Pergamon

Tetrahedron 58 (2002) 8771–8777

TETRAHEDRON

Oligonucleotide conjugates based on acyclonucleosides and their use in DNA hybridization assays

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Received 12 June 2002; revised 26 July 2002; accepted 22 August 2002

Abstract—Synthesis of two oligonucleotide building blocks based on acyclonucleosides (**10**, **11**) which enable the introduction of several nonluminescent and luminescent lanthanide(III) chelates to the oligonucleotide structure is described. They were used in an instrument-assisted DNA synthesis in a standard manner. A modified deprotection procedure was used to ensure metal complexation. Also the applicability of these oligonucleotide conjugates to DNA hybridization assays is demonstrated.

1. Introduction

The fluorescent labels attached to monomers for solid phase oligonucleotide chemistry are most commonly organic dyes, several of those blocks are even commercially available. Although organic chromophores can be utilized in several applications, such labels and labeled biomolecules suffer from many commonly known drawbacks such as Raman scattering, low water solubility and concentration quenching. In addition, the sensitivity is compromized by the phosphorescence of microtitration plates. Instead, the unique properties of lanthanide(III) chelates,[1,2] such as strong long decay-time luminescence, make them ideal labels for microtitration plate based assays.[3] Furthermore, large Stokes shift and very sharp emission bands enable the simultaneous use of four lanthanides (i.e. Eu, Tb, Sm, Dy) in the analysis. Time-resolved fluorimetric assays based on lanthanide chelates have found increasing applications in diagnostics, research and high throughput screening. The heterogeneous DELFIA® (dissociation-enhanced lanthanide fluorescence immunoassay) technique is applied in assays requiring exceptional sensitivity, robustness and a multi-label approach. Development of highly luminescent stable chelates extends the use of time resolution to homogeneous assays, based on fluorescence resonance energy transfer (TR-FRET), fluorescence quenching (TR-FQA) or changes in luminescence properties of a chelate during a binding reaction.[1–6] Luminescent lanthanide(III) chelates have been succesfully exploited in mixed phase DNA hybridization assays,[7,8] high throughput helicase assays[9] as well as in real time PCR.[10]

Quite recently, we reported our strategy for the introduction of lanthanide(III) chelates to oligonucleotide structures on solid phase.[11] We synthesized nucleosidic phosphoramidite blocks by a Mitsunobu reaction between 2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)uridine and primary alcohols containing a conjugate group in their structures (a protected functional group, an organic dye or a precursor of a lanthanide(III) chelate) followed by phosphitylation. They were used in an instrument-assisted DNA synthesis in a standard manner. A slightly modified deprotection procedure was used for the preparation of oligonucleotide conjugates tethered to lanthanide(III) chelates. The main purpose of the present work was to improve our approach for oligonucleotide derivatization. Here, the carbohydrate moiety is replaced by an optically pure alcohol while the base moiety is unchanged. Due to the lack of a carbohydrate moiety, the oligonucleotide probes have enhanced stability towards nucleases.[12–16] The labeling reactant is designed to have minimal hybridization properties, i.e. the label is attached to base moieties at positions needed for Watson–Crick base pairing, as it is desirable for DNA hybridization assays. The label can be attached to the 5′ or 3′-terminus of the coding sequence, and in fact up to 10 labels can be coupled to the oligonucleotide probe if required.

The applicability of the oligonucleotide conjugates synthesized in the present work for microtitration plate based DNA hybridization assays is also demonstrated.

2. Results and discussion

2.1. Introduction of linker arms to nucleosides

Introduction of linker arms to the nucleobase is most commonly performed by allowing a nucleoside with a good leaving group (e.g. *N*-tosyl, halogen, triazole, thiol) at C4 of

Keywords: oligonucleotide; lanthanide(III); DNA synthesis.

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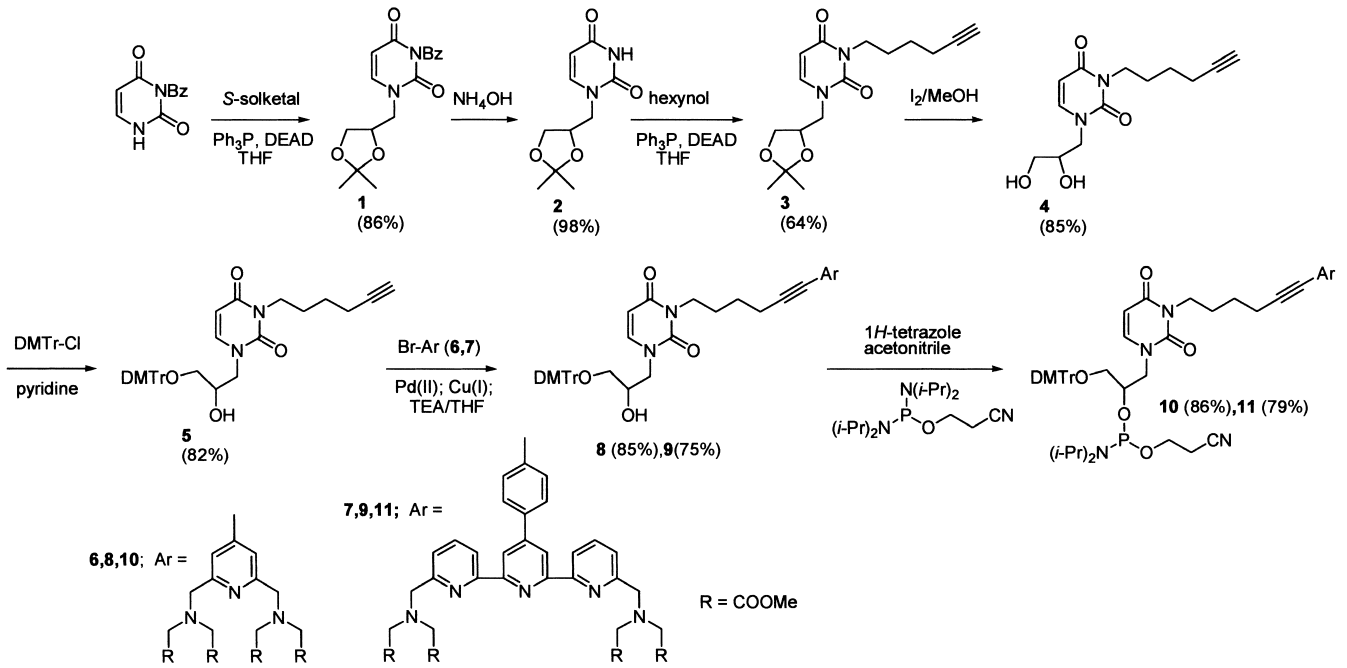
PII: S0040-4020(02)01056-6



Scheme 1.

pyrimidines or C2, C8 or C6 of purines to react with the appropriate nucleophilic linker molecule (e.g. an alkane-α,ω-diamine).[17] Alternatively, cytosine residues can be modified by bisulfite-ion-catalyzed transamination reaction.[18,19] Since normally an excess of linker molecule and rather vigorous reaction conditions has to be used, laborious purification procedures cannot be avoided. The reaction conditions impose additional requirements on the protecting groups in the target molecule. These problems may be overcome by attachment of the linker molecules to C5 of pyrimidine bases by a palladium-catalyzed coupling reaction between 5-halogeno pyrimidine nucleoside and an alkynyl-or allyl linker. Recently, attachment of a linker arm to the *N*3 of 3′,5′-*O* protected thymidine[20] and 2′-deoxy-5′-*O*-(4,4′-dimethoxytrityl)uridine[11] based on the Mitsunobu reaction have been reported. Since the coupling reaction is performed under mild conditions, a wide range of tethers can be introduced. The latest method for nucleoside tethering was also used in the present work.

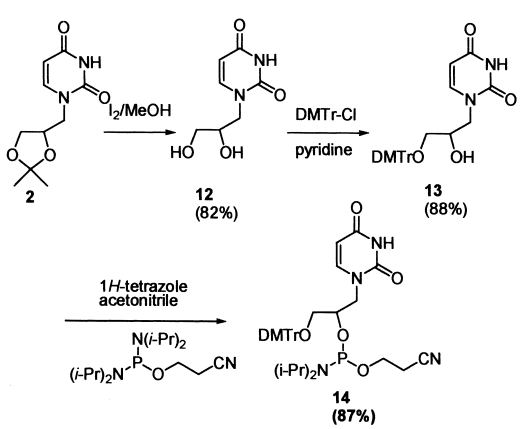
2.2. Synthesis of the phosphoramidites

Synthesis of the oligonucleotide building blocks is depicted in Scheme 1. Accordingly, Mitsunobu reaction between 3-benzoyluracil[21] and *S*-solketal gave **1** in good yield. Although Mitsunobu reaction of unprotected uracil with an alcohol has been reported,[22] protection of *N*3 considerably enhances the overall yield. Furthermore, in contrast to results reported for carbocyclic analogues of 2′,3′-dideoxy-5′-homonucleosides,[23] the reaction could be performed at ambient temperature. After cleavage of the benzoyl group by ammonolysis, a second Mitsunobu reaction with hexynol was performed to give rise to **3**. The isopropylidene protection was removed by iodolysis,[24] and the primary hydroxy function was dimethoxytritylated in the conventional manner. Palladium-catalyzed coupling of tetramethyl 2,2′,2″,2‴-[(4-bromopyridine-2,6-diyl)bis-(methylenenitrilo)]tetrakis(acetate)[11] **6**, or tetramethyl 2,2′, 2″,2‴-{[4′-(4″-bromophenyl)-2,2′:6′,2″-terpyridine-6,6″-diyl]bis-(methylenenitrilo)}tetrakis(acetate)[11] **7**, to compound **5** yielded the ligands **8**, **9**, which were finally phosphitylated with 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite using 1*H* tetrazole as the catalyst to give the oligonucleotide building blocks **10** and **11** as solids after purification using silica gel chromatography.

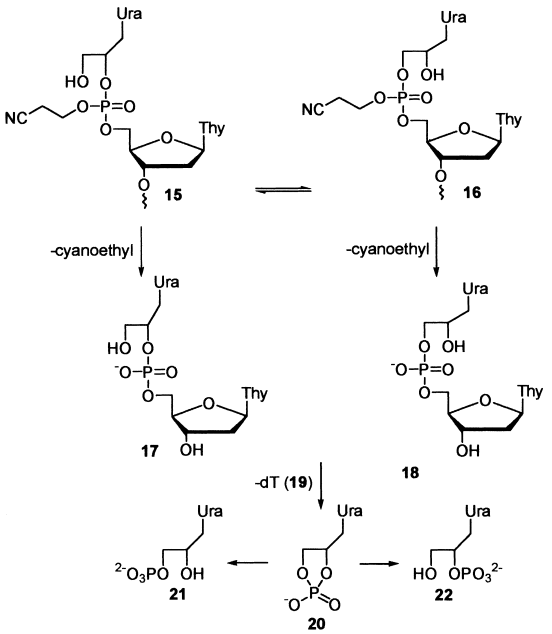
The model phosphoramidite without a ligand structure was obtained from compound **2** by deprotection of the *cis*-diol followed by dimethoxytritylation and phosphitylation of the primary and secondary hydroxyl functions, respectively (Scheme 2).

2.3. Investigation of possible side reactions under basic conditions and in the presence of trivalent lanthanide ions

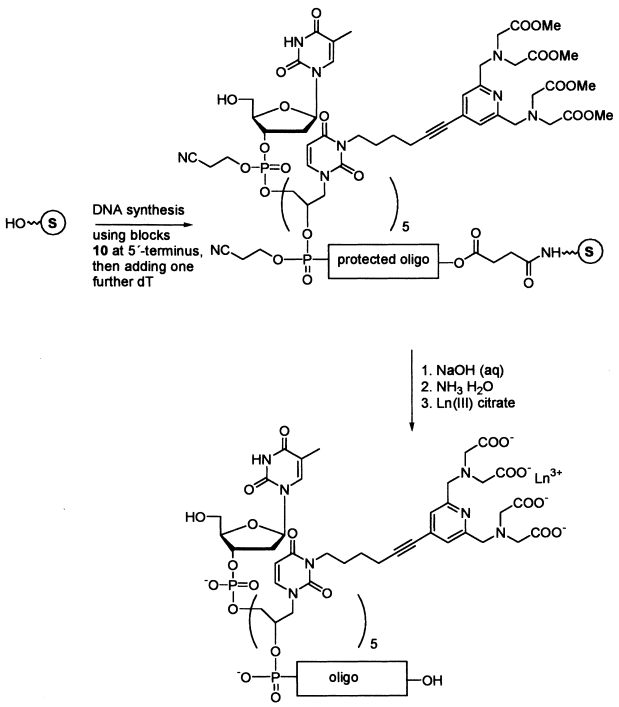
It is known that the phosphodiester bonds of oligoribonucleotides hydrolyze rapidly under basic conditions due to intramolecular nucleophilic attack of the 2′-hydroxyl group



Scheme 2.



Scheme 3.



Scheme 4.

at the phosphorus.[25,26] A similar reaction has also been reported for 1-[(2′*S*)-2′,3′-dihydroxy-propyl]cytosine analogues of dinucleoside monophosphates,[23] which are structurally very similar to the acyclonucleosides synthesized in the present work. Ribonucleoside phosphotriesters, in turn, are highly susceptible to phosphate migration.[27–30] Furthermore, trivalent lanthanide ions are know to promote both RNA hydrolysis and dephosphorylation, although binding lanthanide ions to organic ligands markedly reduces their catalytic activity.[31,32] These reactions may occur also in the present case since direct introduction of lanthanide(III) chelates to oligonucleotides requires a modified deprotection protocol: (i) treatment with 0.1 M NaOH for 4 h at rt. (ii) ammonolysis, and (iii) treatment with lanthanide(III) citrate. To clarify this, two model sequences were synthesized: d(X^{1}T) and d(TX^{1}T), where X^1 is the acyclonucleoside without a tether arm, and the product distribution was analyzed after each step using HPLC–MS. Indeed, after treatment with aq. NaOH, the HPLC trace of d(X^{1}T) showed several peaks assigned as thymidine (**19**), two monophosphates (**21**, **22**), the cyclic monophosphate (**20**) and two dimers (**17**, **18**; major components) (Scheme 3, Table 1). Neither ammonolysis nor treatment with europium citrate dramatically changed the product distribution. In all likelihood, the primary hydroxyl function of **15** reacts extremely rapidly under basic conditions with the phosphate triester giving the two isomers (**15**, **16**) prior to β-elimination of the cyanoethyl group. Prolonged treatment in basic media then results in partial phosphodiester hydrolysis. By contrast, similar treatment of the trimer d(TX^{1}T) gave only a single peak with correct molecular weight ([M−H]$^-$ obs. 793.3, calcd 793.2) in accordance to the stability of oligodeoxyribonucleotides under the reaction conditions employed. Thus, the above mentioned side reactions could result in severe fragmentation of oligonucleotide conjugates synthesized with blocks **10** and **11** if the terminal hydroxyl function of the acyclic structure is present. Fortunately, coupling an additional natural 2′-deoxyribonucleoside unit at the 5′-terminus of the oligonucleotide conjugate enhances dramatically stability of the oligomers.

Table 1. HPLC–MS characterization data for the products of the reactions of protected d(X^{1}T) under basic media and in the presence of Eu (citrate)

Compound[a]	t_R (min)[b]	[M−H]$^-$ (obs)	[M−H]$^-$ (calcd)
17	15.3	489.20	489.10
18	17.8	489.20	489.10
19	14.5	241.13	241.08
20	5.5	247.06	247.06
21	5.3	265.08	265.02
22	5.7	265.08	265.02

[a] Characterization of the isomers is tentative.
[b] For chromatographic conditions, see Section 3.

2.4. Synthesis of the oligonucleotide conjugates

In order to demonstrate the applicability of **10** and **11** for the preparation of oligonucleotide conjugates, several oligomers were synthesized using standard instrument-assisted DNA chemistry. Up to five ligand blocks were coupled to the 5′-terminus of the coding sequences using 10 min coupling time and 0.2 M concentration. No differences in coupling efficiency between the blocks and commercial nucleoside analogues were detected as judged on DMTr-cation assay. After coupling the ligand blocks, a single thymidine was attached to prevent side reactions during the basic conditions employed for deprotection of the oligonucleotide conjugate.

As discussed above, the direct introduction of lanthanide(III) chelates to oligonucleotides requires a modified deprotection strategy[33] as described in Scheme 4. Accordingly, after completion of the oligonucleotide synthesis the fully protected oligomer was initially treated with sodium

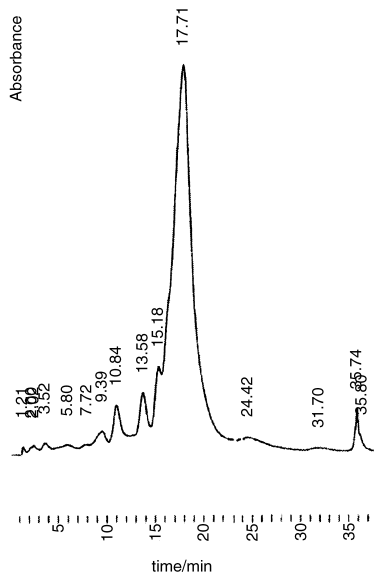


Figure 1. HPLC trace (crude reaction mixture) of an oligonucleotide conjugate (Insulin C) 5′-T(X^2)$_5$CTC CCC GGC CG-3″; Ln=Tb synthesized with block **10**. X^2 is the position of the label. For chromatographic conditions, see Section 3. ESI-TOF MS of the major peak at t_R 17.71 min: $[M-4H]^{-4}$ (obs) 1953; calcd 1953.

hydroxide (0.1 M; 4 h at rt) to ensure total hydrolysis of the ester protecting groups followed by ammonolysis to complete the base deprotection. Treatment of the deblocked oligomer with lanthanide(III) citrate (Ln=Sm, Eu, Tb, Dy) converted the oligonucleotide conjugates to the corresponding lanthanide(III) chelates. After desalting on NAP columns, purification was performed on polyacrylamide gel electrophoresis (PAGE). An HPLC profile of an oligonucleotide conjugate (crude reaction mixture) is shown in Fig. 1 as an illustrative example. All oligonucleotide conjugates were analyzed on ESI-TOF MS and their observed molecular weights were in accordance with the proposed structures.

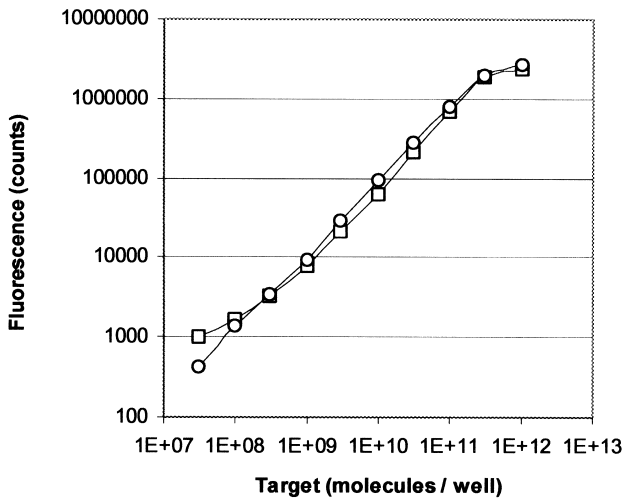


Figure 2. Dual DNA hybridization assay. Specific fluorescence on binding of Tb chelate labeled Ins-C (□) and Eu chelate labeled Ins-T (○) as a function of amount of target sequences in wells. For oligonucleotide sequences, see Section 3.

2.5. DNA hybridization assays

In order to demonstrate the functionality of the oligonucleotide conjugates synthesized in DNA hybridization assays, oligonucleotides tethered to nonluminescent lanthanide(III) chelates were allowed to hybridize with biotinylated target sequences on streptavidin coated microtitration plates. The unhybridized oligonucleotide probes were removed by washing, and the lanthanide content was measured using DELFIA® technology. An illustrative example on simultaneous hybridization of two oligonucleotides (Ins-C and Ins-T) labeled with five nonluminescent Tb and Eu-chelates, respectively, synthesized with block **10** to complementary target sequences is shown in Fig. 2.

The sensitivity and linearity is in accordance with the results obtained with oligonucleotide probes synthesized with nucleosidic phosphoramidites,[11,34] demonstrating the suitability of the acyclic derivatives for the assays employed. It is worth noting that the labels attached to the *N*3 position of uracil residues naturally weaken hydrogen bonds in the duplex. Thus these labels should be used only up- or downstream of the coding sequence.

3. Experimental

3.1. General

Adsorption column chromatography was performed on columns packed with silica gel 60 (Merck). Reagents for oligonucleotide synthesis were purchased from Applied Biosystems. 5′-Biotinylated oligonucleotides (targets for Ins-C and Ins-T probes) were purchased from Sigma Genosys. The time-resolved fluorometer (Victor^{2}V), DELFIA® assay buffer, DELFIA® wash solution and DELFIA® enhancement solution were from PerkinElmer Life Sciences. ^{1}H NMR spectra were recorded on a Brucker 250 or a Jeol LA-400 spectrometer operating at 250.13 and 399.8 MHz, respectively. The signal of TMS was used as an internal reference. Coupling constants are given in Hz. ^{31}P NMR spectra were recorded on a Jeol LA-400 spectrometer operating at 161.9 MHz. H_3PO_4 was used as an external reference. IR spectra were recorded on a Perkin–Elmer spectrum one spectrophotometer. HPLC–MS analyses were performed on an Applied Biosystems Mariner ESI-TOF instrument connected to an Agilent 1100 Series HPLC system. The mobile phase used was the following: Buffer A: 0.005 M TEAA (pH 7.5); Buffer B: A in 50% (v/v) acetonitrile. Gradient: from 0 to 30 min, from 95% A to 70% A; from 30 to 31 min, from 70% A to 100% B. Flow rate was 0.6 mL min^{-1}, UV detection at 260 nm. Column: HyPURITY Elite C18 (5 μm), 150–4.6. HRMS analyses were performed on the Mariner instrument. HPLC analyses were performed on a Merck-Hitachi Instrument consisting of L-7400 detector, L-7100 gradient pump and D-7500 integrator. Mobile phase used was the following: buffer A: 0.02 M TEAA (pH 7.5); buffer B: A in 50% (v/v) acetonitrile. Column LiChrocart 125-3 Purospher RP-18e, 5 μm. Gradient: from 15 to 40% A in 30 min. Flow rate was 0.6 mL min^{-1}.

3.1.1. (*S*)-1-(2,3-*O*-Isopropylidene-2,3-dihydroxypropyl)-3-benzoyluracil (1). 3-Benzoyluracil (16.0 g, 74.0 mmol),

S-solketal (12.0 mL, 96 mmol) and triphenylphosphine (23.3 g, 89 mmol) were dissolved in dry THF (160 mL). Diethylazodicarboxylate (DEAD; 14.0 mL, 89 mmol) was added in five portions during 15 min, and the mixture was stirred at rt for an additional 2 h. After concentration in vacuo the product was purified on silica gel (eluent: diethyl ether). Pure fractions were pooled and concentrated to give 21.0 g (86%) of the title compound as an oil. ^{1}H NMR ($CDCl_3$): δ 7.94 (1H, d, J=7.5 Hz); 7.66 (1H, m); 7.45 (2H, m); 7.35 (2H, d, J=8.1 Hz); 5.82 (1H, d, J=7.5 Hz); 4.38 (1H, m); 4.07 (2H, m); 3.71 (2H, m); 1.46 (3H, s); 1.35 (3H, s). $[M+H]^+$ obs 331.1295 calcd for $C_{17}H_{19}N_2O_5^+$: 331.1288.

3.1.2. (*S*)-1-(2,3-*O*-Isopropylidene-2,3-dihydroxypropyl)-uracil (2). Compound **1** (20.5 g, 62.2 mmol) was suspended in the mixture of 25% aq. ammonia and methanol (200 mL, 1:1, v/v), and stirred at ambient temperature for 2 h, concentrated in vacuo and purified on silica gel (eluent: 20% methanol in dichloromethane, v/v) to give the title compound as a solid. Yield was 13.8 g (98%). Mp 150–2°C. ^{1}H NMR ($CDCl_3$): δ 9.28 (1H, br); 7.30 (1H, d, J=5 Hz); 5.71 (1H, d, J=5 Hz); 4.38 (1H, m); 4.11 (2H, m); 3.68 (2H, m); 1.41 (3H, s); 1.33 (3H, s). $[M+H]^+$ obs 277.1035 calcd for $C_{10}H_{15}N_2O_4^+$: 227.1032.

3.1.3. (*S*)-1-(2,3-*O*-Isopropylidene-2,3-dihydroxypropyl)-3-(5-hexyn-1-yl)uracil (3). Compound **2** (15.3 g, 68 mmol), 5-hexyn-1-ol (8.9 mL; 81 mmol) and triphenylphosphine (21.0 g, 81 mmol) were suspended in dry THF (250 mL). DEAD (12.8 mL, 81 mmol) was added in four portions during 15 min, and the mixture was allowed to stir overnight at ambient temperature. After concentration, purification was performed on silica gel (eluent: 3% methanol in dichloromethane, v/v) to give the title compound as an oil. Yield was 13.4 g (64 %). ^{1}H NMR ($CDCl_3$): δ 7.26 (1H, d, J=8.1 Hz); 5.73 (1H, d, J=8.1 Hz); 4.38 (1H, m); 4.11 (2H, m); 3.96 (2H, t, J=7.0 Hz); 3.69 (2H, m); 2.80 (2H, br); 2.25 (2H, td, J=2.7 and 7.1 Hz); 1.95 (1H, t, J=2.7 Hz); 1.76 (2H, m); 1.61 (2H, m); 1.42 (3H, s); 1.34 (3H, s). IR (film): 3290 cm^{-1} (C≡CH). $[M+H]^+$ obs 307.1659 calcd for $C_{16}H_{23}N_2O_4^+$: 307.1658.

3.1.4. (*S*)-1-(2,3-Hydroxypropyl)-3-(5-hexyn-1-yl)uracil (4). Compound **3** (13.3 g, 43.4 mmol) was dissolved in methanol (500 mL) containing iodine (1%, w/v), and stirred overnight at ambient temperature. Sodium bisulfite was added and the mixture was stirred until the color of iodine disappeared. Solid material was removed by filtration, and the filtrate was concentrated in vacuo. Purification was performed on silica gel (eluent MeOH/CH_2Cl_2 1:9, v/v) to give the title compound as a solid. Yield was 9.83 g (85%). Mp 101–3°C. ^{1}H NMR ($CDCl_3$): δ 7.27 (1H, d, J=7.9 Hz); 5.74 (1H, d, J=7.9); 3.99 (2H, m); 3.96 (2H, t, J=7.3 Hz); 3.84 (1H, m); 3.61 (2H, m); 2.84 (2H, br); 2.25 (2H dt, J=2.7 and 7.0 Hz); 1.95 (1H, t, J=2.7 Hz); 1.75 (2H, m); 1.60 (1H, m). IR (KBr): 3265 cm^{-1} (C≡CH). $[M+H]^+$ obs 267.1339 calcd for $C_{13}H_{19}N_2O_4^+$: 267.1325.

3.1.5. (*S*)-1-[3-(4,4′-Dimethoxytrityl-2,3-dihydroxypropyl)]-3-(5-hexyn-1-yl)uracil (5). Compound **4** (5.44 g, 20 mmol) was dried by coevaporations with dry pyridine and dissolved in the same solvent (40 mL). 4,4′-Dimethoxytrityl chloride (8.3 g, 24.5 mL; dissolved in 25 mL of dry dichloromethane) was added dropwise, and the mixture was stirred for 3 h at rt, after which all volatile materials were removed in vacuo. The residue was dissolved in dichloromethane, washed with sat. $NaHCO_3$ and dried over Na_2SO_4. Purification on silica gel using diethyl ether as the eluent yielded 9.3 g (82 %) of compound **5** as a solid. Mp 46°C. ^{1}H NMR ($CDCl_3$): δ 7.30 (9H, m); 7.22 (1H, d, J=7.9 Hz); 7.17 (4H, d, J=9.0 Hz); 5.74 (1H, d, J=7.9 Hz); 3.98 (4H, m); 3.94 (1H, m); 3.80 (6H, s); 2.45 (1H, br); 2.23 (2H, dt, J=2.7 and 7.2 Hz); 1.95 (1H, t, J=2.7 Hz); 1.76 (2H, m); 1.57 (2H, m). IR (KBr): 3293 cm^{-1} (C≡CH). $[M+Na]^+$ obs 591.2467 calcd for $C_{34}H_{36}N_2NaO_6^+$: 591.2466.

3.1.6. (*S*)-1-[3-(4,4′-Dimethoxytrityl-2,3-dihydroxypropyl)]-3-{tetramethyl 2,2′,2″, 2‴-[(4-(5-hexyn-1-yl)-pyridine-2,6-diyl)bis(methylenenitrilo)]tetrakis-(acetato)}-uracil (8). A mixture of tetramethyl 2,2′,2″,2‴-[(4-bromopyridine-2,6-diyl)bis-(methylenenitrilo)]tetrakis(acetate) (**6**; 3.2 g, 6.3 mmol) and compound **5** (4.7 g, 8.2 mmol) in dry THF (14 mL) and triethylamine (24 mL) was deaerated with argon. Bis(triphenylphosphine)palladium(II) chloride (88 mg, 0.126 mmol) and CuI (48 mg, 0.252 mmol) were 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 (Na_2SO_4) and concentrated. Purification on silica gel (eluent petroleum ether, bp 40–60°C/ethyl acetate/triethylamine, 2:5:1, v/v/v) yielded the title compound as a solid (5.3 g, 85%). Mp 40°C. ^{1}H NMR ($CDCl_3$): δ 7.39 (2H, s); 7.27 (9H, m); 7.12 (1H, d, J=7.9 Hz); 6.83 (4H, d, J=8.8 Hz); 5.61 (1H, d, J=7.9 Hz); 4.11 (2H, m); 3.98 (4H, s); 3.94 (1H, m); 3.79 (6H, s); 3.70 (12H, s); 3.61 (8H, s); 3.17 (2H, d, J=4.9 Hz); 2.74 (1H, br); 2.44 (2H, t, J=7.1 Hz); 1.72 (2H, m); 1.65 (2H, m). IR (KBr): 2236 cm^{-1} (C≡C). $[M+H]^+$ obs 992.4245 calcd for $C_{53}H_{62}N_5O_{14}^+$: 992.4288.

3.1.7. (*S*)-1-[3-(4,4′-Dimethoxytrityl-2,3-dihydroxy-propyl)]-3-(2,2′,2″,2‴-{[4′-(4″-(5-hexyn-1-yl)-phenyl)-2,2′:6′,2″-terpyridine-6,6″-diyl]bis (methylene-nitrilo)tetrakis(acetato)}uracil (9). Synthesis, work up and purification was performed as described in Section 3.1.6 but using tetramethyl 2,2′,2″,2‴-{[4′-(4″-bromophenyl)-2,2′:6′,2″-terpyridine-6,6″-diyl]bis-(methylenenitrilo)}tetrakis(acetate) (**7**; 1.5 g, 2.0 mmol) and compound **5** (1.5 g, 2.5 mmol). Yield was 1.9 g (75%, an oil). ^{1}H NMR (DMSO-d_6): δ 8.63 (2H, s); 8.55 (2H, d, J=7.7 Hz); 8.02 (2H, t J=7.7 Hz); 7.86 (2H, t, J=8.5 Hz); 7.62 (4H, t, J=7.3 Hz); 7.53 (1H, d, J=8.1 Hz); 7.42 (2H, d, J=7.4 Hz); 7.28 (4H, m); 6.88 (4H, d, J=8.8 Hz); 5.64 (1H, d, J=7.7 Hz); 5.32 (1H, d, J= 5.5 Hz); 4.10 (4H, s); 3.86 (2H, m); 3.72 (8H, s); 3.68 (6H, s); 3.51 (1H, m); 2.97 (2H, m); 2.88 (2H, m); 2.51 (6H, m). IR (film): 2227 cm^{-1} (C≡C). $[M+H]^+$ obs 1222.5059 calcd for $C_{69}H_{72}N_7O_{14}^+$: 1222.5132.

3.1.8. (*S*)-1-(2,3-Dihydroxypropyl)uracil (12). Iodolysis of compound **2** (2.4 g, 10.6 mmol) as described in Section 3.1.4 yielded the title compound as an oil after silica gel column chromatography (eluent MeOH/CH_2Cl_2, 1:5, v/v). Yield was 1.63 g (82%). ^{1}H NMR (DMSO-d_6): δ 11.21 (1H, br); 7.50 (1H, d, J=7.8 Hz); 5.50 (1H, d, J=7.8 Hz); 5.03 (1H, br); 4.72 (1H, br); 3.93 (1H, m); 3.66 (2H, m); 3.35

(2H, m). $[M+H]^+$ obs 187.0713 calcd for $C_7H_{11}N_2O_4^+$: 187.0736.

3.1.9. (*S*)-1-[3-(4,4′-Dimethoxytrityl-2,3-dihydroxypropyl)]uracil (13). Compound **12** (11.8 mmol) was dimethoxytritylated as described in Section 3.1.5. Purification was performed on silica gel (eluent $MeOH/CH_2Cl_2$, 3:97, v/v). Yield was 5.1 g (88%; a solid). Mp 51°C. ^1H NMR ($CDCl_3$): δ 9.21 (1H, br); 7.42–7.28 (9H, m); 7.20 (1H, d, J=7.8 Hz); 6.83 (4H, d; J=8.8 Hz); 5.56 (1H, d, J=7.8 Hz); 4.08 (2H, m); 3.79 (6H, s); 3.63 (1H, m); 3.18 (2H, m). $[M+Na]^+$ obs 511.1833 calcd for $C_{28}H_{29}N_2O_6Na^+$: 511.1840.

3.2. Synthesis of the phosphoramidites. General procedure

Predried alcohol (**8**, **9**, **13**) and 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (1.5 equiv.) were dissolved in dry acetonitrile. 1*H* tetrazole (1 equiv.; 0.45 M in acetonitrile) was added, and the mixture was stirred for 30 min at rt before being poured into 5% $NaHCO_3$, extracted with dichloromethane and dried over Na_2SO_4. Purification was performed on silica gel column (eluent petroleum ether, bp 40–60°C/ethyl acetate/triethylamine, 2:5:1, v/v/v).

3.2.1. (*S*)-1-[3-(4,4′-Dimethoxytrityl-2,3-dihydroxypropyl-2-*O*-(2-cyanoethyl *N*,*N*-diisopropyl) phosphoramidato)]-3-{tetramethyl 2,2′,2″,2‴-[(4-(5-hexyn-1-yl)pyridine-2,6-diyl)bis-(methylenenitrilo)-tetrakis(acetato)}uracil (10). ^{31}P NMR ($CDCl_3$): δ 152.65 (0.5 P); 152.47 (0.5 P). $[M+H]^+$ obs 1192.5366 calcd for $C_{62}H_{79}N_7O_{15}P^+$: 1192.5326.

3.2.2. (*S*)-1-[3-(4,4′-Dimethoxytrityl-2,3-dihydroxypropyl)-2-*O*-(2-cyanoethyl *N*,*N*-diisopropyl) phosphoramidato)]-3-[3-(2,2′,2″,2‴-{[4′-(4″-(5-hexyn-1-yl)phenyl)-2,2′:6′,2′-terpyridine-6,6″-diyl]bis (methylenenitrilo)tetrakis(acetato)}uracil (11). ^{31}P NMR ($CDCl_3$): δ 152.97 (0.5 P); 152.81 (0.5 P). $[M+H]^+$ obs 1444.6141 calcd for $C_{78}H_{89}N_9NaO_{15}P^+$: 1444.6130.

3.2.3. (*S*)-1-[3-(4,4′-Dimethoxytrityl-2,3-dihydroxy-propyl)-2-*O*-(2-cyanoethyl *N*,*N*-diisopropyl) phosphoramidato]uracil (14). ^{31}P NMR ($CDCl_3$): δ 152.87 (0.5 P); 152.73 (0.5 P). $[M+Na]^+$ obs 711.2922 calcd for $C_{37}H_{46}N_4NaO_7P^+$: 711.2918.

3.3. Synthesis of the oligonucleotide conjugates

The oligonucleotides were synthesized on an Applied Biosystems Expedite instrument in 1.0 μmol scale using phosphoramidite chemistry and recommended protocols (DMTr-Off synthesis). Phosphoramidites **10** or **11** were coupled to the 5′-terminus (coupling time 10 min, conc. 0.2 M) followed by a single thymidine phosphoramidite. As the chain assembly was completed, the oligonucleotides were deprotected by first treating the solid support with 0.1 M sodium hydroxide for 4 h 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 55°C, after which aqueous lanthanide citrate (5 equiv. per ligand) was added, and the mixture was kept for 90 min at ambient temperature. Desalting by gel filtration followed by denaturing PAGE yielded in the desired oligonucleotide conjugates containing lanthanide(III) chelates in their structure.

3.4. Hybridizations

DNA hybridizations were performed on streptavidin coated microtitration plates as described previously in detail.[35] For DELFIA®-based hybridization assays, the following oligonucleotide probes were synthesized:

Insulin C: $T(X^2)_5$CTC CCC GGC CG; Ln=Tb.
Insulin T: $T(X^2)_5$CTC CCT GGC CG; Ln=Eu.

where X^2 is the 5′-label synthesized using block **10**.

The 5′-biotinylated targets were the following (the complementary parts on italics):

Insulin C target: 5′-biotin GTT GTC GGC CGG GGA GCT GAG A.
Insulin T target: 5′-biotin GTT GTC GGC CAG GCA GCT GAG A.

Acknowledgements

J. H. wish to thank Professors Mikko Oivanen (University of Helsinki) and Harri Lönnberg (University of Turku) for valuable discussions.

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