

EXPERIMENTAL PROCEDURES

Reagents for machine assisted oligonucleotide synthesis were purchased from Applied Biosystems (Foster City, CA). 2-Cyanoethyl-*N,N,N',N'*-tetraisopropylphosphodiamidite, *N*6-trifluoroacetamidohexanol and 2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)uridine were synthesized according to published procedures. Adsorption column chromatography was performed on columns packed with silica gel 60 (Merck). DELFIA™ enhancement solution and europium standards were products of PerkinElmer Life Sciences (Wallac). NMR spectra were recorded on a Jeol LA-400 spectrometer operating at 399.8, 350.0, 161.9 and 100.5 MHz for ¹H, ¹⁹F, ³¹P and ¹³C, respectively, or on a Jeol GX 500 instrument operating at 500.00 and 125.65 MHz for ¹H and ¹³C, respectively. Me₄Si was used as an internal (¹H and ¹³C) and H₃PO₄ (³¹P) and trifluoroacetic acid (¹⁹F) as external references. Coupling constants are given in Hz. When reported, signal characterization is based on ¹H, ¹H, ¹H, ¹³C and ¹³C, ¹³C COSY experiments. IR spectra were recorded on a Perkin Elmer 2000 FT-IR spectrophotometer. Oligonucleotides were assembled on an Applied Biosystems 932 DNA Synthesizer using phosphoramidite chemistry and recommended protocols.

The synthesis of 2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-*N*3-(*N*6-trifluoroacetamidohex-1-yl)uridine (2a).

2'-Deoxy-5'-*O*-(4,4'-dimethoxytrityl)uridine (8.0 g, 15.1 mmol), Ph₃P (4.7 g, 17.9 mmol) and *N*6-trifluoroacetamidohexan-1-ol (4.1 g, 18.1 mmol) were dissolved in dry THF (80 mL). DEAD (2.85 mL) was added in five portions during 15 min, after which the mixture was stirred 2h at ambient temperature and concentrated. Purification on silica gel (eluent diethyl ether) yielded 95% of **2**. ¹H NMR (DMSO-*d*₆; 500 MHz): δ 9.36 (1H, br, NH); 7.68 (1H, d, *J* 8.1, H-6); 7.35 (2H, DMTr); 7.25 (7H, DMTr); 6.87 (4H, d, DMTr); 6.17 (1H, t, *J* 6.2, H-1'); 5.47 (1H, d, *J* 8.1, H-5), 5.38 (1H, d, *J* 4.7, 3'-OH), 4.31 (1H, m, H-3'), 3.90 (1H, m, H-4); 3.8 (2H, t, NCH₂), 3.71

(6H, s, 2 • OMe) 3.25 (1H, H-5'), 3.20 (1H, dd, H-5''); 3.15 (2H, q, CH₂NH), 2.23 (2H, H-2'; H-2''), 1.47 (4H, m); 1.25 (4H, m). ¹³C NMR (DMSO-d₆): δ 161.7 (C4), 158.0 (C=O), 156.5 (q, CF₃); 150.3 (C2); 144.8 (DMT); 138.8 (C6); 129.7, 127.8, 127.7, 126.7, 113.1 (DMT); 100.7 (C-5), 85.7 (DMT); 85.5 (C4'); 85.2 (C1'); 69.8 (C3'); 63.3 (C5'); 55.5 (2 • OMe); 40.1 (NCH₂); 39.7 (C2'); 39.0 (CH₂NHCO); 28.0, 25.9, 25.8 (CH₂) ¹⁵N NMR (DMSO-d₆): δ -294.5 (NHCOCF₃); -264.0 (N1); -244.6 (N3). ¹⁹F NMR (DMSO-d₆): δ -75.17.

The synthesis of tetramethyl 2,2',2'', 2''''-[(6-hydroxy-1-hexyn-2-yl)pyridine-2,6-diyl]bis(methylenenitrilo)]tetrakis(acetate) (5).

A mixture of tetramethyl 2,2',2'',2''''-[4-bromopyridine-2,6-diyl]bis(methylenenitrilo)tetrakis(acetate) (**4**; 2.0 g, 4.0 mmol), bis(triphenyl-phosphinepalladium(II) chloride (56 mg, 0.08 mmol) and CuI (30 mg, 0.16 mmol) in dry THF (25 mL) and triethylamine (8 mL) was deaerated with argon. 5-hexynol was added and the mixture was stirred for 7 h at 55 °C. The cooled solution was filtered, the filtrate was evaporated and redissolved in dichloromethane. The solution was washed with water, dried and concentrated. Purification on silica gel yielded the title compound as an oil (1.70 g, 81%). Compound **6**: ¹H NMR (CDCl₃; 400 MHz): 7.46 (2H, s); 3.99 (4H, s); 3.71 (12H, s, 4 CH₃); 3.62 (8H, s, 4 CH₂); 2.53 (4H, m, CH₂); 1.70 (4H, m, 2 CH₂) IR (neat): 2242 cm⁻¹(C≡C).

The synthesis of 2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-N3 {tetramethyl 2,2',2'', 2''''-[(4-(hex-5-yn-1-yl)pyridine-2,6-diyl]bis(methylene-nitrilo)}tetrakis(acetato)uridine (3b).

2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)uridine was allowed to react with compound **5** as described for compound **2a**. Purification on silica gel (eluent CH₂Cl₂: MeOH 95:5, v/v) yielded the title compound as a foam. Yield was 70%. Compound **3b**: ¹H NMR (DMSO-d₆; 500 MHz): δ 7.67 (1H, d, J 8.2, H-6); 7.36 (2H, s, pyridine); 7.35

(2H, DMTr); 7.25 (7H, DMTr); 6.85 (4H, d, DMTr); 6.16 (1H, t, H-1, J 6.3); 5.48 (1H, d, J 8.1, H-5); 5.34 (1H, d, J 4.8, 3'-OH); 4.29 (1H, m, H-3'); 3.89 (1H, m, H-4'); 3.86 (4H, s, 2 · CH₂); 3.82 (2H, t, J 5.6 Ar-CH₂); 3.58 (8H, s, 4 · CH₂), 3.24 (1H, dd, J 10.7 and 5.2, H-5'); 3.19 (1H, dd, J 3.1 and 10.7, H-5''); 2.50 (2H, t, CH₂); 2.20 (2H, t, H-2' and H-2''); 2.72 (1H, br, OH) 1.73 (2H, m, CH₂); 1.52 (2H, m, CH₂).

The synthesis of 6-[4-(dimethylamino)azobenzene-4'-sulfonamido]hexan-1-ol (7).

To a stirred solution of 6-aminohexan-1-ol (0.50 g, 4.27 mmol) in dichloromethane (10 mL) was added dropwise a solution of dabsyl chloride (0.5 g, 1.54 mmol) in dichloromethane (10 mL). After 1h the mixture was washed with sat aq. NaHCO₃. The organic layer was dried over Na₂SO₄ and concentrated. Purification on silica gel (eluent CH₂Cl₂ containing 1% (v/v) MeOH) yielded the title compound as red solid. Compound **7**: ¹H NMR (CDCl₃): δ 7.97-7.89 (6H, m); 6.76 (2H, d, J 9.3); 4.50 (1H, br t, J 6.3); 3.60 (2H, t, J 6.2); 3.13 (6H, s); 2.98 (2H, q, J 6.9); 1.63 (1H, br); 1.50 (4H, m); 1.30 (4H, m).

The synthesis of 2'-deoxy-N3-[6-[4-(dimethylamino)azobenzene-4'-sulfonamido]hex-1-yl-5'-O-(4,4'-dimethoxytrityl)uridine (2c).

The title compound was synthesised by Mitsunobu alkylation of Compound **1** and Compound **6** using procedures described as for compound **2a**. The yield was 74%. Compound **3c**: ¹H NMR (400 MHz, DMSO-d₆): δ 7.98-7.88 (6H, m, dabsyl); 7.74 (1H, d, J 8.2; H-6); 7.57 (1H, J 1.2 H-6); 7.40 (2H, d, DMT); 7.30-7.24 (7H, m, DMT); 6.83 (4H, d, J , 9.0, DMT), 6.75 (2H, d, J 7.3, dabsyl); 6.48 (1H, dd, J 7.8), 5.45 (1H, d, J 8.2, H-5); 5.03 (1H, t, J 6.1, NH); 4.55 (1H, m, H-3'); 4.07 (1H, m, H-4'); 3.92 (2H, m, NCH₂); 3.79 (6H, s, 2 ·

OCH₃); 3.46 (1H, dd, *J* 3.2 and 10.5, H-5'); 3.36 (1H, dd, *J* 2.9 and 10.5, H-5''); 3.12 (6H, s, N(CH₃)₂); 2.99 (2H, m, CH₂NH); 2.46 (1H, m, H-2''); 2.31 (1H, m, H-2' and 3'-OH); 1.59 (4H, m, 2 · CH₂); 1.38-1.25 (4H, m, 2 · CH₂).

The synthesis of (E)-3-(4'-Bromophenyl)-1-pyrid-2'-yl)prop-2-enone (8)

4-Bromobenzaldehyde (50 g, 0.27 mol) was added in the ice-cold mixture methanol (540 mL) and water (110 mL) containing potassium hydroxide (15.2 g). After all aldehyde was dissolved 2-acetylpyridine (30.3 mL, 0.27 mol) was added and the reaction was allowed to proceed overnight at ambient temperature. The precipitation formed was filtered, washed with cold methanol and dried. Yield was 64 g (82%). ¹H NMR (CDCl₃): δ 8.75 (1H, br. d); 8.31 (1H, d, *J* 12 Hz); 8.20 (1H, br d); 7.90 (1H, m); 7.87 (1H, d, *J* 12); 7.59 (5H, m). MS (EI⁺) 288, 289 [M⁺].

The synthesis of 4''-(4'''-Bromophenyl)-2,2':6',2''-terpyridine (10).

A mixture of compound 8 (20.6 g, 71 mmol), dry ammonium acetate (137 g) and freshly prepared *N*-[2-(pyrid-2'-yl)-2-oxoethyl]pyridinium iodide (9; 23.3 g, 71 mmol) in dry methanol (650 mL) was heated at reflux overnight. The mixture was cooled to rt and refrigerated. The precipitation was separated by filtration, washed with cold methanol and dried. Yield was 12.5 g (45%). ¹H NMR (dms-*d*₆) δ: 8.77 (2H, br d, *J* 4); 8.71 (2H, s); 8.69 (2H, d *J* 7.9); 8.06 (2H, td, *J* 2.5 and 7.5); 7.92 (2H, d, *J* 7.5); 7.79 (2H, d, *J* 7.5); 7.55 2H, m). MS (EI⁺) 388, 390 [M⁺].

The synthesis of 4''-(4'''-Bromophenyl)-2,2':6',2''-terpyridine N,N''-Dioxide (11).

3-Chloroperbenzoic acid (29.1 g, 121 mmol) was added to compound **10** (12.4 g, 32 mmol) in dichloromethane (500 mL) and the mixture was stirred overnight at ambient temperature. The mixture was washed with 10 % sodium carbonate (300 mL), dried (Na_2SO_4) and concentrated. Purification on silica gel (eluent 10% methanol in dichloromethane) gave 11.4 g (85%) of product. ^1H NMR ($\text{dms}\text{-}d_6$) δ : 9.06 (2H, s); 8.43 (2H, m); 8.24 (2H, m); 7.80 (4H, s); 7.54 (4H, m). MS (EI^+) 419, 421 [M^+].

The synthesis of 4''-(4'''-Bromophenyl)-2,2':6',2''-terpyridine-6,6''-dicyanonitrile (12).

Trimethylsilylcyanide (13.7 mL, 110 mmol) was added to compound **11** (4.6 g, 11 mmol) in dichloromethane (170 mL). After 5 min, benzoyl chloride (5.1 mL, 44 mmol) was added within 20 min. After stirring overnight, the mixture was evaporated to half volume, 10% solution of K_2CO_3 (100 mL) was added, the mixture was stirred for 15 min, and the precipitate filtered and washed with water and cold dichloromethane. Yield was 3.69 g (77%). ^1H NMR ($\text{dms}\text{-}d_6$) δ : 8.98 (2H, d, J 8.0); 8.68 (2H, s); 8.31 (2H, t, J 7.6); 8.21 (2H, d, J 7.6); 7.97 (2H, d, J 8.4); 7.80 (2H, d, J 8.4). IR (KBr): 2237 cm^{-1} (CN). MS (EI^+) 437, 439 [M^+].

The synthesis of tetramethyl 2,2',2'',2'''-{[4'-(4''-bromophenyl)-2,2':6',2''-terpyridine-6,6''-diyl]bis(methylenenitrilo)}tetrakis (acetate) (14)

A suspension of compound **12** (3.65 g, 8.3 mmol) in dry THF (100 mL) was deaerated with argon. $\text{BH}_3 \cdot \text{THF}$ was added during 20 min. After stirring for 2.5 h at ice-bath, the excess of borane was destroyed by addition of methanol. The mixture was evaporated, and the residue was dissolved in methanol saturated with HCl (50 mL). After stirring for 2h at room temperature, the mixture was concentrated. The residue was suspended in THF, filtered, washed with THF and dried. This material was suspended in dry DMF (50

mL). Diisopropylethylamine (21 mL), methyl bromoacetate (3.1 mL, 33.3 mmol) and KI (1.51 g, 9.1 mmol) were added, and the mixture was stirred overnight at room temperature and concentrated. The residue was dissolved in dichloromethane (80 mL), washed with sat NaHCO₃ (3 · 40 mL) and dried. Purification was performed on silica gel (eluent Pet. Ether: ethyl acetate:triethylamine 5:2:1, v/v/v) Yield was 6.6 g. ¹H NMR (CDCl₃) δ: 8.68 (2H, s); 8.55 (2H, d, *J* 6); 7.87 (2H, t, *J* 6); 7.81 (2H, d, *J* 6); 7.68 (2H, d, *J* 6); 7.62 (2H, d, *J* 6); 4.19 (4H, s); 3.73 (8H, s); 3.70 (12H, s).

The synthesis of tetramethyl 2,2',2'',2'''-{[4'-(4''-(6-hydroxy-2-hexyn-1-yl)phenyl)-2,2':6',2''-terpyridine-6,6''-diyl]bis (methylenenitrilo)}tetrakis(acetate) (15).

Compound **14** (2.0 g, 2.72 mmol) and 5-hexyn-1-ol (360 µL; 3.28 mmol) were dissolved in the mixture of dry THF (15 mL) and triethylamine (4 mL) and the mixture was deaerated with argon for 10 min. Pd(Ph₃P)₂Cl₂ (37.5 mg, 0.053 mmol) and CuI (21.9 mg, 0.11 mmol) were added and the mixture was stirred overnight at 60 °C. The cooled mixture was filtered and the filtrate was concentrated in vacuo. The residue was dissolved in dichloromethane (50 mL), washed with water (2 · 20 mL) and dried. Purification on silica gel (eluent 10% methanol in dichloromethane (v/v)) gave 1.63 g (80%) of product. IR (film) 2232 cm⁻¹ (C≡C, weak). ¹H NMR (CDCl₃) δ: 8.70 (2H, s); 8.55 (2H, d, *J* 7.9); 7.87 (2H, t, *J* 7.9); 7.85 (2H, d, *J* 8.6); 7.61 (2H, d, *J* 7.6); 7.56 (2H, d, *J* 8.2); 4.19 (4H, s); 3.73 (8H, s); 3.75 (2H, m); 3.70 (12H, s); 2.52 (2H, t, 6.7); 1.77 (6H, m); 1.74 (1H br). MS (FAB⁺) 752.

The synthesis of 2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-3-(2,2',2'',2'''-{[4'-(4''-(5-hexyn-6-yl)phenyl)-2,2':6',2''-terpyridine-6,6''-diyl]bis (methylenenitrilo)}tetrakis(acetato) uridine (2d)

2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)uridine (1) was allowed to react with compound 15 under Mitsunobu conditions as described for compound 2a. Purification was performed on silica gel (eluent petr. ether: ethyl acetate: triethylamine; 2:5:1; v/v/v). Yield was 61%. ¹H NMR (CDCl₃) δ: 8.70 (2H, s.); 8.55 (2H, d); 7.86 (4H, m); 7.76 (1H, d); 7.57 (4H, m); 7.37 (nH, d); 6.83 (4H, d); 6.37 (1H, t); 5.45 (1H, d); 4.59 (1H, m); 4.21 (4H, s); 4.09 (1H, m); 3.99 (2H, t); 3.79 (8H, s); 3.70 (12H, s); 3.49 (2H, m); 2.79 (1H, br s); 2.53 (2H, m and t); 2.29 (1H, m); 1.78 (4H, m).

Synthesis of the phosphoramidites. General procedure.

Predried alcohol and 2-cyanoethyl N,N,N',N'-tetraisopropylphosphordiamidite (1.5 eq) were dissolved in dry acetonitrile. 1H tetrazole (1eq; 0.45 M in acetonitrile) was added, and the mixture was stirred for 30 min at room temperature before being poured into 5% NaHCO₃ and extracted with dichloromethane and dried over Na₂SO₄. Purification was performed either on silica gel column (eluent petr. ether: ethyl acetate: triethylamine; 2:5:1, v/v/v) or by precipitation from cold (-70 °C) hexanes.

2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-N3-(N6-trifluoroacetamido-hexyl)uridine 3'-O-(2-cyanoethyl N,N-diisopropyl)phosphoramidite (3a). Could be purified on silica or by precipitation. White powder. ³¹P NMR (CDCl₃): δ 148.6 (0.5 P), 148. 4 (0.5 P).

2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-N3 {tetramethyl 2,2',2'', 2'''-[(4-(hex-5-yn-1-yl)pyridine-2,6-diyl)bis(methylene-nitrilo)]tetrakis(acetato) uridine 3'-O-(2-cyanoethyl N,N-diisopropyl) phosphoramidite (3b). Purified on silica gel. White powder. ³¹P NMR (CDCl₃): δ 149.4 (0.5 P), 149. 1 (0.5 P).

2'-Deoxy-N3-[6-[4-(dimethylamino)azobenzene-4'-sulfonamido]hex-1-yl-5'-O-(4,4'-dimethoxytrityl)uridine 3'-O-(2-cyanoethyl N,N-

diisopropyl) phosphoramidite (3c). Purified on silica gel. Red solid. ^{31}P NMR (CDCl_3): δ 148.5 (0.5 P), 148.2 (0.5 P).

2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-3-(2,2',2'',2'''-{[4'-(4''-(5-hexyn-6-yl)phenyl)-2,2':6',2''-terpyridine-6,6''-diyl]bis(methylenenitrilo)}tetrakis(acetato)uridine 3'-O-(2-cyanoethyl N,N-diisopropyl) phosphoramidite (3d). Purified on silica gel. White powder ^{31}P NMR (CDCl_3): δ 148.7 (0.5 P), 148.3 (0.5 P).

Tetramethyl 2,2',2'',2'''-{[4'-(4''-(6-oxy-2-hexyn-1-yl)phenyl)-2,2':6',2''-terpyridine-6,6''-diyl]bis(methylenenitrilo)}tetrakis(acetato)-(2-cyanoethyl N,N-diisopropyl) phosphoramidite (16). Purified on silica gel. Colorless oil ^{31}P NMR (CDCl_3): δ 147.7 (1P).

The synthesis of 2'-deoxy-5'-O-(4,4'-dimethoxytrityl)-N3-(N6-trifluoroacetamidohex-1-yl)uridine 3'-O-succinate(18).

Compound 2a (0.50 g, 0.67 mmol) was dissolved in dry pyridine (5 mL). Succinic anhydride (135 mg, 1.35 mmol) and cat. amount of DMAP were added, and the mixture was stirred overnight at room temperature and concentrated. The residue was dissolved in dichloromethane, washed with aqueous triethylamine and dried. Purification was performed on silica gel (eluent:10% MeOH in dichloromethane) ^1H NMR (CDCl_3): δ 7.70 (1H, d, J 8.2); 7.28 (9H, m); 6.83 (4H, d J 8.9); 6.20 (1H, t, J 7.3); 5.80 (1H, d, J 8.2); 5.40 (1H, m); 4.13 (1H, m); 3.91 (4H, m); 3.80 (6H, s); 3.40 (2H, m); 2.69 (5H, m); 2.43 (1H, m); 1.61 (4H, m); 1.38 (4H, m).

The synthesis of the solid support 19.

Long chain alkylamine controlled pore glass was treated with a mixture of 10% triethylamine in 80% aq. Ethanol, washed with acetonitrile and dried. Compound 18 (0.5 mmol), N,N'-diisopropylcarbodiimide (1.0 mmol, 157 μL); and N-hydroxysuccinimide (0.5 mmol, 58 mg) were added to the suspension

of the solid support in dry pyridine (5 mL) and the mixture was shaken overnight at ambient temperature. The suspension was filtered, washed with dry pyridine, kept in a mixture of Ac₂O:pyridine:*N*-methylimidazole (1:5:1; v/v/v) for 10 min, and finally washed with ether. Loading as judged on DMTr-cation assay was 34 $\mu\text{mol g}^{-1}$.

OLIGONUCLEOTIDE SYNTHESIS

Introduction of primary amino groups to the oligonucleotide structure in the aid of compound 3a.

Model sequences were synthesized on an ABI instrument, and up to 10 phosphoramidites **3a** were coupled to its 5'-terminus using standard conditions (concentration 0.1 M in acetonitrile; coupling time 30 s). No difference in coupling efficiency between **3a** and normal nucleosidic building blocks were detected as judged on DMTr-cation response. After standard ammonolytic deprotection, the oligonucleotide prepared was isolated on PAGE and desalted on NAP columns. This oligonucleotide was finally labeled with the non-luminescent europium(III) chelate as described in reference 8.

Introduction of lanthanide(III) chelates to the oligonucleotide structure in the aid of compound 3b

Model sequences were synthesized as described above. One or 10 phosphoramidites **3b** were coupled to its 5'-terminus using standard conditions. No difference in coupling efficiency between **3b** and normal nucleosidic building blocks were detected. When the chain assembly was completed, the oligonucleotides were deprotected by first treating the solid support with 0.1 M sodium hydroxide for 4h at ambient temperature. 1.0 M ammonium chloride was then added, and the solution was concentrated in vacuo. The residue was treated with conc. ammonia for 16 h at 60 °C, after which europium citrate (10 eq. per ligand) was added, and the mixture was kept 90

min at room temperature. Desalting by NAP followed by RP HPLC yielded the desired oligonucleotide conjugates containing one or ten europium(III) chelates in their structure.

Luminescent chelates were introduced to the oligonucleotide structure in the aid of blocks **3d** and **17** analogously to block **3a**, except in the case of block **17** DMTr-On synthesis was applied.

Purification of oligonucleotide conjugates on HPLC

The oligonucleotide conjugates were purified on reversed phase techniques using either HyPURITY™ Elite (ThermoQuest), Purospher RP-18e (Merck) or Inertsil ODS-3 (GL Sciences) columns and TEAA buffer and acetonitrile gradients as mobile phase.

Characterization of the oligonucleotides prepared

Measurement of lanthanide(III) ion content by DELFIA

Oligonucleotides containing terpyridine based chelates.

- Oligonucleotide concentration was initially measured on UV spectrophotometry taking into account the absorptivity of the terpyridine ligand. Then the europium ion is released to solution by treatment with 0.1 M HCl for 1 h at room temperature. The Eu(III) content is measured using commercial DELFIA™ protocol (see ref. 2 of the article).

Oligonucleotides containing pyridine based chelates.

- measurement was performed as above but initial treatment with 0.1 M HCl was not needed.

Mass spectrometric analyses were performed on an Applied Biosystems Mariner instrument (API-TOF).

An example. Characterization of an oligonucleotide d(GCA CTG CTA TCG CAT CGT ATG ACG GAC ACC TCA CAA TAG AAC CT) tethered to

terpyridine chelate at its 5'-terminus (See **Scheme 7** in the article). Oligonucleotide concentration and its Eu(III) content were measured using UV and DELFIA as described above. Ratio between oligonucleotide and lanthanide was 1. Mass analysis yielded the molecular weight 14 336.0 (found); 14 336.4 (calc.).