2'-O-[2-(Amino)-2-oxoethyl] Oligonucleotides

Thazha P. Prakash, Andrew M. Kawasaki, Elena A. Lesnik, Stephen R. Owens and
Muthiah Manoharan*

Department of Medicinal Chemistry, Isis Pharmaceuticals Inc., 2292 Faraday Ave.,
Carlsbad, CA 92008

Supporting information

***N*³-Benzyloxymethyl-2'-O-(2-methoxy-2-oxoethyl)-5-methyluridine (2a).** *N*³-Benzyloxymethyl-5-methyluridine (9.70 g, 25.6 mmol) was dissolved in anhydrous DMF (250 mL), the reaction mixture was cooled to 5 °C and NaH (60% oil dispersion) (1.025 g, 25.6 mmol) was added. The reaction mixture was allowed to warm to ambient temperature and it was stirred for 1 h. The suspension was cooled to -40 °C in a CH₃CN-CO₂ bath and methyl 2-bromoacetate (2.36 mL, 25.6 mmol) was added slowly. The reaction mixture was allowed to slowly warm to ambient temperature over 1 h, and then stirred for 16 h at ambient temperature. MeOH (10 mL) was added followed by addition of glacial AcOH (5 mL), and the reaction mixture was stirred for 5 min. The solvent was evaporated *in vacuo* to give an oil, which was dissolved in EtOAc (500 mL), and the organic phase was washed with water (3 x 50 mL) and brine (50 mL). After drying the organic layer with Na₂SO₄, the solvent was evaporated *in vacuo* to give an oil, which was dissolved in 2M NEt₃ in anhydrous MeOH (64 mL). After stirring at ambient temperature for 1 h the solvent was evaporated *in vacuo* to give an oil that was purified by column chromatography using CH₂Cl₂-MeOH, 95:5, to give the title compound as a hygroscopic white foam (8.56 g, 74%). ¹H NMR (CDCl₃): 7.63 (s, 1H), 7.30 (m, 5H), 5.72 (d, 1H, J_{1',2'} = 3.0 Hz), 5.47 (s, 2H), 4.69 (s, 2H), 4.39 (q_{a,b} 2H, J_{a,b} = 17.6 Hz), 4.2-4.0 (m, 3H), 3.8-3.6 (m, 2H), 3.74 (s, 3H), 1.89 (s, 3H). ¹³C NMR (CDCl₃) 171.7, 163.3, 150.8, 137.6, 135.9, 128.1, 127.5, 127.4, 109.7, 90.3, 84.5, 83.5, 72.1, 70.3, 68.2, 67.9, 60.9, 52.2, 12.9. HRMS (FAB) calcd for C₂₁H₂₆N₂O₉Na⁺: 473.1536; found: 473.1549. 2D-¹H NMR (TOCSY) confirmed 2'-O-alkylation.

2'-O-(2-Methoxy-2-oxoethyl)-5-methyluridine (3a). Compound **2a** (2.99 g, 6.64 mmol) was dissolved in anhydrous MeOH (80 mL) and added, under an argon atmosphere, to a pressure bottle containing 10% Pd on carbon (750 mg). The vessel was pressurized to 30 psi of hydrogen and shaken for 16 h. The solids were filtered through a Celite pad, the solvent was evaporated *in vacuo* to give an oil that was dissolved in 1 M NEt₃ in anhydrous MeOH, and the solution was heated at reflux temperature for 2 h. The solvent was evaporated *in vacuo* to give the title compound as a white solid (2.735 g, 95%). ¹H NMR (DMSO-d₆): 11.4 (br s, 1H), 7.75 (s, 1H), 5.92 (d, 1H, J_{1',2'} = 5.2 Hz), 5.19 (m, 2H), 4.24 (m, 2H), 3.87 (m, 1H), 3.63 (s, 3H), 1.78 (s, 3H). ¹³C NMR (pyridine-d₅): 171.3, 165.0, 152.1, 136.7, 110.2, 88.1, 85.9, 84.1, 69.6, 68.0, 61.0, 51.7, 12.7. LRMS (ES): m/z 352.8 (M + Na)⁺.

5'-O-(4,4'-dimethoxytrityl)-2'-O-(2-methoxy-2-oxoethyl)-5-methyluridine (4).

Compound **3a** (2.74 g, 6.34 mmol) was dried by evaporation with anhydrous pyridine twice, and then dried *in vacuo* (0.1 torr) for 12 h at ambient temperature. Compound **3** and dimethylaminopyridine (39 mg, 0.32 mmol) were dissolved in anhydrous pyridine (60 mL), 4,4'-dimethoxytritylchloride (2.79 g, 8.24 mmol) was added, and the reaction mixture was stirred for 12 h. MeOH (10 mL) was added and, after stirring for 30 min, the solvent was evaporated *in vacuo* to give an oil, which was dissolved in EtOAc (500 mL). The organic was washed with water (3 x 50 mL), then with brine (50 mL). NEt₃ (5 mL) was added to the organic, it was dried with Na₂SO₄, and the solvent was evaporated *in vacuo* to give a foam that was purified by column chromatography using CH₂Cl₂-EtOAc (60:40), to afford the title compound as a foam (2.445 g, 59%). ¹H NMR (CDCl₃): 7.69 (s, 1H), 7.43-6.81 (m, 13H), 5.93 (d, 1H, J_{1',2'} = 2.6 Hz), 4.46 (q_{a,b} 2H, J = 17.2 Hz), 4.14 (m, 2H), 3.79 (s, 9H), 3.51 (m, 2H), 1.35 (s, 3H). ¹³C NMR (CDCl₃): 171.6, 164.2, 158.6, 150.5, 144.3, 135.3, 135.2, 130.0, 128.0, 127.9, 127.0, 113.2, 110.8, 87.9, 86.7, 84.5, 83.3, 68.4, 67.8, 61.7, 55.1, 52.2, 11.7. HRMS (FAB) calcd for C₃₄H₃₆N₂O₁₀Cs⁺: 765.1424. Found: 765.1452. Anal. Calcd for: C₃₄H₃₆N₂O₁₀: % C, 64.55; % H; 5.74; % N, 4.43. Found: % C, 65.01; % H; 5.79; % N, 4.54.

5'-O-(4,4'-dimethoxytrityl)-2'-O-(2-methoxy-2-oxoethyl)-5-methyluridine-3'-(2-cyanoethyl-N, N-diisopropylphosphoramidite) (5). Compound **4** (1.29 g, 2.04 mmol)

was dissolved in CH₂Cl₂ (20 mL), and diisopropylethylamine (0.78 mL, 4.48 mmol) and 2-cyanoethyl diisopropylchlorophosphoramidite (0.5 mL, 2.24 mmol) were added dropwise at ambient temperature. After stirring 1.5 h at ambient temperature, additional diisopropylethylamine (0.78 mL, 4.48 mmol) and 2-cyanoethyl diisopropylchlorophosphoramidite (0.501 mL, 2.24 mmol) were added dropwise at ambient temperature. After an additional 1 h EtOAc (200 mL) was added to the reaction mixture, the organic was washed with 5% aqueous NaHCO₃ twice, then washed with brine, and the organic was dried with MgSO₄. The solvent was evaporated *in vacuo* to give an oil that was dissolved in CH₂Cl₂, and the product was purified by column chromatography using CH₂Cl₂-EtOAc-NEt₃ (70:30:0.5), to yield the title compound as an oil (1.43 g). Further elution with EtOAc-NEt₃ (100:0.5), provided a second fraction of product. The product was dissolved in CH₂Cl₂ (14 mL) and added slowly to vigorously stirring hexanes (200 mL) to yield a white gum and a slightly turbid supernatant. The mixture was allowed to settle for several hours, the supernatant was decanted off, and the gum was washed with hexanes three times. The hexane washes were decanted off and the product was dried *in vacuo* (0.1 torr) for 12 h to give the title compound as white foam (0.92 g, 54%). The second fraction of product was isolated as a white solid (0.12 g, 7%). ³¹P NMR (CDCl₃): 151.1, 151.0. HRMS (FAB) calcd for C₄₃H₅₃N₄O₁₁PCs⁺: 965.2503. Found: 965.2489.

5'-O-(4,4'-dimethoxytrityl)-2'-O-(2-methoxy-2-oxoethyl)-5-methyluridine-3'-O-succinate (6). The nucleoside **4** (0.25 g, 0.38 mmol) was mixed with succinic anhydride (0.06 g, 0.57 mmol) and DMAP (0.03 g, 0.2 mmol) and dried in vacuum at 40 °C overnight. The mixture was dissolved in anhydrous CH₂Cl₂ (1 mL), (C₂H₅)₃N (0.08 mL, 0.8 mmol) was added and the solution stirred at room temperature under argon atmosphere for 4 h. It was then diluted with CH₂Cl₂ (30 mL) and washed with ice-cold aqueous citric acid (10 wt %, 30 mL) and water (30 mL). The organic phase was dried over anhydrous Na₂SO₄ and concentrated to dryness. The residue obtained was purified by flash silica gel column chromatography and eluted with 10% MeOH in CH₂Cl₂ to afford compound **6** in 82% isolated yield (0.95 g): *R*_f 0.51 (10% MeOH in CH₂Cl₂); ¹H NMR (200 MHz, DMSO-d₆) 1.40 (s, 3H), 3.19-3.36 (m, 4H), 3.56 (s, 3H), 3.73 (s, 6H), 4.18 (m, 1H), 4.23 (s, 2H), 4.50 (m, 1H), 5.33 (m, 1H), 5.93 (d, *J* = 6.62 Hz, 1H), 6.91 (d, *J* = 8.78 Hz, 4H), 7.23-7.40 (m, 9H), 7.48 (s, 1H), 11.46 (s, 1H); ¹³C NMR (50 MHz,

CDCl₃) 11.51, 29.57, 30.15, 51.62, 54.99, 62.78, 67.04, 70.52, 79.91, 81.26, 86.06, 87.03, 111.12, 113.12, 123.98, 127.22, 127.85, 129.89, 134.90, 136.39, 143.93, 148.85, 150.96, 158.54, 163.99, 170.18, 172.25, 175.57; HRMS (FAB) Calcd for C₃₈H₄₀N₂O₁₃Na⁺ 755.2428 found 755.2448.

5'-O-(4,4'-dimethoxytrityl)-2'-O-(2-methoxy-2-oxoethyl)-5-methyluridine-3'-O-succinyl-controlled pore glass (7). Compound **6** was loaded on to the aminoalkyl controlled pore glass (CPG) according to the standard synthetic procedure (TBTU mediated synthesis of functionalized CPG synthesis: Bayer, E.; Bleicher, K.; Maier, M.A.; *Z. Naturforsch.* 1995, 50b, 1096-1100) to yield the functionalized solid support **7** (40.23 mol/g).

5'-O-(4,4'-dimethoxytrityl)-2'-O-[2-(dimethylamino)-2-oxoethyl]-5-methyluridine (24). Compound **4** (1.00 g, 1.58 mmol) was dissolved in 2.0 M dimethylamine in THF, the reaction flask was sealed with a septum, and the reaction mixture was stirred at ambient temperature for 18 h. The solvent was evaporated *in vacuo* at ambient temperature to give the title compound as a foam (0.97 g, 95%). ¹H NMR (CDCl₃): 7.73 (s, 1H), 7.45-6.80 (m, 13H), 5.87 (d, 1H, J_{1',2'} = 1.0 Hz), 4.57 (q_{ab}, 2H), 4.46 (m, 1H), 4.23 (m, 1H), 4.02 (m, 1H), 3.77 (s, 6H), 3.52 (m, 2H), 2.96 (s, 3H), 2.91 (s, 3H), 1.35 (s, 3H). ¹³C NMR (CDCl₃): 170.0, 164.2, 158.5, 150.7, 144.4, 135.4, 135.3, 135.0, 130.0, 128.0, 127.8, 127.5, 126.8, 113.1, 110.5, 89.0, 86.5, 85.7, 83.4, 68.5, 61.5, 55.0, 35.5, 35.3, 11.7. HRMS (FAB) calcd for C₃₅H₃₉N₃O₉Na⁺: 668.2584. Found: 668.2561.

5'-O-(4,4'-dimethoxytrityl)-2'-O-[2-(dimethylamino)-2-oxoethyl]-5-methyluridine-3'-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (25). Compound **24** (914 mg, 1.42 mmol) was dissolved in CH₂Cl₂ (14 mL), and then diisopropylamine tetrazolide (146 mg, 0.86 mmol) and 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite (0.54 mL, 1.70 mmol) were added. Additional 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite (3 x (0.54 mL, 1.70 mmol)) was added three more times over the next 10 h. EtOAc (120 mL) was added and about 15 mL of solvent was evaporated *in vacuo*. The organic was washed with saturated NaHCO₃ (3 x 12 mL), then with brine (2 x 12 mL), and dried with MgSO₄. The solvent was evaporated *in vacuo* at 27 °C to give an oil, which was dissolved in CH₂Cl₂ (4 mL), and

then slowly added to rapidly stirred hexanes (200 mL) to yield a wax. The supernatant was decanted, the wax was washed with hexanes three times and the washes were decanted. The precipitation was repeated two additional times to give a white wax which was dried *in vacuo* at ambient temperature to give the title compound as a foam (1.09 g, 91%). ^{31}P NMR (CD_2Cl_2): 152.9, 152.8. HRMS (FAB) calcd for $\text{C}_{44}\text{H}_{56}\text{N}_5\text{O}_{10}\text{P Na}^+$: 868.3663. Found: 868.3691.

5'-O-(4,4'-dimethoxytrityl)-2'-O-[2-(dimethylamino)-2-oxoethyl]-5-methyluridine-3'-O-succinyl-controlled pore glass (26). Compound **26** was synthesized according to the procedure used for the synthesis of **6** and **7** from compound **24** (0.2 g, 0.30 mmol), DMAP (0.04 g, 0.38 mmol), succinic anhydride (0.06 g, 0.61 mmol) and triethyl amine (0.11 mL, 0.81 mmol) and dichloroethane (1 mL) 3'-O-succinate derivative of **24** was obtained in 96 % (0.22 g) yield. R_f 0.42 (10% MeOH in CH_2Cl_2); ^1H NMR (200 MHz, CDCl_3) 1.38 (s, 3H), 2.67 (m, 4H), 2.95 (s, 3H), 2.97 (s, 3H), 3.34 (dd, $J = 2.06$ Hz, 9.06 Hz, 1H), 3.59 (d, $J = 9.28$ Hz, 1H), 3.80 (s, 6H), 4.28-4.54 (m, 4H), 5.42 (t, $J = 5.38$ Hz, 1H), 6.01 (d, $J = 3.48$ Hz, 1H), 6.84 (d, $J = 8.30$ Hz, 4H), 7.23-7.40 (m, 9H), 7.71 (s, 1H), 9.18 (br s, 1H); ^{13}C NMR (50 MHz, CDCl_3) 11.57, 29.11, 29.26, 35.41, 36.05, 55.13, 62.17, 68.37, 70.39, 79.83, 81.02, 87.01, 111.04, 113.23, 127.06, 128.04, 130.01, 135.07, 135.87, 143.97, 150.81, 158.62, 164.51, 168.81, 171.49, 175.30; HRMS (FAB) Calcd for $\text{C}_{39}\text{H}_{43}\text{N}_3\text{O}_{12}\text{Na}^+$ 768.7738 found 768.7658. 3'-O-succinyl derivative of **24** was loaded on to CPG by following a reported procedure (TBTU mediated synthesis of functionalized CPG synthesis: Bayer, E.; Bleicher, K.; Maier, M.A.; *Z. Naturforsch.* 1995, 50b, 1096-1100.) to yield the functionalized solid supports **26** (57.89 mol/g).

Oligonucleotide synthesis 8-11. 0.1 M solution of amidite **5** in anhydrous acetonitrile was used for the synthesis of the modified oligonucleotides. Functionalized CPG **7** was used when necessary. For incorporation of **5**, phosphoramidite solutions were delivered in two portions, each followed by a 5 min coupling wait time. Oxidation of the internucleotide phosphite to phosphate was carried out using 0.5 M solution of CSO [1-S-(+)-(10-camphorsulfonyl)oxaziridine] in acetonitrile for 4 min for 2- mol synthesis. All other steps in the protocol supplied by Millipore were used without modification. The coupling efficiencies were more than 97%. After completion of the synthesis, solid

support bound oligonucleotides **8-11** were used for the synthesis of oligonucleotides **12-23**.

Oligonucleotide synthesis 12-15. Solid support bound oligonucleotides **8-11** (2 mol) were treated with 50% *N,N*-dimethylethylenediamine in ethanol (2 mL) and kept at room temperature for 24 h. The solid support was filtered through a syringe filter (0.45 μ m, Pall Gelman Laboratories, Ann Arbor, MI, USA), the solid support was washed with water (2 x 1 mL). Combined filtrate and washings were dried. Crude oligonucleotides were purified by High Performance Liquid Chromatography (HPLC, Waters, C-4, 7.8 x 300 mm, A = 100 mM ammonium acetate, pH = 6.5, B = acetonitrile, 5 to 60 % B in 55 Min, Flow 2.5 mL min⁻¹, λ = 260 nm). Detritylation was carried out by adjusting the pH of the solution to 4.2 with acetic acid and keeping the resulting solution at room temperature for 16-18 h. Oligonucleotides were desalted by HPLC. Oligonucleotides were characterized by ES MS and purity was assessed by HPLC and Capillary Gel Electrophoresis. The isolated yields for modified oligonucleotides were 30-35%.

Oligonucleotide synthesis 16-19. Solid support bound oligonucleotides **8-11** (2 mol) were treated with 40% aqueous methylamine (2 mL) and kept at room temperature for 24 h. The solid support was filtered through a syringe filter washed the solid support with water (2 x 1 mL). Combined filtrate and washing were concentrated to dryness. The crude oligonucleotides were purified by HPLC, detritylated and analyzed according to the procedure described for **12-15**. Oligonucleotides were isolated in 29-34 % yield.

Oligonucleotide synthesis 20-23. Solid support bound oligonucleotides **8-11** (2 mol) were treated with saturated methanolic ammonia (2 mL, 37 wt%) and kept at room temperature for 24 h. The solid support was filtered through a syringe filter. The solid support was washed with water (2 x 1 mL). Combined filtrate and washing were dried. The crude oligonucleotides were purified by HPLC, detritylated and analyzed according to the procedure described for **12-15**. Oligonucleotides were isolated in 35-37 % yield.

Oligonucleotide synthesis 27-30. Oligonucleotides **27-30** were synthesized using the amidite **25** according to the procedure used for the synthesis of oligonucleotides **8-11**. After the completion of the synthesis, the solid support was suspended in aqueous ammonia (38 wt%, 1.8 ml for 2 mol synthesis) and kept at room temperature for 2 h to

remove the N^4 -benzoyl group from C residue. After 2 h, aqueous methyl amine (40 wt% solution, 0.2 mL) was added and kept at room temperature for additional 22 h. The solid support was filtered and washed with water. The washes were combined with the filtrate and evaporated to dryness. The crude oligonucleotides were purified by HPLC (Waters, C-4, 7.8 $\times$ 300 mm, A = 100 mM ammonium acetate, pH = 6.5, B = acetonitrile, 5 to 60 % B in 55 Min, Flow 2.5 mL min⁻¹, λ = 260 nm). Detritylation was carried out by adjusting the pH of the solution to 4.2 with acetic acid and keeping the resulting solution at room temperature for 16-18 h. Oligonucleotides were then purified by HPLC (conditions as above). Oligonucleotides were characterized by ES Ms analysis and HPLC and Capillary Gel Electrophoresis assessed purity. The isolated yields for modified oligonucleotides were 20-24 %.

Table 1. ES MS and HPLC data for oligonucleotides 12-23 and 27-30

No	Sequence 5'-3'	ES MS		HPLC Retention Time, min ^a .
		Calcd	found	
12	d (t ₁ CC AGG t ₁ Gt ₁ CCG CA _{t₁} C)	5411.50	5410.00	18.72
13	d (GCG t ₁ t ₁ t ₁ t ₁ t ₁ t ₁ t ₁ t ₁ t ₁ t ₁ GC G)	6317.64	6318.12	19.39
14	d (CTC GTA Ct ₁ t ₁ t ₁ t ₁ C CGG TCC)	5970.97	5969.30	19.51
15	d (TTT TTT TTT TTT TTT t ₁ t ₁ t ₁ t ₁)	6295.21	6294.82	20.51
16	d (t ₂ CC AGG t ₂ Gt ₂ CCG CA _{t₂} C)	5182.10	5180.01	18.02
17	d (GCG t ₂ t ₂ t ₂ t ₂ t ₂ t ₂ t ₂ t ₂ t ₂ t ₂ GC G)	5746.64	5745.68	18.23
18	d (CTC GTA Ct ₂ t ₂ t ₂ t ₂ C CGG TCC)	5741.57	5739.58	18.12
19	d (TTT TTT TTT TTT TTT t ₂ t ₂ t ₂ t ₂)	6066.77	6065.76	1.98
20	d (t ₃ CC AGG t ₃ Gt ₃ CCG CA _{t₃} C)	5126.02	5124.97	17.78
21	d (GCG t ₃ t ₃ t ₃ t ₃ t ₃ t ₃ t ₃ t ₃ t ₃ t ₃ GC G)	5606.44	5607.10	17.03
22	d (CTC GTA Ct ₃ t ₃ t ₃ t ₃ C CGG TCC)	5685.49	5684.66	17.70
23	d (TTT TTT TTT TTT TTT t ₃ t ₃ t ₃ t ₃)	6010.73	6010.45	19.25
27	d (t ₄ CC AGG t ₄ Gt ₄ CCG CA _{t₄} C)	5238.22	5237.60	27.54
28	d (GCG t ₄ t ₄ t ₄ t ₄ t ₄ t ₄ t ₄ t ₄ t ₄ t ₄ GC G)	5886.34	5885.60	28.57
29	d (CTC GTA Ct ₄ t ₄ t ₄ t ₄ C CGG TCC)	5797.45	5796.80	27.87

30	d (TTT TTT TTT TTT TTT t ₄ t ₄ t ₄ t ₄)	6122.69	6122.40	24.51
-----------	--	---------	---------	-------

12-15 t₁ = 2'-O-DMAEAc-5-methyluridine, **16-19** t₂ = 2'-O-NMAc-5-methyluridine, **20-23** t₃ = 2'-O-NAc-5-methyluridine, **27-30** t₄ = 2'-O-DMAc-5-methyluridine

^a Water C-4, 3.9 x 300 mm, A = 50 mM triethylammonium acetate, pH 7, B = acetonitrile, 5 to 60 % B in 55 min, flow 1.5 mL min⁻¹, λ = 260 nm.

T_m analysis. Absorbance versus temperature curves were measured at 260 nm using a Gilford Response II spectrophotometer interfaced to an IBM PC computer. The buffer contained 100 mM Na⁺, 10 mM phosphate, 0.1 mM EDTA, pH 7. Oligonucleotide concentration was 4 M; and concentration of each strand was determined from the absorbance at 85 °C and extinction coefficients calculated according to Puglisi and Tinoco (*Methods Enzymol*, **1989**, 180, 304-325). Oligonucleotide solutions were heated at a rate of 0.7 °C/min in 1 cm path length cells and then cooled to confirm reversibility and lack of evaporation. T_m values were obtained from the absorbance versus temperature curves. Standard deviations did not exceed 0.5 °C. Each T_m reported was an average of two experiments. T_m per modification was calculated by subtracting the T_m of the unmodified DNA-RNA parent duplex and dividing by the number of modified residues in the sequence.

Nuclease stability studies. Oligonucleotides were 5' end-labeled using [⁻³²P]ATP and T4 polynucleotide kinase. After the labeling reaction, the T4 polynucleotide kinase was heat inactivated at 95 °C for 3 min and oligonucleotides were used without any further purification. The snake venom phosphodiesterase (USB, Cleveland, OH) assay was performed using 1 M oligonucleotide at 37 °C in a buffer of 50 mM Tris-HCl, pH 8.5, 72 mM NaCl and 14 mM MgCl₂ at an enzyme concentration of 5 x 10⁻³ U mL⁻¹. The enzyme was shown to maintain its activity under these condition for 24 h. Aliquots of the reaction mixture were removed at the indicated times, quenched by addition to an equal volume of 80% formamide gel loading buffer containing tracking dyes, heated for 2 min at 95 °C, and then stored until analysis by denaturing polyacrylamide electrophoresis. Quantitation was performed on a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA).