutes. The reaction mixture was left stirring at room temperature for 5 hours, at which point TLC revealed consumption of the starting material 5 (Rf = 0.72 in EtOAc). The reaction mixture was concentrated in vacuo which produced a dark yellow oil which was further purified using silica gel chromatography eluting with a gradient of MeOH/CH₂Cl₂ (2% MeOH in CH₂Cl₂ to 10% MeOH in CH₂Cl₂) to afford the title compound as a thick yellow oil (0.82 g, 93%); ¹H NMR (400 MHz, CDCl₃) δ 2.23 (s, 2H), 2.74 (m, 8H), 3.49 (s, 4H), 3.62 (t, 4H, J = 5.1 Hz); ¹³C NMR (125 MHz, CDCl₃) δ 42.38, 49.75, 54.60, 58.80, 72.96, 77.78; ESI-HRMS (ES⁺) m/z calculated for C₁₂H₂₀N₂O₂: 225.1598, found 225.1598 [M+H]⁺.

Synthesis of 2,2′-(ethane-1,2-diylbis(allylazanediyl))bis(ethan-1-ol) – Compound (13)

To a solution of 10 (0.59 g, 1.42 mmol) in 10 ml of THF was added 1.0M tetrabutylammonium fluoride (4.96 mL, 4.96 mmol) over 3 minutes. The reaction mixture was left stirring at room temperature for 4.5 hours at which point TLC revealed consumption of the starting material (Rf = 0.92 in 15% MeOH in CH₂Cl₂). The reaction mixture was concentrated in vacuo which produced a thick yellow oil which was further purified using silica gel chromatography eluting with a gradient of MeOH/CH₂Cl₂ (2% MeOH in CH₂Cl₂ to 5% MeOH in CH₂Cl₂) to afford the title compound as a thick yellow oil (0.30 g, 92%); ¹H NMR (400 MHz, CDCl₃) δ 2.70 (m, 8H), 3.27 (d, 4H, J = 6.6 Hz), 3.66 (t, 4H, J = 5.1 Hz), 5.23 (dm, 4H, J = 11.7 Hz), 5.91 (m, 2H): ¹³C
NMR (125 MHz, CDCl$_3$) $\delta$ 51.62, 55.61, 58.18, 59.86, 118.44, 134.58; ESI-HRMS (ES$^+$) m/z calculated for C$_{12}$H$_{24}$N$_2$O$_2$: 229.1911, found 229.1920 [M+H]$^+$.  

**Synthesis of N,N'-(ethane-1,2-diyl)bis(N-(2-hydroxyethyl)acetamide) – Compound (14)**

To a solution of 11 (1.02 g, 2.21 mmol) in 10 mL of THF was added triethylamine trihydrofluoride (0.51 mL, 3.10 mmol) drop-wise over 5 minutes. The resulting reaction mixture was allowed to stir at room temperature for 3 hours at which point TLC analysis indicated the consumption of the starting material ($R_f$ = 0.79 in 15% MeOH in CH$_2$Cl$_2$). The reaction was concentrated *in vacuo* to afford an pale yellow oil which was further purified using silica gel chromatography eluting with a gradient of MeOH/CH$_2$Cl$_2$ (5% MeOH in CH$_2$Cl$_2$ to 20% MeOH in CH$_2$Cl$_2$) to afford the title compound as a pale golden coloured oil (0.47 g, 92%); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 2.11 (s, 6H), 3.50 (m, 8H), 3.79 (t, 4H, $J = 5.5$ Hz); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 21.87, 46.19, 53.40, 60.27; ESI-HRMS (ES$^+$) m/z calculated for C$_{10}$H$_{20}$N$_2$O$_4$: 233.1496 found 233.1500 [M+H]$^+$.  

**Synthesis of 2-((2-((2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethyl)(prop-2-yn-1-yl)amino)ethyl)(prop-2-yn-1-yl)amino)ethan-1-ol – Compound (15)**

A solution of 12 (0.54 g, 2.41 mmol) in 30 ml of CH$_2$Cl$_2$ was added diisopropylethylamine (0.34 mL, 1.93 mmol) which was left to stir. Another solution of 4,4’-dimethoxytrityl chloride in 5 mL of CH$_2$Cl$_2$ was prepared and was subsequently added to the solution being stirred drop-wise over 10 minutes. The reaction was left to stir overnight at which point it was extracted with a saturated NaHCO$_3$ solution. The organic layer was collected and dried with Na$_2$SO$_4$ and concentrated *in vacuo* to produce a dark oil which was further purified using silica gel chromatography eluting with a gradient of hexanes/EtOAc (20% EtOAc in hexanes to 100%
EtOAc) to afford the title compound as a dark oil (0.56 g, 46%); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.27 (s, 1H), 2.19 (dt, 2H, \(J = 14.85\) Hz), 2.67 (m, 6H), 2.79 (t, 2H, \(J = 5.9\) Hz), 3.24 (t, 2H, \(J = 5.7\) Hz), 3.41 (m, 2H), 3.47 (m, 2H), 3.52 (t, 2H, \(J = 5.3\) Hz), 3.80 (s, 6H), 6.84 (dt, 4H, \(J = 8.6\) Hz), 7.20-7.35 (m, 7H), 7.44-7.48 (m, 2H); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 42.24, 42.70, 50.26, 51.49, 53.20, 54.38, 55.01, 58.80, 61.67, 72.42, 76.13, 85.89, 112.73, 126.33, 127.42, 127.87, 129.70, 136.03, 144.75, 158.06; ESI-HRMS (ES\(^{+}\)) m/z calculated for C\(_{33}\)H\(_{38}\)N\(_2\)O\(_4\): 527.2904, found 527.2899 [M + H]\(^{+}\).

