Efficient stabilization of oligonucleotide duplexes through terpyridine metal complexes

Fabrice Freville, Nathalie Pierre, and Serge Moreau

Abstract: A large increase in the melting temperature of complementary oligonucleotides was obtained through the conjugation of terpyridine ligands at their nearby 5′ or 3′ ends and the addition of stoichiometric amounts of various transition metals. We describe here a short efficient synthetic route for the conjugation of terpyridine moieties and report new data on the stabilizing contribution of metal-assisted hybridization of oligonucleotides through UV-monitored melting temperature experiments.

Key words: oligonucleotides, metal complexes, terpyridine.

Résumé : Une augmentation importante de la température de fusion d'oligonucléotides complémentaires peut être obtenue par la conjugaison des ligands terpyridine à leurs extrémités adjacentes (5′ ou 3′) et l'addition de quantités stœchiométriques de divers métaux de transition. On décrit ici une courte voie de synthèse efficace pour la conjugaison des groupes terpyridine. On présente également de nouvelles données expérimentales sur l'effet stabilisant des métaux dans l'hybridation des oligonucléotides par des mesures de température de fusion soumises en spectroscopie UV.

Mots clés : oligonucléotides, complexes métalliques, terpyridine.

Introduction

We previously reported a high stabilization of complementary DNA strands association through the addition of a coordination link between the oligomers (1). Such a link was obtained using terpyridine moieties as chelating agents and serinol as a deoxyribose mimic to allow easy conjugation of the chelator to oligonucleotides at nearby 3′ and 5′ ends.

We report here a new way to access the efficient conjugation of terpyridine moieties on both 3′ and 5′ ends of oligonucleotides. To allow structural studies, we chose to use a pantolactone-based ribose mimic developed by Dioubankova et al. (2). This synthetic route was used to extend our studies on metal-assisted hybridization of oligonucleotides, providing an easy way to increase nucleic acid duplex stability.

Results

We chose to use two short complementary DNA sequences to evaluate the contribution of the metallic bridge on duplex stability. This data was obtained by UV-thermal denaturation experiments. The introduction of the terpyridine chelators at nearby 5′ and 3′ ends required the synthesis of a phosphoramidite monomer bearing the terpyridine moiety and a specific solid-supported reagent for 3′ conjugation.

Chemical synthesis

Taking into account the strategy develop by Dioubankova et al., we designed a new synthetic route involving pantolactone as a precursor for a nonnucleosidic monomer. The new monomer was derivatized as a phosphoramidite unit and used in a solid-supported terpyridine linker. The whole synthetic scheme is depicted in Fig. 1.

Pantolactone **1** was treated with an excess of 1,3 diaminopropane. The side chain aminogroup was transiently protected through trifluoroacetylation. The primary hydroxyl group was then selectively tritylated using 4,4′-dimethoxytrityl chloride, yielding compound **3**. **3** was purified by silica gel flash chromatography using a dichloromethane – ethyl acetate gradient. The overall yield from **1** was 68%. The N-protecting group was then removed by sodium carbonate treatment leading to **4** (yield of 74%). The terpyridine monomer **5** was obtained by the reaction with the *N*-hydroxysuccinimide ester **9** in high yield (82%), after chromatography over aluminum oxide using an ethyl acetate – hexane gradient. This monomer **5** was used either to synthesize the phosphoramidite **6** through phosphitylation with 2-cyanoethyl-*N*,*N*-diisopropylchloro phosphoramidite or to yield a solid-supported reagent **7** by reaction with succinylated LCAA-CPG. **6** was purified by reverse-phase chromatography and elution with neat acetonitrile (yield 43%). The overall yield from **1** was 17%. The solid-supported reagent **7** was obtained by known procedure (3). The loading range of the CPG reagent was found to be 25–35 μmol/g. The terpyridine moiety **8** was synthesized according to published procedures (4) and the *N*-hydroxysucciimide ester **9** was obtained in high yield (93%).

Duplex formation

To evaluate the stability of various metallic bridges be-

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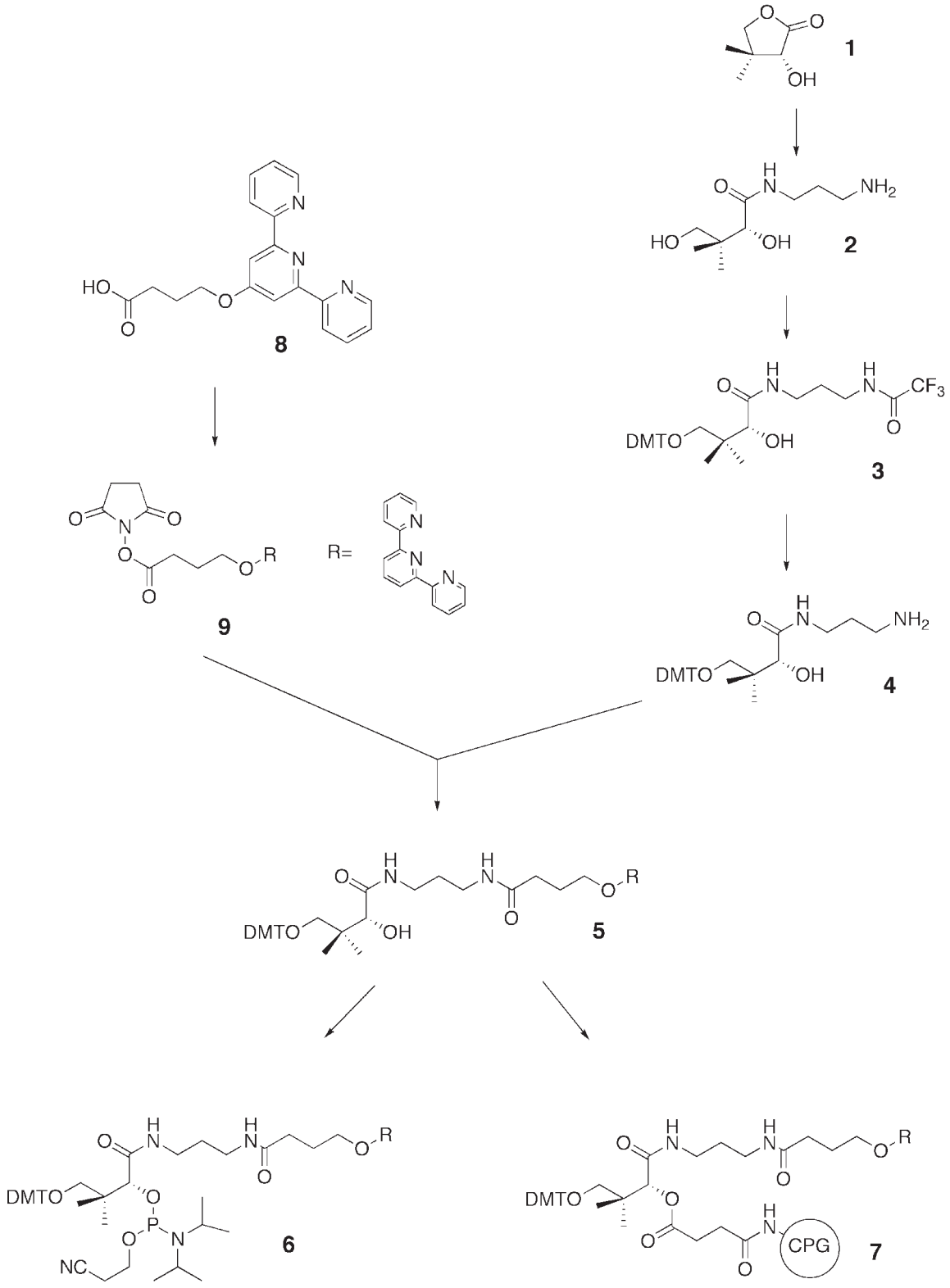
Fig. 1. Chemical synthetic pathway for the various monomer units.



tween complementary oligomers, we designed specific strand combinations. Oligonucleotide sequences are gathered in Table 1, together with the MS analysis of TPY-conjugated strands by MALDI-Q-TOF in negative mode. We thus confirmed the chemical structure of modified oligomers with the monoisotopic peaks in the MS analysis. Using the previously mentioned linkers, the terpyridine chelator was introduced into the 3′ or 5′ ends of complementary

Table 1. Sequences of the duplexes used in the melting temperature experiments.

No.	Duplex sequences	Calcd. (*m*/*z*)	Expt. $(M - H)^-$ (*m*/*z*)
I	5′ CCTTTCTTG 3′		
	3′ GGAAAGAAC 5′		
II	5′ CCTTTCTTG-TPY 3′	3237.68	3236.68
	3′ GGAAAGAAC 5′		
III	5′ CCTTTCTTG 3′		
	3′ GGAAAGAAC-TPY 5′	3362.76	3361.75
IV	5′ CCTTTCTTG-TPY 3′		
	3′ GGAAAGAAC-TPY 5′		
V	5′ CCTTTCTTG-TPY 3′		
	3′ TPY-GGAAAGAAC 5′	3362.76	3361.75

Note: TPY accounts for the terpyridine ligand. Columns 3 and 4 show the results of MS analysis (MALDI-Q-TOF, negative mode) of TPY-conjugated strands, calculated and experimental data, respectively.

Table 2. Effect of terpyridine ligands on values of Tm of oligonucleotide duplexes.

	Tm[a] (ΔTm)[b]					
Duplexes	EDTA	Zn^{2+}	Fe^{2+}	Co^{2+}	Ni^{2+}	Cu^{2+}
I	26.5	26.4	26.4	Nd[c]	Nd	Nd
II	30.6	30.0	32.3	Nd	Nd	Nd
III	32.0	33.0	32.6	Nd	Nd	Nd
IV	37.0	61.8 (35.3)	63.5 (37)	63.6 (37.1)	65.2 (38.7)	57.5 (31)
V	34.5	34.0	34.4	Nd	Nd	Nd

Note: Oligomers concentrations are 1 μmol/L.
[a]Melting temperature (Tm, °C, ±1 °C) (mean values from at least three experiments).
[b]ΔTm: Tm duplex IV – Tm duplex I (ΔTm indicated inside parentheses).
[c]Nd: not determined.

strands. Five different duplexes were studied, allowing comparisons between unmodified double-stranded oligomers and either mono- or bis-terpyridine conjugated oligonucleotides.

Thermal denaturation experiments were conducted in two different buffers. Data from nonmetalated species were obtained in a buffer containing EDTA to avoid contamination from traces of metal ions. As can be deduced from Table 2, the addition of at least 1 equiv. of Zn^{2+} ions (relative to duplex concentration) to the terpyridine-containing duplex IV resulted in a large increase in Tm (ΔTm = + 35.3 °C) in comparison with that of the unmodified duplex I. All other combinations of terpyridines moieties did not produce this stabilizing contribution. Duplexes II and III, although revealing some slight stabilization compared with I, did not reveal any stabilizing effect when compared with the data obtained from an unmetallated incubation medium (see column 2, Table 2). The presence of the two nearby terpyridine ligands is a prerequisite for the stabilization through added metal ions; duplex V, which exhibits noncontiguous terpyridine ligands, was not stabilized. The expected stoichiometry of the ligation reaction was obtained from a titration experiment with Zn^{2+} ions using the specific absorption band (320 nm) of the metalated terpyridine determined in a previous study (1) (Fig. 2).

Data from the nonmetalated buffer revealed a slight contribution from the terminal terpyridine in the stabilization of the duplexes (see column 2, Table 2). This stabilizing effect might arise from the formation of stacked aromatic pyridines on double-stranded oligonucleotide ends. The intercalation abilities of the terpyridine nucleus was discarded from previously observed data.

Fig. 2. Titration experiment showing the variation of the absorbance at 320 nm as a function of added $ZnCl_2$ (equiv. refers to the duplex concentration (1 μmol/L)).

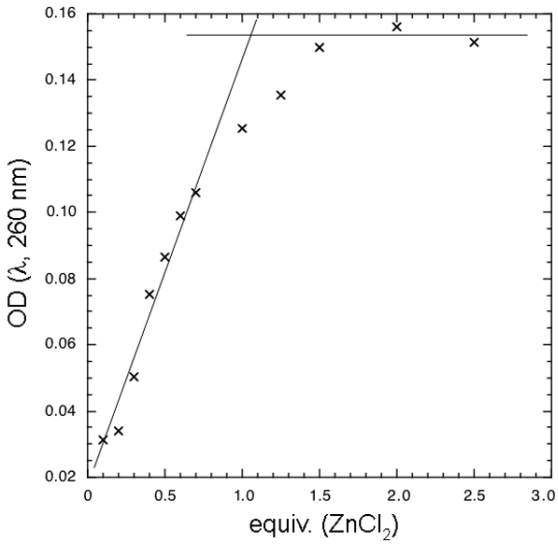


Table 2 also showed data from various chelating metals. All produced large increases in the Tm values of metalated duplexes; ΔTm from the unmodified duplex I ranged from 31 to 38.7 °C. The observed variations in the stabilizing contributions of the various chelated metals might arise from the coordination properties of the metals. All are known to form octahedral terpyridine complexes (5), however, other coordination numbers of 4 and 5 are also observed for copper (6,

7) and zinc–nickel complexes (8, 9). We cannot, therefore, exclude the occurrence of different coordination patterns in the metal-assisted hybridization of the oligonucleotides with coordination links between the nearby terpyridines varying from one to three.

Experimental section

General

Thin layer chromatography was performed on Merck silica gel 60F254 aluminum-backed plates. Flash chromatography refers to column chromatography performed with a Biotage system. NMR spectra were recorded with a Bruker Avance 300 spectrometer working at 300 MHz for ^{1}H, 75.45 for ^{13}C, and 121.49 for ^{31}P. The chemical shifts are expressed in ppm using TMS as internal standard (for ^{1}H and ^{13}C NMR data) and 85% H_3PO_4 as external standard for ^{31}P NMR data. Mass spectra (MALDI-Q-TOF, negative mode) were recorded on a Waters Ultima spectrometer. A special preparation technique was designed to analyse the TPY-modified oligonucleotides. The stainless steel target was first covered with a thin layer of matrix (0.5 μL of 2,4,6-trihydroxyacetophenone (THAP), 10 mg/mL in acetone) and this layer was washed with a saturated EDTA solution (2 × 1 μL) and water (2 × 1 μL). The oligonucleotides (0.5 μL of a 20 μmol/L solution in water) were then deposited on this matrix layer and allowed to dry. The final preparation step was the application of a second layer of matrix (0.5 μL of a 1:1 solution of THAP (30 mg/mL in ethanol) and 100 mmol/L aqueous ammonium citrate) that was allowed to dry at room temperature.

Chemical synthesis

Compound 3

A solution of (*R*)-(–)-pantolactone (1 g, 7.68 mmol) and 1,3-diaminopropan (1.67 mL, 38.42 mmol) in 6 mL EtOH was kept at 55 °C for 48 h, to which was added 13.7 mL (115.3 mmol) of ethyl trifluoroacetate. The mixture was kept at room temperature overnight, evaporated, then co-evaporated with toluene and dry pyridine. The residue was dissolved in dry pyridine (40 mL), cooled in an ice bath, and 4,4′-dimethoxytrityl chloride (2.86 g, 8.45 mmol) was slowly added. The reaction was stopped after 2 h by the addition of 0.5 mL of water and then diluted with 200 mL of $CHCl_3$. The organic phase was washed with 2.5% $NaHCO_3$ (3 × 60 mL), water (2 × 60 mL), and 20% NaCl (60 mL), then dried ($MgSO_4$). The residue was purified by flash chromatography using a stepwise gradient of EtOAC (0%–15%) in CH_2Cl_2–Et_3N (0.25%) to yield **3** as a colorless oil (3.14 g, 68%). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ: 8.03 (t, 1H, N*H*COCF$_3$), 7.36–6.82 (m, 13H, H_{DMTr}), 6.66 (t, 1H, NHCOCH(OH), $^{3}J_{NH-CH_2}$ = 6 Hz), 4.07 (s, 1H, C*H*OH), 3.77 (s, 6H, OCH_3), 3.27 (m, 2H, C*H*$_2$NHCOCF$_3$), 3.16 (m, 3H, C*H*$_2$NHCO and CH_{2a}ODMTr), 3.01 (d, 1H, CH_{2b}ODMTr), 1.57 (m, 2H, $CH_2CH_2CH_2$), 1.12 (s, 3H, CH_{3a}), 1.02 (s, 3H, CH_{3b}). ^{13}C NMR (75.45 MHz, $CDCl_3$, ppm) δ: 173.8 (*C*ONH), 158.7 (*C*OCH$_3$), 157.5 (q, *C*OCF$_3$, $^{2}J_{C-F}$ = 36.5 Hz), 144.1–113.4 (7C, DMTr), 116.0 (q, CO*C*F$_3$, $^{1}J_{C-F}$ = 285.9 Hz), 86.8 (O*C*$_{DMTr}$), 77.8 (*C*HOH), 71.2 (*C*H$_2$ODMTr), 55.3 (OCH_3), 38.9 (*C*(CH$_3$)$_2$), 36.0 (*C*H$_2$NHCOCF$_3$) 35.4 (*C*H$_2$NHCO), 29.0 ($CH_2CH_2CH_2$), 22.0 (CH_{3a}), 20.9 (CH_{3b}).

Compound 4

Compound **3** (3 g, 5.9 mmol) was dissolved in MeOH (40 mL), then an aqueous solution of Na_2CO_3 (10%, 11 mL) was added. The reaction mixture was stirred overnight at 65 °C, evaporated, diluted with 200 mL of EtOAc, washed with 1.5% $NaHCO_3$ (3 × 150 mL), then dried (Na_2SO_4), evaporated, and co-evaporated with toluene (3 × 30 mL) and CH_2Cl_2 (2 × 30 mL) to yield 2.2 g of **4** (74% yield). This compound was used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ: 7.36–6.82 (m, 14H, H_{DMTr} and NHCO), $^{3}J_{NH-CH_2}$ = 6 Hz), 4.08 (s, 1H, C*H*OH), 3.78 (s, 6H, OCH_3), 3.16 (m, 3H, C*H*$_2$NHCO and CH_{2a}ODMTr), 3.01 (d, 1H, CH_{2b}ODMTr), 2.62 (t, 2H, C*H*$_2$NH$_2$, $^{3}J_{H-H}$ = 6.6 Hz), 1.57 (m, 2H, $CH_2CH_2CH_2$), 1.12 (s, 3H, CH_{3a}), 1.02 (s, 3H, CH_{3b}). ^{13}C NMR (75.45 MHz, $CDCl_3$, ppm) δ: 172.4 (*C*ONH), 158.6 (*C*OCH$_3$), 144.1–113.4 (7C, DMTr), 86.4 (O*C*$_{DMTr}$), 77.1 (*C*HOH), 70.8 (*C*H$_2$ODMTr), 55.2 (OCH_3), 39.3 (CH_2NH_2), 39.0 (*C*(CH$_3$)$_2$), 36.4 (*C*H$_2$NHCO), 33.0 ($CH_2CH_2CH_2$), 22.0 (CH_{3a}), 20.9 (CH_{3b}).

Compound 5

4 was carefully dried by three co-evaporations with pyridine and then dissolved in anhydrous pyridine. The NHS ester **9** was added and the solution stirred at RT and monitored by TLC. The reaction medium was evaporated and purified by chromatography over aluminum oxide with a gradient of ethyl acetate in hexane (50%–80%) in the presence of 0.25% triethylamine (1.35 g, yield 82%). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ: 8.68 (ddd, 2H, $^{3}J_{ortho}$ = 4.8 Hz, $^{4}J_{meta}$ = 1.8 Hz, and $^{5}J_{para}$ = 0.9 Hz, H6 and H6″), 8.62 (ddd, 2H, $^{3}J_{ortho}$ = 7.5 Hz, $^{4}J_{meta}$ = 1.0 Hz and $^{5}J_{para}$ = 0.9 Hz, H3 and H3″), 8.02 (s, 2H, H3′ and H5′), 7.85 (ddd, 2H, $^{3}J_{ortho}$ = 7.8 Hz, $^{3}J_{ortho}$ = 7.5 Hz, and $^{4}J_{meta}$ = 1.5 Hz, H4 and H4″), 7.41–6.85 (m, 15H, H_{DMTr}, H5 and H5′), 6.64 (t, 1H, $^{3}J_{H-H}$ = 6 Hz, NHCO), 6.52 (t, 1H, $^{3}J_{H-H}$ = 5.7 Hz, NHCO), 4.29 (t, 2H, $^{3}J_{H-H}$ = 5.9 Hz, CH_2CH_2O), 4.09 (s, 1H, C*H*OH), 3.80 (s, 6H, CH_3O), 3.19 (m, 5H, C*H*$_2$NHCO and CH_{2a}ODMTr), 3.02 (d, 1H, CH_{2b}ODMTr), 2.45 (t, 2H, $^{3}J_{H-H}$ = 7.2 Hz, CH_2CO), 2.22 (m, 2H, NHCH$_2$C*H*$_2$CH$_2$NH), 1.50 (m, 2H, CH$_2$C*H*$_2$CH$_2$), 1.07 (s, 3H, CH_{3a}), 1.00 (s, 3H, CH_{3b}). ^{13}C NMR (75.45 MHz, $CDCl_3$, ppm) δ: 172.8 (CONH), 172.2 (CONH), 166.9 (C4′), 158.5 (*C*OCH$_3$), 156.9 (C2′, C6′), 155.9 (C2,C2″), 148.9 (C6, C6″), 144.3–113.2 (12C, DMTr), 136.6 (C4, C4″), 123.7 (C5, C5″), 121.2 (C3, C3″), 107.4 (C3′, C5′), 86.4 (O*C*$_{DMTr}$), 77.3 (*C*HOH), 70.8 (*C*H$_2$ODMTr), 66.2 (O*C*H$_2$), 55.2 (OCH_3), 38.8 (*C*(CH$_3$)$_2$), 35.8 (*C*H$_2$NHCO) 35.6 (*C*H$_2$NHCO), 32.8 ($OCH_2CH_2CH_2CONH$), 29.6 (NHCH$_2$*C*H$_2$CH$_2$NH), 25.1 (NHCH$_2$*C*H$_2$CH$_2$O), 22.0 (CH_{3a}), 20.9 (CH_{3b}). MS (FAB$^-$) calcd.: 823.97; found: 822 (M – H)$^-$.

Compound 6

5 (1 g, 1.2 mmol) was dissolved in anhydr. CH_2Cl_2 (10 mL). Diisopropylethylamine (520 μL, 3 mmol) and 2-cyanoethyl diisopropylchlorophosphoramidite (1.8 mmol, 430 mg) were then added to the previous solution. The reaction was stopped after 1 h at RT with 500 mL of CH_3OH and diluted with 100 mL of EtOAc, washed with 2.5%

$NaHCO_3$ (200 mL), and then dried over Na_2SO_4. The residue was purified over reverse phase silica gel (Lichroprep RP-18 (40–63 μmol/L)) and eluted with CH_3CN–Et_3N (0.25%) to yield 534 mg of solid **5** (43%). ^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ: 8.67 (d, 2H, $^3J_{ortho}$ = 4.2 Hz, H6 and H6″), 8.60 (d, 2H, $^3J_{ortho}$ = 7.8 Hz, H3 and H3″), 8.01 (s, 2H, H3′ and H5′), 7.85 (td, 2H, $^3J_{ortho}$ = 7.5 Hz, and $^4J_{meta}$ = 0.9 Hz, H4 and H4″), 7.41–6.85 (m, 15H, H_{DMTr}, H5 and H5″), 6.56 (t, 1H, $^3J_{H–H}$ = 6.0 Hz, NHCO), 6.43 (t, 1H, $^3J_{H–H}$ = 5.7 Hz, NHCO), 4.28 (t, 2H, $^3J_{H–H}$ = 6.0 Hz, $COCH_2CH_2CH_2O$), 4.09 (d, 1H, $^2J_{H–P}$ = 12.6 Hz, C*H*OP), 3.78 (s, 6H, CH_3O), 3.70–3.4 (m, 4H, CH_2NHCO), 3.4–2.9 (m, 6H, CH_2ODMTr, CH_2CH_2OP, NC*H*), 2.43 (m, 4H, $OCH_2CH_2CH_2CO$, $POCH_2CH_2CN$), 2.21 (m, 2H, $NHCH_2CH_2CH_2NH$), 1.55 (m, 2H, $OCH_2CH_2CH_2CO$), 1.11 (m, 15H, CH_3), 1.00 (s, 3H, CH_{3b}). ^{13}C NMR (75 MHz, $CDCl_3$, ppm) δ: 171.4 (CONH), 170.0 (CONH), 167.0 (C4′), 158.6 ($COCH_3$), 157.1 (C2′, C6′), 156.1 (C2, C2″), 149.0 (C6, C6″), 145.2–112.9 (7C, DMTr), 136.7 (C4, C4″), 123.8 (C5, C5″), 121.3 (C3, C3″), 117.9 (CH_2CN), 107.4 (C3′, C5′), 85.8 (OC_{DMTr}), 77.3 (CHOP), 70.8 (CH_2ODMTr), 67.3 ($OCH_2CH_2CH_2CO$), 58.2 ($NCCH_2CH_2OP$), 55.2 (OCH_3), 43.4 (NCH), 38.8 ($C(CH_3)_2$), 35.9 (CH_2NHCO) 35.7 (CH_2NHCO), 32.8 ($OCH_2CH_2CH_2CONH$), 29.6 ($NHCH_2CH_2CH_2NH$), 25.1 ($COCH_2CH_2CH_2O$), 24.6 ($NCH(CH_3)_2$), 22.7 and 22.3 ($C(CH_3)_2$), 20.4 (CH_2CN). ^{31}P NMR (120 MHz, $CDCl_3$, ppm) δ: 156.0, 152.5. MS (FAB^-) calcd.: 1023.19 $(M – H)^-$; found: 1022 $(M – H)^-$.

Compound 7

A solution of succinic anhydride (300 mg, 3 mmol) and DMAP (80 mg, 0.66 mmol) in 10 mL of dry pyridine was added to 3000 mg of LCAA-CPG (500 Å) and the mixture was left at RT for 24 h with occasional swirling. After filtration, successive washes with 10 mL portions of pyridine, CH_2Cl_2, Et_2O, and drying, the succinylated LCAA-CPG was suspended in 4 mL of DMF pyridine (1:1 *v*/*v*); compound **5** (206 mg, 0.25 mmol), 1,3-diisopropylcarbodiimide (0.28 mL, 1.8 mmol)), and DMAP (20 mg) were added. The suspension was left for 48 h at RT. To block the remaining carboxylic groups, a solution of pentachlorophenol (100 mg) was added and the mixture was kept at RT for further 12 h. The support was filtered, resuspended in 5% *v*/*v* solution of piperidine (3 mL), reacted for 10 min, filtered again and washed successively with 10 mL portions of $CHCl_3$, CH_3OH, CH_3CN, and dried in vacuo. Loading was determined by treating a portion (5 mg) of **7** with 1 mL of 3% *w*/*v* CCl_3CO_2H in 1,2-dichloroethane and measuring the absorbance of DMTr cation at 504 nm (ε = 75 000 L mol^{-1} cm^{-1}). Loading found: 25–35 μmol/g.

Compound 9

Compound **8** (5.36 mmol, 2 g), obtained according to previously published procedures (4), was dissolved in dimethylformamide (40 mL), and *N*,*N*-diisopropylcarbodiimide (1.1 equiv., 5.9 mmol, 923 mL) and *N*-hydroxysuccinimide (1.1 equiv., 5.9 mmol, 678 mg) were added. The reaction was stirred at 40 °C overnight. The crude mixture was concentrated and crystallization from methanol yielded **9** (2.17 g, 93%).

Oligonucleotide synthesis

All oligonucleotides were synthesized on a 0.2 μmol scale with a Millipore Expedite 9809 synthesizer using conventional β-cyanoethyl phosphoramidite chemistry. The standard bases were dissolved in anhydrous acetonitrile (0.1 mol/L final concentrations). The modified phosphoramidites were dissolved in anhydrous acetonitrile and coupled manually with a coupling time of 15 min. The coupling efficiency was the same as that of unmodified amidites. All oligomers were synthesized "trityl off". The solid support was treated overnight at 55 °C with fresh concd. NH_4OH (1 mL). The crude oligomers were purified by electrophoresis on denaturated polyacrylamide gels (20%) and visualized by UV shadowing. The pure samples were desalted through reverse-phase set-pak cartridges (C18, Waters)

UV-monitored melting experiments

Purified oligonucleotides (each strand was 1 μmol/L) were diluted in 200 mL of the appropriate buffer. The mixture was boiled for 2 min and the hybridization was assured by low-temperature cooling of the sample. Melting experiments were performed with a Cary 1E UV–vis spectrophotometer with a temperature controller unit. Samples were kept at 5 °C for at least 30 min and then heated from 4 to 90 °C at a rate of 0.4 °C/min. The absorbance (λ = 260 nm) was measured every minute. The melting temperature was determined from the maxima of the first derivative. Two different buffers were used. The studies on the metalated species were performed in a buffer containing 10 mmol/L sodium phosphate (10 mmol/L, pH 7) and sodium chloride (150 mmol/L). The metal-free experiments were measured in an EDTA-containing buffer: EDTA (200 mmol/L), sodium phosphate (10 mmol/L, pH 7), and sodium chloride (150 mmol/L).

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