**Synthesis of 2-(allyl(2-(allyl(2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethyl)amino)ethyl)amino)ethan-1-ol-Compound (16)**

To a solution of 13 (0.61 g, 2.61 mmol) in 30 mL of CH\(_2\)Cl\(_2\) was added diisopropylethylamine (0.37 mL, 2.09 mmol) followed by the drop-wise addition of 4,4’-dimethoxytrityl chloride (0.71 g, 2.10 mmol) in 5 mL of CH\(_2\)Cl\(_2\) over 10 minutes. The reaction was left to stir overnight at which time the reaction became bright yellow. The reaction was extracted with saturated NaHCO\(_3\) solution and the organic layer was collected and dried with Na\(_2\)SO\(_4\). The organic layer was concentrated \textit{in vacuo} to produce an orange- yellow oil which was further purified using silica gel chromatography eluting with a gradient of hexanes/EtOAc (50% hexanes in EtOAc to 10% hexanes in EtOAc) to afford the title compound as an oil (0.63 g, 46%); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.23 (s, 1H), 2.67 (m, 4H), 2.76 (t, 2H, \(J = 5.5\) Hz), 2.87 (t, 2H, \(J = 5.7\) Hz), 3.20 (m, 4H), 3.26 (t, 2H, \(J = 5.6\) Hz), 3.55 (t, 2H, \(J = 4.7\) Hz), 3.75 (s, 6H), 5.14 (dd, 4H, \(J = 10.2\) Hz), 5.83 (m, 2H), 6.8 (dt, 4H, \(J = 9.4\) Hz), 7.17-7.27 (m, 7H), 7.39 (m, 2H); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 29.64, 50.45, 51.44, 52.78, 55.18, 55.58, 57.25, 57.84, 59.03, 60.39, 86.55, 113.11, 126.78, 127.76, 127.82, 129.92, 133.62, 135.89, 144.68, 158.46; ESI-HRMS (ES\(^{+}\)) m/z calculated for C\(_{33}\)H\(_{42}\)N\(_2\)O\(_4\): 530.3113, found 530.3183 [M+H]\(^{+}\).
Synthesis of \( N-(2-(\text{bis}(4\text{-methoxyphenyl})(\text{phenyl})\text{methoxy})\text{ethyl})-N-(2-(\text{2-hydroxyethyl})\text{acetamido})\text{ethyl})\text{acetamide} \) – Compound (17)

A solution of 14 (1.12 g, 4.82 mmol) in 30 mL of CH\(_2\)Cl\(_2\) had triethylamine (0.34 mL, 3.35 mmol) added to it and the resulting mixture was allowed to stir for 10 minutes to dissolve the starting material. Meanwhile, another solution of 4,4'-dimethoxytrityl chloride (0.98 g, 2.89 mmol) in 5 ml of CH\(_2\)Cl\(_2\) was prepared and added drop-wise to the first solution over a period of 10 minutes. The reaction was then left to stir at room temperature overnight at which point the reaction was extracted with a saturated NaHCO\(_3\) solution. The organic layer was collected and dried with Na\(_2\)SO\(_4\) and concentrated \textit{in vacuo} to afford a yellow oil. This oil was further purified using silica gel chromatography eluting with a gradient of MeOH/CH\(_2\)Cl\(_2\) (2% MeOH in CH\(_2\)Cl\(_2\) to 10% MeOH in CH\(_2\)Cl\(_2\)) to afford the title compound as a bright yellow oil (1.13 g, 44%); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 2.13 (m, 6H), 3.27 (m, 2H), 3.37-3.64 (m, 8H), 3.70-3.84 (m, 8H), 6.84 (m, 4H), 7.17-7.33 (m, 7H), 7.36-7.41 (m, 2H); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 21.96, 46.90, 53.95, 55.23, 60.44, 81.37, 86.68, 113.12, 127.02, 127.76, 129.12, 129.95, 135.73, 139.51, 147.37, 158.52, 172.92; ESI-HRMS (ES\(^+\)) m/z calculated for C\(_{31}\)H\(_{38}\)N\(_2\)O\(_6\): 557.2622, found 557.2611 [M+H]\(^+\).

Synthesis of \( 2-((2-((2-(\text{bis}(4\text{-methoxyphenyl})(\text{phenyl})\text{methoxy})\text{ethyl})(\text{prop-2-yn-1-yl})\text{amino})\text{ethyl})(\text{prop-2-yn-1-yl})\text{amino})\text{ethyl} \) (2-cyanoethyl) diisopropylphosphoramidite – Compound (18)

A solution of 15 (0.36 g, 0.68 mmol) in 10 mL of dry CH\(_2\)Cl\(_2\) was prepared using flame dried glassware under a N\(_2\) \((g)\) atmosphere. To this solution was added freshly distilled
diisopropylethylamine (0.59 mL, 3.40 mmol). 2-cyanoethyl-\(N,N\)-diisopropyldiisopropylchlorophosphoramidite (0.45 mL, 2.04 mmol) was added over 15 seconds and then the reaction was stirred for 2.5 hours at which point TLC analysis showed consumption of the starting material (R\(_f\) = 0.88 in EtOAc). The reaction was concentrated in vacuo affording a yellow oil which was further purified using silica gel chromatography eluting with a gradient of hexanes/EtOAc (20% EtOAc in hexanes to 50% EtOAc in hexanes at 2% TEA with each mobile phase) affording the title compound as a clear oil (0.35 g, 70.5%); ¹H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 1.29 (m, 12H), 2.19 (dt, 2H, J = 10.94 Hz), 2.64 (m, 8H), 2.78 (m, 6H), 3.16 (t, 2H, J = 5.9 Hz), 3.46 (m, 5H), 3.79 (s, 6H), 6.82 (dt, 4H, J = 8.6 Hz), 7.20–7.35 (m, 7H), 7.44–7.48 (m, 2H); ¹³C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 20.32, 21.20, 22.96, 24.52, 25.89, 43.07, 45.24, 47.65, 52.04, 53.87, 55.16, 58.51, 62.36, 72.93, 79.03, 86.01, 113.00, 117.62, 126.57, 128.15, 129.98, 136.40, 145.13, 158.32; ³¹P NMR (125 MHz, CDCl\(_3\)) \(\delta\) 147.91; ESI-HRMS (ES\(^+\)) m/z calculated for \(C_{42}H_{55}N_4O_5P\) 726.3911, found 644.2881 [M+H]\(^+\) (hydrolyzed).

**Synthesis of 2-(allyl(2-(allyl(2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethyl)amino)ethyl)amino)ethyl (2-cyanoethyl)diisopropyldiisopropylphosphoramidite – Compound (19)**

A solution of ¹⁶ (0.33 g, 0.63 mmol) in 10 mL of dry CH\(_2\)Cl\(_2\) was prepared in a flame dried round bottom flask under a N\(_2\) (g) atmosphere. Freshly distilled diisopropylethylamine (0.55 ml, 3.16 mmol) was added via an inert transfer. 2-cyanoethyl-\(N,N\)-diisopropyldiisopropylchlorophosphoramidite (0.42 ml, 1.89 mmol) was added to the reaction over 15 seconds. The reaction proceeded for 3 hours at which point TLC analysis indicated consumption of the starting material (R\(_f\) = 0.18 in EtOAc). The reaction mixture was concentrated in vacuo producing a yellow oil which was further purified using silica gel chromatography eluting with a gradient of Hexanes/EtOAc (20%
EtOAc in hexanes to 50% EtOAc in hexanes, with each mobile phase having 2% triethylamine) to afford the title compound as a clear oil (0.45 g, 71%); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.18 (m, 12H), 2.57 (m, 6H), 2.70 (m, 5H), 3.14 (m, 6H), 3.61 (m, 3H), 3.80 (s, 6H), 5.16 (m, 4H), 5.83 (m, 2H), 6.82 (d, 4H, J = 9 Hz), 7.20 – 7.35 (m, 7H), 7.45 (m, 2H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 20.27, 24.62, 42.93, 52.66, 54.14, 55.18, 58.27, 61.65, 62.23, 85.98, 112.99, 117.25, 126.58, 127.69, 128.19, 130.00, 135.79, 136.51, 145.21, 158.33; $^{31}$P NMR (125 MHz, CDCl$_3$) $\delta$ 147.64; ESI-HRMS (ES$^+$) m/z calculated for C$_{43}$H$_{62}$N$_3$O$_5$P: 730.4124, found 647.3183 [M+H]$^+$ (Hydrolyzed product).

**Synthesis of 2-(N-(2-(N-(2-(bis(4-methoxyphenyl)(phenyl)methoxy)ethyl)acetamido)ethyl)acetamido)ethyl (2-cyanoethyl) diisopropylphosphoramidite – Compound (20)**

A solution of 17 (0.12 g, 0.21 mmol) in 8 mL of dry CH$_2$Cl$_2$ was prepared in flame dried glassware. To this was added freshly distilled triethylamine (0.15 ml, 1.03 mmol) and the reaction was placed under a N$_2$ (g) atmosphere. 2-cyanoethyl-$N,N$-diisopropylchlorophosphoramidite (0.14 ml, 0.56 mmol) was added over 15 seconds and then the reaction was stirred at room temperature for 3.5 hours at which point TLC analysis indicated consumption of the starting material ($R_f$ = 0.28 in EtOAc with 2% TEA). The reaction was then concentrated *in vacuo* which produced a yellow oil. This oil was then further purified using silica gel chromatography eluting with a gradient of hexanes/EtOAc (50% hexanes in EtOAc to 100% EtOAc keeping 2% TEA for each mobile phase) to afford the title compound as a clear oil (0.11 g, 71%); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.26 (m, 12H), 2.13 (m, 6H), 3.27 (m, 2H), 3.36-3.66 (m, 8H), 3.72 (m, 1H), 3.81 (s, 6H), 6.79-6.86 (m, 4H), 7.17-7.33 (m, 7H), 7.37 (m, 2H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 17.01, 20.18, 21.97, 22.54, 23.61, 24.91, 25.52, 42.77, 42.93, 46.30,
47.06, 49.74, 51.13, 53.54, 54.18, 55.70, 58.78, 58.94, 62.01, 62.86, 72.44, 78.31, 86.08, 113, 59, 118.29, 126.97, 127.95, 128.04, 129.67, 136.72, 145.83, 158.49, 172.41; ESI-HRMS (ES+) m/z calculated for C_{40}H_{55}N_{4}O_{7}P: 734.3881, found 651.2703 [M+H]^+ (hydrolyzed).

(2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)phenyl)methanol- compound (21)

White solid 1,2-benzenedimethanol (304.65 mg, 2.20 mmol) was dissolved in 10 mL (123.64 mmol) of anhydrous pyridine. 4,4’-dimethoxytrityl chloride (592.20 mg, 1.75 mmol) was then added to the reaction solution which was then purged with nitrogen gas stirred overnight. The reaction was extracted in 10 mL of DCM, washed five times with water, and dried with Na$_2$SO$_4$. The organic layer was concentrated in vacuo to yield a thick yellow oil. This oil was purified by silica using flash chromatography with mobile phase hexane:EtOAc (Gradient: 3:1→2:1 hexane:EtOAc), to give product as a clear colourless oil (0.52 g, 1.16 mmol). Yield: 66.5%. $^1$H NMR (400 MHz, CDCl$_3$) δ3.74 (s, 6H), 4.08 (s, 2H), 4.53 (s, 2H), 5.20 (m, 4H), 7.37 (m, 12H) and 7.50 (m, 3H).$^{13}$C NMR (125 MHz, CDCl$_3$) δ55.22, 63.52, 65.14, 87.52, 113.34, 126.91, 127.92, 128.05, 128.48, 129.41, 129.94, 135.74, 136.40, 140.42, 144.53, 149.06, 158.59. ESI-HRMS (ES+) m/z calculated for C$_{29}$H$_{28}$O$_4$= 440.1874, found 463.1880 [M+Na]$^+$.  

2-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)benzyl (2-cyanoethyl) diisopropylphosphoramidite- Compound (22)

Compound 21 (487.60 mg, 1.11 mmol) was dissolved in 10 mL of anhydrous THF. Triethylamine (TEA) (1.50 mL, 10.76 mmol), 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (1.4 mL, 6.28 mmol), and DMAP 70.13 mg, 0.57 mmol) were added, respectively. The reaction was purged with N$_2$(g) and monitored using TLC. After 4 hours the reaction was concentrated in vacuo to yield a thick yellow oil and white solid. The
phosphoramidite was purified by silica using flash chromatography with mobile phase hexane:acetone in a 2:1 ratio with 2%TEA. The resulting product was a clear, colourless solid (0.45g, 0.71 mmol). Yield: 64.0%. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$1.05-1.32 (m, 17H), 1.40-1.41 (m, 1H), 2.45-2.48 (m, 2H), 2.72-2.74 (m, 1H),3.49-3.58 (m, 3H), 3.64-3.69 (m, 2H), 3.78 (s, 6H), 4.2 (m, 2H), 4.5-4.64 (m, 1H), 6.82-6.85 (m, 4H), 7.19-7.43 (m, 11H), 7.49-7.61 (m, 2H). $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$20.48, 20.54, 24.74, 24.81, 24.92, 43.3, 43.43, 45.55, 45.61, 55.48, 58.65, 58.83, 62.99, 63.17, 63.71, 86.82, 113.41, 117.87, 127.03, 127.88, 127.96, 128.12, 128.42, 129.41, 130.33, 136.46, 136.89, 145.30, 158.75. $^{31}$P NMR (167 MHz, CDCl$_3$) $\delta$147.89. ESI-HRMS (ES$^+$) m/z calculated for C$_{39}$H$_{46}$NO$_5$P = 639.3113, found 606.2977 [M+H]$^+$ (hydrolysed).

$(4'$-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-[1,1'$'$-biphenyl]-4-yl)methyl 2-cyanoethyl diisopropylphosphoramidite- Compound (23)

4,4-bis(hydroxymethyl)biphenyl (243.4 mg, 1.14 mmol) was dissolved in 10 mL (123.6 mmol) of anhydrous pyridine. 4,4'-dimethoxytrityl chloride (308.3 mg, 0.91 mmol) was then added to the reaction solution which was then purged with N$_2$ (g) stirred overnight. The reaction was extracted in 10 mL of CH$_2$Cl$_2$, washed five times with water, and dried with Na$_2$SO$_4$. The organic layer was concentrated in vacuo to yield a yellow oil. This oil was partial purified by silica using flash chromatography with mobile phase hexane:acetone (Gradient: 30% acetone in hexanes to 80% acetone in hexanes), to give a clear colourless oil. This oil decayed in vacuo and could not be characterized, thus it was reacted immediately to form compound 23. The oil was dissolved in 10 mL of anhydrous CH$_2$Cl$_2$. Triethylamine (TEA) (0.55 mL, 3.95 mmol), 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.50 mL, 2.24 mmol), and DMAP (26.57mg, 0.22 mmol) were added, respectively. The reaction was purged with N$_2$ (g) and monitored using
TLC. After 4 hours the reaction was concentrated in vacuo to yield a thick yellow oil. The phosphoramidite was purified by silica using flash chromatography with mobile phase hexane:acetone in a 2:1 ratio with 2% TEA. The resulting product was a clear colourless solid (0.19 g, 0.26 mmol). Yield: 69.4%. $^1$H NMR (400 MHz, CDCl$_3$), $\delta$1.20-1.34 (m, 18H), 2.64-2.67 (m, 2H), 3.66-3.71 (m, 2H), 3.81-3.92 (m, 8H), 4.23 (s, 2H), 4.70-4.84 (m, 1H), 6.83-6.88 (m, 4H), 7.24-7.62 (m, 17H). $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$20.41, 24.63, 43.27, 45.34, 55.21, 58.40, 58.59, 65.17, 65.29, 86.40, 113.12, 117.62, 126.74, 126.91, 127.38, 127.83, 128.21, 129.11, 130.08, 136.29, 137.99, 138.41, 139.64, 140.25, 145.06, 158.46. $^{31}$P NMR (167 MHz, CDCl$_3$) $\delta$148.09. ESI-HRMS (ES$^+$) m/z calculated for C$_{44}$H$_{49}$N$_2$O$_5$P= 739.3271, found 762.3279 [M+H$^+$].
Experimental Nucleic Acid and Biological Procedures:

Procedure for Nucleic Acid Synthesis and Purification

All β-cyanoethyl 2’-O-TBS protected phosphoramidites, reagents and solid supports were purchased from ChemGenes Corporation and Glen Research. Anti-luciferase and anti-GAPDH strands including the sense and 5’-phosphorylated antisense strand, were purchased from and purified by Integrated DNA Technologies (IDT). All commercial phosphoramidites were dissolved in anhydrous acetonitrile to a concentration of 0.1 M. All sequences were synthesized via solid support phosphoramidite chemistry using the Applied Biosystems 394 DNA/RNA synthesizer. Oligonucleotides were synthesized on 0.2 μM dT solid supports. 1.0 μM synthesis cycles were used and all reactions were performed under an inert N₂ (g) atmosphere maintained at 55 psi. All phosphoramidites were synthesized using coupling times of 15 minutes. Antisense sequences were chemically phosphorylated at the 5’-end by using 2-[2-(4,4’-dimethoxytrityloxy)ethylsulfonyl]ethyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite. Upon completion, columns were removed from the synthesizer, dried under a stream of N₂ (g), sealed and stored at 4 °C. Oligonucleotides were removed from solid support upon exposure to 1.5 mL of EMAM (methylamine 40% wt. in H₂O and methylamine 33% wt. in ethanol, 1:1 (Sigma)). Columns were incubated with EMAM for 1 hour at room temperature with the solution
in full contact with the controlled pore glass. The oligonucleotides were then transferred into screw cap microcentrifuge tubes and incubated overnight at room temperature in EMAM to deprotect the nitrogenous bases. The following day, samples were evaporated on a Speedvac evaporator overnight and resuspended in a solution of dimethylsulfoxide (DMSO): 3HF/triethylamine (TEA) (100 μl: 125 μl) (Sigma). The samples were then incubated at 65 °C for 2.5 hours to remove the 2’-O-TBS protecting groups. Crude oligonucleotides were precipitated in EtOH and desalted through Millipore Amicon Ultra 3000 MW cellulose filters. Oligonucleotides were purified using a 20% denaturing polyacrylamide gel. Pure oligonucleotides were excised from the gel and were purified in a microcentrifuge tube by crushing and soaking the excised gel in an elution buffer (3 mM sodium acetate, 10 μM EDTA, pH 7). The resulting oligonucleotides were desalted a second time through Millipore Amicon Ultra 3000 MW cellulose filters. At 10 μM, equimolar amounts of complimentary RNAs were combined and dried down in a Speedvac overnight. The RNAs were then suspended in a binding buffer (75 mM KCl, 50 mM Tris-HCl, 3 mM MgCl2, pH 8.3) and incubated at 95 °C for 2 minutes and then cooled slowly to room temperature to generate siRNA duplexes used for biological assays.

**Procedure for Characterizing Oligonucleotides through ESI Q-TOF**

All single-stranded RNAs were gradient eluted through a Zorbax Extend C18 HPLC column with a MeOH/H2O (5 : 95) solution containing 200 mM hexafluoroisopropyl alcohol and 8.1 mM triethylamine, and finally with 70% MeOH. The eluted RNAs were subjected to ESI-MS (ES-), producing raw spectra of multiply-charged anions and through resolved isotope deconvolution, the molecular weights of the resultant neutral oligonucleotides were confirmed. The final neutral mass of the RNAs were confirmed using this method.
Procedure for the Annealing of siRNAs for Biophysical Measurements

10 µM of each RNA were combined in a microcentrifuge tube and dried down in a Speedvac evaporator. On the following day, the samples were resuspended in 500 µl of a sodium phosphate buffer system (90mM NaCl, 10 mM Na$_2$HPO$_4$, 1 mM EDTA, pH 7). The solution was then heated to 95 °C for 2 minutes to denature the siRNA duplex. They were then slowly cooled to room temperature for 5 hours to ensure proper duplex formation. Once annealed, 300 µl of the siRNAs were transferred into a 1 mm path length quartz cuvette.

Procedure for Circular Dichroism of siRNA Duplexes

The prepared siRNA was placed in a spectropolarimeter (Jasco J-815) and circular dichroism (CD) measurements of each siRNA were recorded in quadruplicate. Absorbance was recorded from 200 to 300 nm at a temperature of 20 °C with a screening rate of 10 nm/min and a 0.2 nm data pitch. The average of the quadruplicates was quantified via Jasco’s Spectra Manager v.2 software and automatically attuned against the baseline sodium phosphate buffer sample.

Procedure for Melting Temperature of siRNA Duplexes

The prepared siRNA was placed in a spectropolarimeter (Jasco J-815) and the melting temperature ($T_m$) of each siRNA was measured via UV absorbance at 260 nm by increasing the temperature of the sample from 10 to 95 °C at a rate of 0.5 °C/min with an absorbance measurement taken at each 0.5 °C increment. The absorbance reading was adjusted to the baseline blank buffer sample. The $T_m$ values were calculated by using Meltwin v.3.5 software. Four independent experiments were run for each siRNA and the reported $T_m$ values represents
the average of the four experimental measurements using Meltwin version 3.5 software assuming the two-state model.²

**Procedure for Waking Frozen HeLa Cells**

Cryopreserved cells, stored in 1.5 ml of Eagle's Minimum Essential Medium (EMEM) (ATCC®) media supplemented with 5% DMSO, were removed from CryoPro® liquid nitrogen dewar (VWR) and slowly thawed to 25 °C. Cells were reconstituted in 10 ml of EMEM and transferred into a 50 ml polystyrene tissue culture treated incubation flask (Falcon) containing 20 ml of EMEM. Cells were then incubated overnight in a Forma Series II CO₂ Incubator (ThermoScientific) at 37 °C under 5% CO₂ atmosphere. Once cells obtained a confluency of 80-90% they were passaged normally and transferred into a 250 ml polystyrene tissue culture treated incubation flask (Falcon) containing 25 ml of EMEM supplemented with 10% fetal bovine serum (FBS) (Perbio) and 1% Penicillin-Streptomycin (Sigma).

**Procedure for Sub-Culturing of HeLa Cells (Passaging)**

1x10⁶ cells were seeded in a 250 ml polystyrene tissue culture treated incubation flask (Falcon) containing 25 ml of EMEM supplemented with 10% (v/v) fetal bovine serum (FBS) (Perbio) and 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma). Cells were incubated in a Forma Series II CO₂ Incubator at 37 °C under 5% CO₂ atmosphere until 80-85% confluency. Once 80-85% confluency was reached, cells were washed 3 times with 10 ml of phosphate buffered saline (NaCl 137 mM, KCl 2.7 mM, PO₄³⁻ 10 mM, pH 7.4) (PBS). Cells were then treated with 3 ml of 0.25% Trypsin (SAFC Bioscience) to disperse the cells. The cells were then pelleted and resuspended in 5 ml of EMEM for counting. The cells were then diluted in EMEM to a final
concentration of 1x10^6 cells/ml for further sub-culturing or biological assays. Cells were sub-cultured no more than 18 times for biological assays.

**Procedure for Cryopreservation of HeLa cells**

Cells were passaged normally until final resuspension of cells; cells were suspended in EMEM supplemented with 5% DMSO (Sigma) to a concentration of 1x10^7 cells/ml. A 1ml aliquot of cells were transferred into 2ml Fisherbrand cryogenic vials (Fisher) and cooled to 4 °C, then frozen at -20 °C and finally stored in CryoPro® liquid nitrogen dewar (VWR) until use.

**Procedure for siRNA Transfections**

50 µl of cells (total of 5x10^4 cells) were added to each well of a 24-well plate (Falcon®) with 350 µl of growth media and incubated at 37 °C with 5% CO₂. After 24 hours of incubation, cells were treated with 1, 10 and 20 nM concentrations of siRNAs using Lipofectamine 2000 (Invitrogen) in 1X Opti-Mem (ATCC®). The desired volume of siRNAs was mixed with 25 µl of Opti-Mem in a microcentrifuge tube on ice, and incubated for 5 minutes. 1 µl of Lipofectamine was mixed with 25 µl of Opti-Mem in a microcentrifuge tube and incubated for 5 minutes at room temperature. The contents of the tubes were combined and incubated for 20 minutes at room temperature and transferred into respective wells of the 24-well cell culture plate.

**Procedure for RNA isolation and cDNA synthesis**

Achieving a cellular concentration of no more than 2.5 x 10^5 cells/well after a total incubation time of 48 hours, cells were lysed using TRIzol® Reagent (Ambion®). RNA was purified following manufacturers procedure. RNA integrity was visualized using a 1% denaturing agarose
gel in 1X TBE buffer. RNA was quantified by measuring absorbance at 260 nm using GENESYS™ 10S UV-Vis Spectrophotometer (Thermo). 1 µg of RNA was used for cDNA synthesis using the iScript reverse transcription kit (Biorad). Reverse transcription was performed at 25 °C for 5 minutes, 42 °C for 30 minutes, and 85 °C for 5 minutes. cDNA samples were then stored at -20 °C for real time polymerase chain reaction (qPCR) using a PX2 thermocycler (Thermo).

**Procedure for qPCR**

Relative transcript levels were quantified by real time quantitative PCR (qPCR) using CFX Connect™ Real-Time PCR Detection System (Biorad). Standard curves were generated for each gene (18s and GAPDH) to evaluate primer efficiency. The qPCR was performed in triplicate using the following conditions: 2 µl of cDNA, and 10 µl of SsoFast EvaGreen Supermix (Biorad) with respective primer concentrations and topped up to a final volume of 20 µl using DEPC treated water. 1.6 µl of GAPDH forward primer: 5’-ACTTTGTGAAGCTCATTTCCTGGTA -3’ and reverse primer: 5’-GTGGTTTGAGGGCTCTTACTCCTT -3’ were used for a final concentration of 800 nM. 2 µl of 18s forward primer: 5’- CGGCTACCACATCCAAGGAAG- 3’ and reverse primer: 5’- CGCTCCCAAGATCCAACTACTAC- 3’ for a final concentration of 100 nM. Thermocycle conditions included an initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 2 seconds, 52 °C for 15 seconds, and 72 °C for 5 seconds. Fluorescence was measured during the end of each extension phase. After 40 cycles, a melting curve was generated by slowly increasing the temperature from 65 °C to 95 °C at a rate of 0.1 °C/s, while fluorescence was measured at each increment. To ensure no genomic contamination, a control lacking reverse transcriptase was used for each cDNA sample. No template controls were run for every set of
primers. Normalized relative gene expression (ΔΔCq) of GAPDH was calculated against reference gene 18s and corrected for primer efficiency using CFX Manager Software (BIORAD).

**Procedure for in vitro Dual-Reporter Luciferase Assay**

Prior to transfection, HeLa cells were seeded on 12-well plates (Greiner Bio-One) at a density of 100,000 cells per well and incubated at 37 °C with 5% CO2 in EMEM containing 10% FBS. After 24 hours, varying concentrations of anti-luciferase siRNAs were co-transfected with both pGL3 (Promega) and pRLSV40 luciferase-expressing plasmids using Lipofectamine 2000 (Invitrogen) in Gibco’s Opti-Mem Reduced Serum Medium 1X (Invitrogen) according to the manufacturer’s protocol. After an additional 24 hours, cells were incubated in 1X passive lysis buffer (Promega) for 20 minutes at room temperature, and the cell lysates were loaded onto white and opaque, 96-well plates (Costar). Using the Dual-Luciferase Reporter Assay kit (Promega), Lar II and Stop & Glo® luciferase substrates were added to the lysates and enzymatic activity of firefly and Renilla luciferase vectors were measured respectively using a Synergy HT (Bio-Tek) plate luminometer. The ratio of firefly/Renilla luminescence expressed as a percentage relates the reduction in firefly expression to siRNA efficacy when compared to untreated controls. Each value is the average of at least three independent experiments with the indicated error (SDOM).
**Figures and Tables:**

**Table S1:** Predicted and recorded masses for chemically-modified RNAs

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Sense RNAs</th>
<th>Predicted Neutral Mass</th>
<th>Observed Neutral Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>ss24</td>
<td>5'- CUUACGCUP₃AGUACUUCGAtt -3'</td>
<td>6546.9</td>
<td>6545.1</td>
</tr>
<tr>
<td>ss25</td>
<td>5'- CUUACGCUGP₃GUACUUCGAtt -3'</td>
<td>6562.9</td>
<td>6562.2</td>
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<tr>
<td>ss26</td>
<td>5'- CUUACGCUX₅AGUACUUCGAtt -3'</td>
<td>6958.6</td>
<td>6959.2</td>
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<tr>
<td>ss27</td>
<td>5'- CUUACGCUGX₈GUACUUCGAtt -3'</td>
<td>6955.2</td>
<td>6953.2</td>
</tr>
<tr>
<td>ss28</td>
<td>5'- CUUACGCUAA₈GUACUUCGAtt -3'</td>
<td>6306.8</td>
<td>6305.1</td>
</tr>
<tr>
<td>ss29</td>
<td>5'- CUUACGCUL₈GUACUUCGAtt -3'</td>
<td>6302.9</td>
<td>6304.1</td>
</tr>
<tr>
<td>ss30</td>
<td>5'- CUUACGCUPP₈GUACUUCGAtt -3'</td>
<td>6298.8</td>
<td>6298.1</td>
</tr>
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<td>ss31</td>
<td>5'- GGUCAUCCDD₂GACAACUUUt -3'</td>
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</tr>
<tr>
<td>ss32</td>
<td>5'- GGUCAUCCBB₁₆GACAACUUUt -3'</td>
<td>6311.8</td>
<td>6310.1</td>
</tr>
</tbody>
</table>
ESI Q-TOF were recorded in a negative electrospray mode after HPLC elution using two mobile phases; MeOH/H₂O 5:95 (v/v) with 200 mM hexafluoroisopropyl alcohol and 8.1 mM triethylamine, and 70% MeOH. \( P_5 \) corresponds to the single propargyl modification; \( X_5 \) corresponds to the single cholesterol modification; \( AA_8 \) corresponds to the double acetyl modification; \( LL_8 \) corresponds to the double allyl modification; \( PP_8 \) corresponds to the double propargyl modification. \( DD_5 \) corresponds to the benzene modification; \( BB_{10} \) corresponds to the biphenyl modification.
**Figure S1.** RNA duplex conformation of wtLuc and RNAs 24-27 displayed through circular dichroism spectroscopy. RNAs were suspended (~2.4 nmol/duplex) in 500 µL of a sodium phosphate buffer (90 mM NaCl, 10 mM Na₂HPO₄, 1 mM EDTA, pH 7) and the solution was scanned from 200-310 nm at 20 C. All scans were performed in quadruplicate and averaged using version 2 of Jasco’s Spectra Manager software.

![Circular Dichroism Spectrum of wtLuc and RNAs 24-27](image)

**Figure S2.** RNA duplex conformation of wtLuc and RNAs 28-30 displayed through circular dichroism spectroscopy. RNAs were suspended (~2.4 nmol/duplex) in 500 µL of a sodium phosphate buffer (90 mM NaCl, 10 mM Na₂HPO₄, 1 mM EDTA, pH 7) and the solution was scanned from 200-310 nm at 20 C. All scans were performed in quadruplicate and averaged using version 2 of Jasco’s Spectra Manager software.

![Circular Dichroism Spectrum of wtLuc and RNAs 28-30](image)
Figure S3. RNA duplex conformation of \textbf{wtBcl2} and RNAs 31 and 32 displayed through circular dichroism spectroscopy. RNAs were suspended (~2.4 nmol/duplex) in 500 µL of a sodium phosphate buffer (90 mM NaCl, 10 mM Na$_2$HPO$_4$, 1 mM EDTA, pH 7) and the solution was scanned from 200-310 nm at 20°C. All scans were performed in quadruplicate and averaged using version 2 of Jasco’s Spectra Manager software.

$^{1}$H/$^{13}$C NMR Spectra of Compounds
$^1$H NMR Spectrum of 1

$^{13}$C NMR Spectrum of 1

$^1$H NMR Spectrum of 2
$^{13}$C NMR Spectrum of 2

$^1$H NMR Spectrum of 3
$^{13}$C NMR Spectrum of 3
$^1$H NMR Spectrum of 6

$^{13}$C NMR Spectrum of 6

$^1$H NMR Spectrum of 7
$^{13}$C NMR Spectrum of 7

$^1$H NMR Spectrum of 8
$^{13}$C NMR Spectrum of 8

$^1$H NMR Spectrum of 9
$^{13}$C NMR Spectrum of 9

$^1$H NMR Spectrum of 10
$^{13}$C NMR Spectrum of 10

$^1$H NMR Spectrum of 11
$^{13}$C NMR Spectrum of 11

$^1$H NMR Spectrum of 12
$^{13}$C NMR Spectrum of 12

$^1$H NMR Spectrum of 13
$^{13}$C NMR Spectrum of 13

$^1$H NMR Spectrum of 14
$^{13}$C NMR Spectrum of 14

$^1$H NMR Spectrum of 15
$^{13}$C NMR Spectrum of 15

$^1$H NMR Spectrum of 16
$^{13}$C NMR Spectrum of 16

$^1$H NMR Spectrum of 17
$^1$H NMR Spectrum of 18

$^1$C NMR Spectrum of 17
$^{13}$C NMR Spectrum of 18

$^1$H NMR Spectrum of 19
$^{13}$C NMR Spectrum of 19

$^1$H NMR Spectrum of 20
$^{13}$C NMR Spectrum of 20

$^1$H NMR Spectrum of 21
\(^{13}\text{C}\) NMR Spectrum of 21

\(^{1}\text{H}\) NMR Spectrum of 22
$^{13}$C NMR Spectrum of 22
$^1$H NMR Spectrum of 23

$^{13}$C NMR Spectrum of 23
References: