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Pd²⁺-Mediated Base Pairing in Oligonucleotides

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ABSTRACT: Two short glycol nucleic acid (GNA) oligonucleotides, having either a terminal or an intrachain nucleobase replaced by the pyridine-2,6-dicarboxamide chelate of Pd²⁺, have been synthesized and their hybridization properties studied by melting temperature measurements. In the termini of a double-stranded oligonucleotide, the Pd²⁺ chelates provided dramatic stabilization of the duplex relative to its metal-free counterpart, in all likelihood owing to formation of Pd²⁺-mediated base pairs between pyridine-2,6-dicarboxamide and the opposing nucleobase. In contrast, no stabilization was observed when the Pd²⁺ chelate was placed in the middle of the chain. Furthermore, the results could not be reproduced by adding a Pd²⁺ salt *in situ* to the dilute oligonucleotide solutions but the palladated oligonucleotides had to be synthesized and purified prior to the hybridization studies. This behavior, presumably attributable to the relatively slow ligand-exchange reactions of Pd²⁺, differs greatly from what is usually observed with more labile metal ions. The present results offer an explanation for the failure of previous attempts to incorporate Pd²⁺-mediated base pairs into oligonucleotides.

1. Introduction

Metal-ion-mediated base pairing of nucleic acids has been studied extensively for more than a decade owing to its potential in DNA nanotechnology and in expanding the genetic alphabet.[1-9] So far, the most promising results have been obtained with the kinetically labile Ag⁺, Hg²⁺ or Cu²⁺ as the bridging metal ion. For expanding the range of applications of metal-ion-mediated base pairing to chemotherapy, however, the low intracellular concentrations of these metal ions present a severe limitation. DNA cross-linking by the kinetically inert Pt²⁺, in turn, is a widely employed strategy in cancer chemotherapy[10-12] but suffers from low sequence-specificity. Oligonucleotides capable of metal-ion-mediated hybridization even under the metal-deficient conditions of the cell would offer an elegant solution to this problem.

Along with Pt^{2+} , Pd^{2+} appears an ideal candidate for metal-ion-mediated base pairing. Both of these metal ions prefer nitrogen over oxygen ligands and form highly stable square-planar complexes that are compatible with the steric requirements of the base stack of a double-helical nucleic acid. At monomeric level, several Pd^{2+} -mediated base pairs have been described in the literature.[13-19] In fact, the very first artificial metal-ion-mediated base pair was formed through coordination of Pd^{2+} . [20] Incorporation of a Pd^{2+} -mediated base pair into an oligonucleotide, however, remains to be convincingly demonstrated. The apparent discrepancy between the results obtained at monomer and oligomer levels may be understood in terms of the relatively slow ligand exchange reactions of Pd^{2+} . A double-helical oligonucleotide presents many potential donor atoms and a Pd^{2+} ion introduced *in situ* may well remain kinetically trapped at a nonproductive binding site.

In this article we present compelling evidence for Pd^{2+} -mediated base pairing within an oligonucleotide. K_2PdCl_4 was allowed to react with short glycol nucleic acid (GNA) oligonucleotides incorporating a single high-affinity ligand. The GNA backbone was chosen as a test bed because of the relatively easy and facile synthesis of modified GNA building blocks. The metallated oligonucleotides were purified by RP HPLC before hybridization experiments. Coordination of a single Pd^{2+} ion by the modified oligonucleotides was verified by mass spectrometric analysis. Thermal melting studies with these oligonucleotides revealed the Pd^{2+} -mediated base pair to be better tolerated in a terminal than in an internal position.

2. Results and discussion

2.1. Building block synthesis

The GNA phosphoramidite building blocks of the four canonical nucleobases were prepared as described previously.[21-23] For preparation of a respective building block of the metal-ion-chelating residue (**1**), the phenolic OH function of diethyl 4-hydroxypyridine-2,6-dicarboxylate was

first derivatized by treatment with (*R*)-2,2-dimethyl-1,3-dioxolane-4-methanol *p*-toluenesulfonate (Scheme 1). The isopropylidene protection was then removed under acidic conditions and the liberated primary OH function was protected as a 4,4'-dimethoxytrityl ether. The secondary OH of the protected intermediate **3** was phosphitylated by conventional methods. Finally, the ethoxycarbonyl substituents of the pyridine ring were converted to aminocarbonyl substituents by treatment with methanolic ammonia to afford the phosphoramidite building block **1**. It is worth pointing out that GNA oligonucleotides with free OH termini will undergo cleavage under these conditions.

< Scheme 1 >

2.2. Preparation of solid supports for the synthesis of GNA oligonucleotides

For immobilization on solid support, protected GNA counterparts of the canonical 2'-deoxynucleosides were first succinylated by treatment with succinic anhydride in anhydrous pyridine (Scheme 2). The crude products were then attached to long chain alkylamine-functionalized controlled pore glass (LCAA-CPG) by conventional HBTU-promoted peptide coupling. Based on trityl response on treatment with 3% dichloroacetic acid in CH₂Cl₂, loadings of the solid supports thus obtained were found to be in the typical range (30 – 50 μmol g⁻¹).

< Scheme 2 >

2.3. Oligonucleotide synthesis

The oligonucleotides (Table 1) were assembled by an automated synthesizer on a CPG-supported succinyl linker. Standard phosphoramidite strategy with 300 s coupling time (with 5-benzylthio-1*H*-tetrazole as the activator) was used throughout the sequences. Based on the trityl

response, the coupling yields were 44% for the modified building block **1** and more than 98% for the other building blocks. The oligonucleotides were released from the supports and the phosphate and base protections removed by treatment with either 16% aq. ammonia (for **ON1** and **ON2**) or 20% aq. methylamine (for **ON3**) for 35 min at 55 °C. To minimize cleavage of the phosphodiester linkages, the aminolyses were carried out with the 3'-O-DMTr protection in place. This protecting group was finally removed in 80% aq. acetic acid (60 min at room temperature).

< Table 1 >

The crude oligonucleotides were purified by RP-HPLC and the purified products characterized by electrospray ionization mass spectrometry (ESI-MS). The concentrations of the oligonucleotides were estimated UV-spectrophotometrically based on absorbance at 260 nm. The molar absorptivities were calculated by an implementation of the nearest-neighbors method assuming the hypochromicities of GNA and DNA oligonucleotides to be equal.[24, 25] For determination of the molar absorptivity of the modified residue, compound **3** was deprotected and converted into the diamide **5** by successive treatments with hydrochloric acid and methanolic ammonia (Scheme 3). Molar absorptivity of **5** at 260 nm was determined to be 2000 M⁻¹ cm⁻¹ (UV spectrum presented as supporting information).

< Scheme 3 >

2.4. Preparation of Pd²⁺-carrying oligonucleotides

Oligonucleotide **ON1**, bearing the modified residue **5** in its 5'-terminus, was dissolved in 2 mM aq. K₂PdCl₄, to a final concentration of 1.3 mM. This mixture was incubated overnight at room temperature, after which it was diluted with phosphate buffer (pH 7.2). The product mixture was fractionated by RP-HPLC eluting with an MeCN gradient in phosphate buffer and the collected fractions desalted by RP-HPLC eluting with an MeCN gradient in water. Based on the HPLC profile,

ON1 had been converted nearly quantitatively to products of lower mobility (Fig. 1). ESI-MS analysis revealed the main product, eluting at 12.5 min, to be a complex of two **ON1** molecules and two Pd^{2+} ions (Fig 2., $\text{ON1}_2:\text{Pd}_2$). Each of the Pd^{2+} ions had displaced two protons, consistent with the binding mode reported previously for pyridine-2,6-dicarboxamide.[18] Given the self-complementarity of the tetranucleotide sequence in the middle of **ON1**, a homoduplex with terminal Pd^{2+} -mediated base pairs between the pyridine-2,6-dicarboxamide moiety of the modified residue **5** and thymine appears the most likely structure for $\text{ON1}_2:\text{Pd}_2$. Coordination of Pd^{2+} to the N3 atom of thymine is accompanied by deprotonation,[18] resulting in a net charge of -1 for each of the Pd^{2+} -mediated base pairs. Based on ESI-MS, the second-most abundant product, eluting at 13.8 min, appeared to consist of three **ON1** molecules and three Pd^{2+} ions (data not shown). The other minor products remained unidentified.

< Figure 1 >

< Figure 2 >

Oligonucleotide **ON2**, incorporating the modified residue **5** in the middle of the sequence, was dissolved in 2 mM aq. K_2PdCl_4 , to a final concentration of 1.8 mM. This mixture was incubated overnight at room temperature, after which it was freeze-dried. An equimolar amount of the complementary oligonucleotide **ON3** was then added and the mixture diluted with phosphate buffer (pH 7.2). The RP-HPLC chromatogram of the product mixture (Fig. 3) consisted of two main peaks, of which the slower eluting one ($t_R = 13.0$ min) was identified as **ON3** by HPLC-MS analysis, as well as spiking with an authentic sample. The faster-eluting peak ($t_R = 10.3$ min), in turn, did not correspond to either of the starting oligonucleotides. In fact, hardly any unreacted **ON2** could be detected in the product mixture. When small volumes of the mixture were injected, the areas of the two peaks were approximately equal but upon injection of larger volumes the first peak grew at the expense of the second one (chromatograms presented as supporting information). Evidently the two peaks represented two compounds in equilibrium and increasing the concentration shifted this equilibrium

to favor the faster-eluting compound. The most likely explanation for such behavior is that the first peak, eluting faster than either **ON2** or **ON3**, corresponded to a heterodimer of these oligonucleotides. This interpretation was confirmed by reinjection of the collected peak – in this chromatogram, peaks of the fast-eluting product and oligonucleotide **ON3**, but not **ON2**, were detected (Fig. 3D). The absence of the peak of **ON2** could be explained if **ON2** eluted as the Pd²⁺ complex **ON2**:Pd and the retention time of this metallo-oligonucleotide was very similar to that of the heterodimer product. Attempts to desalt the product fraction were unsuccessful, presumably due to denaturation of the **ON2**:Pd:**ON3** duplex and subsequent loss of the bridging Pd²⁺ ion at the low ionic strength employed. A small sample was, however, treated as described above for **ON1**₂:Pd₂ and analyzed by ESI-MS (data presented as supporting information). Oligonucleotides **ON2**, **ON3** and **ON2**:Pd were detected, suggesting that Pd²⁺ is coordinated at the desired site but the **ON2**:Pd:**ON3** duplex is not quite as stable as **ON1**₂:Pd₂.

< Figure 3 >

2.5. Thermal stability of the modified oligonucleotide duplexes

Melting temperatures for the Pd²⁺-containing duplexes **ON1**₂:Pd₂ and **ON2**:Pd:**ON3**, as well as their non-metallated counterparts **ON1**₂ and **ON2**:**ON3**, were measured in either a 20 mM cacodylate buffer (pH = 7.4, for **ON1**₂ and **ON1**₂:Pd₂) or a 20 mM phosphate buffer (pH = 7.2, for **ON2**:**ON3** and **ON2**:Pd:**ON3**). The oligonucleotide concentrations were 6.0 μM for **ON1** (3.0 μM for **ON1**₂) and 3.0 μM for **ON2** and **ON3**. The ionic strength of the samples was adjusted to 0.1 M with NaClO₄. With both of the non-metallated oligonucleotide duplexes, typical sigmoidal melting curves were obtained, the *T*_m being 45.9 °C for **ON1**₂ and 36.3 °C for **ON2**:**ON3** (Fig. 4). The palladated duplexes, on the other hand, behaved quite differently: the melting temperature of **ON1**₂:Pd₂ was too high to be determined whereas with **ON2**:Pd:**ON3** no stabilization over **ON2**:**ON3** was observed. Similar results have previously been obtained with 2'-*O*-methyl-RNA oligonucleotides incorporating

2,6-bis(3,5-dimethylpyrazol-1-yl)purine as the metal-ion-chelating nucleobase. In that case, 10 °C stabilization upon addition of 1 eq. of K_2PdCl_4 was obtained with uracil as the opposite base, but only when the metal-ion-mediated base pair was placed at a terminal position.[26] Presumably both of these Pd^{2+} -mediated base pairs deviate from the optimal geometry required by the base stack and this deviation is better tolerated in a terminal than in an intrastrand position.

< Figure 4 >

While impossible to quantify accurately, it is evident that the difference between the thermal stabilities of **ON1**₂:Pd₂ and **ON1**₂ is vastly greater than what has been previously reported for any oligonucleotide structure with Pd^{2+} as the bridging metal ion. The reason for this discrepancy could be the different preparation of the samples. In the present study, Pd^{2+} -carrying duplexes were synthesized and purified prior to the measurements, in contrast to the more common method of simply adding Pd^{2+} salt to a dilute sample containing both strands of the duplex. As discussed above, this method may fail to afford the thermodynamic product due to the relatively slow ligand-exchange reactions of Pd^{2+} . To test this hypothesis, the melting temperature of **ON1**₂ was measured in the presence of 2 eq. (6.0 $\mu\text{mol L}^{-1}$) of K_2PdCl_4 (Fig. 4A). The T_m was somewhat lower than the one obtained in the absence of Pd^{2+} (41.5 and 45.9 °C, respectively). Clearly, mixing dilute solutions of **ON1** and K_2PdCl_4 at neutral pH does not result in significant formation of the desired **ON1**₂:Pd₂ duplex.

2.6. Secondary structure of the modified oligonucleotide duplexes

To probe the secondary structure of the modified duplexes and the possible changes induced by Pd^{2+} coordination, CD spectra of **ON1**₂, **ON2:ON3**, **ON1**₂:Pd₂ and **ON2**:Pd:**ON3** were recorded under the same conditions as described above for the T_m measurements (Figs. 5 and 6). In all cases, a negative band at 275 nm was observed at low temperatures, consistent with an (S)-GNA

double helix.[27] The other previously reported negative band (at 220 nm) was hardly detectable but **ON1₂** exhibited a prominent negative band at 247 nm. This band was not observed with **ON1₂:Pd₂** and the band at 275 nm was also less prominent, suggesting partial unwinding of the double helix upon formation of the terminal Pd²⁺-mediated base pairs. In contrast, the spectra of **ON2:ON3** and **ON2:Pd:ON3** were very similar. As expected, heating resulted in gradual decrease of the CD signals with all the oligonucleotides. In the case of **ON1₂:Pd₂**, however, some ellipticity persisted even at 90 °C, in line with the high thermal stability of this oligonucleotide. In other words, while the terminal Pd²⁺-mediated base pairs of **ON1₂:Pd₂** disrupt the helical secondary structure, they still greatly stabilize the duplex. This stabilization is attributable to the two Pd-N bonds formed between the terminal thymine residues and the Pd²⁺:pyridine-2,6-dicarboxamide chelates. Evidently their bond energy is greater than the energy cost for (partial) unwinding of the double helix.

< Figure 5 >

< Figure 6 >

2.7. Conclusions

Formation of Pd²⁺-mediated base pairs within an oligonucleotide duplex has been demonstrated. At the termini of a short duplex, such base pairs may be highly stabilizing even if they are not easily accommodated in the base stack of the double helix. For realization of its potential as the bridging metal ion, however, Pd²⁺ has to be firmly attached to a predetermined site of one strand of the duplex prior to hybridization. Attempts to introduce Pd²⁺ to mixtures of oligonucleotides *in situ* under typical conditions of hybridization studies are not likely to afford the desired Pd²⁺-mediated duplex in a practical yield.

3. Experimental part

3.1 . General

Preparation of the GNA phosphoramidite building blocks of the four canonical nucleobases has been described in the literature.[21-23] The other reagents were commercial products that were used as received. Solvents were dried over 3 Å molecular sieves and trimethylamine over calcium hydride. ^1H , ^{13}C and ^{31}P NMR spectra: *Bruker Avance* 500- or 400-MHz NMR spectrometer; δ in ppm rel. to Me_4Si (for ^1H and ^{13}C) or orthophosphoric acid (for ^{31}P) as external standard, J in Hz. HR-ESI-MS: *Bruker Daltonics micrOTOF-Q* mass spectrometer; in m/z . UV spectra: *Perkin-Elmer Lambda 35* UV-vis spectrophotometer. CD spectra: *Applied Photophysics Chirascan* spectropolarimeter.

3.2. Syntheses

Diethyl (S)-4-[(2,2-dimethyl-1,3-dioxolan-4-yl)methoxy]pyridine-2,6-dicarboxylate (2). 4-Hydroxypyridine-2,6-dicarboxylate (0.50 g, 2.09 mmol), (*R*)-2,2-Dimethyl-1,3-dioxolane-4-methanol *p*-toluenesulfonate (0.72 g, 2.5 mmol) and potassium carbonate (1.03 g, 7.45 mmol) were suspended in anhydrous DMF (20 mL). The mixture was stirred at 70 °C for 96 h, after which it was evaporated to dryness. The residue was dissolved in CH_2Cl_2 (100 mL) and washed with water (100 mL). The organic layer was dried with Na_2SO_4 and evaporated to dryness. The crude product thus obtained was purified on a silica gel column eluting with a mixture of MeOH and CH_2Cl_2 (6:94, *v/v*). Yield 0.61 g (82%). ^1H -NMR (400 MHz, CDCl_3): 7.77 (s, 2H, Pyr-H3 & -H5), 4.48 (m, 1H, dioxolane-H5), 4.43 (q, J = 7.1, 4H, $\text{CO}_2\text{CH}_2\text{CH}_3$), 4.20-4.09 (m, 3H, dioxolane-H4 & PyrOCH_2), (dd, J = 5.8, 8.6, 1H, dioxolane-H4), 1.42 (s, 3H, dioxolane- CH_3), 1.41 (t, J = 7.1, 6H, $\text{CO}_2\text{CH}_2\text{CH}_3$), 1.36 (s, 3H, dioxolane- CH_3). ^{13}C -NMR (100 MHz, CDCl_3): 166.5 (2C, CONH_2), 164.5 (Pyr-C4), 150.3 (2C, Pyr-C2 & -C6), 114.3 (2C, Pyr-C3 & -C5), 110.1 (dioxolane-C2), 73.4 (dioxolane-C5), 69.3 (PyrOCH_2), 66.3 (dioxolane-C4), 62.3 (2C,

CO₂CH₂CH₃), 26.7 (dioxolane-CH₃), 25.2 (dioxolane-CH₃), 14.2 (2C, CO₂CH₂CH₃). HR-ESI-MS: 354.1547 ([M + H]⁺, C₁₇H₂₄NO₇⁺; calc. 354.1547).

(*S*)-1-[(4,4'-Dimethoxytrityl)oxy]-3-[[2,6-bis(ethoxycarbonyl)pyridin-4-yl]oxy]propan-2-yl (2-cyanoethyl) diisopropylphosphoramidite (**4**). Compound **2** (0.61 g, 1.73 mmol) was dissolved in a mixture of 37% aq. HCl (0.40 mL), MeCN (4.0 mL) and water (4.0 mL). The mixture was stirred at room temperature for 16 h, after which it was evaporated to dryness. The residue was coevaporated from toluene (10 mL) and anhydrous pyridine (3 × 10 mL) and finally redissolved in anhydrous pyridine (10 mL). 4,4'-Dimethoxytrityl chloride (0.65 g, 1.9 mmol) was added and the resulting mixture stirred at room temperature for 16 h, after which it was concentrated under reduced pressure. The oily residue was diluted with CH₂Cl₂ (100 mL) and washed with saturated aq. NaHCO₃ (100 mL). The organic layer was dried with Na₂SO₄ and evaporated to dryness. The crude product thus obtained was purified on a silica gel column eluting with a mixture of Et₃N, MeOH and CH₂Cl₂ (1:3:96, v/v). The purified intermediate **3** (0.274 g, 0.445 mmol), anhydrous Et₃N (311 μL, 2.23 mmol) and 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (120 μL, 0.496 mmol) were dissolved in anhydrous CH₂Cl₂ (1.5 mL). The resulting mixture stirred under N₂ at room temperature for 2 h, after which it was diluted with CH₂Cl₂ (100 mL) and washed with saturated aq. NaHCO₃ (100 mL). The organic layer was dried with Na₂SO₄ and evaporated to dryness. NMR analysis revealed the product thus obtained to be of sufficient purity. Yield 0.3484 g (25%) as two diastereomers in approximately 1:1 ratio. ¹H-NMR (400 MHz, CDCl₃, diastereomer A): 7.78 (s, 2H, Pyr-H3 & -H5), 7.44 (m, 2H, Ph-H2 & -H6), 7.31 (m, 4H, MeOPh-H2 & -H6), 7.27 (m, 2H, Ph-H3 & -H5), 7.23 (t, *J* = 5.0, 1H, Ph-H4), 6.80 (d, *J* = 8.5, 4H, MeOPh-H3 and & -H5), 4.48 (q, *J* = 7.1, 4H, CO₂CH₂CH₃), 4.37-4.18 (m, 3H, PyrOCH₂ & CH₂CH(OP)CH₂), 3.78 (s, 6H, OCH₃), 3.74 (m, 1H, POCH₂), 3.70 (m, 1H, POCH₂), 3.66-3.47 (m, 2H, NCHCH₃), 3.36 (dd, *J* = 9.6, 5.1, 1H, DMTrOCH₂), 3.29 (dd, *J* = 9.4, 6.2, 1H, DMTrOCH₂), 2.61 (t, *J* = 6.4, 2H, CH₂CN), 1.46 (t, *J* = 7.0, 6H, CO₂CH₂CH₃), 1.14 (d, *J* = 5.6, 6H, NCHCH₃), 1.07 (d, *J* = 6.7, 6H, NCHCH₃). ¹H-NMR (400 MHz, CDCl₃, diastereomer B): 7.74 (s, 2H, Pyr-H3 & -H5), 7.42 (m, 2H, Ph-H2 & -H6), 7.31 (m, 4H, MeOPh-H2 & -H6), 7.27 (m, 2H, Ph-H3 & -H5), 7.19 (t, *J* = 6.3, 1H, Ph-H4), 6.82

(d, $J = 8.1$, 4H, MeOPh-H3 and & -H5), 4.48 (q, $J = 7.1$, 4H, CO₂CH₂CH₃), 4.37-4.18 (m, 3H, PyrOCH₂ & CH₂CH(OP)CH₂), 3.82 (m, 1H, POCH₂), 3.78 (s, 6H, OCH₃), 3.75 (m, 1H, POCH₂), 3.66-3.47 (m, 2H, NCHCH₃), 3.40 (m, 2H, DMTrOCH₂), 2.47 (t, $J = 6.5$, 2H, CH₂CN), 1.46 (t, $J = 7.2$, 6H, CO₂CH₂CH₃), 1.18 (d, $J = 7.2$, 6H, NCHCH₃), 1.16 (d, $J = 6.1$, 6H, NCHCH₃). ¹³C-NMR (100 MHz, CDCl₃, diastereomer A): 166.8 (2C, CONH₂), 164.7 (Pyr-C4), 158.5 (2C, MeOPh-C4), 150.2 (2C, Pyr-C2 & -C6), 144.7 (Ph-C1), 135.9 (MeOPh-C1), 135.8 (MeOPh-C1), 130.0 (2C, MeOPh-C2 & -C6), 130.0 (MeOPh-C2 & -C6), 128.1 (2C, Ph-C3 & -C5), 127.8 (2C, Ph-C2 & -C6), 126.8 (Ph-C4), 117.6 (CN), 114.5 (2C, Pyr-C3 & -C5), 113.1 (4C, MeOPh-C3 & -C5), 86.2 (Ar₃C), 71.5 (d, $J = 16.9$, CH₂CH(OP)CH₂), 69.4 (d, $J = 4.5$, PyrOCH₂), 63.1 (d, $J = 4.1$, DMTrOCH₂), 62.4 (2C, CO₂CH₂CH₃), 58.2 (d, $J = 18.4$, POCH₂), 55.2 (2C, OCH₃), 43.2 (d, $J = 12.7$, 2C, NCHCH₃), 24.6 (2C, NCHCH₃), 24.5 (2C, NCHCH₃), 20.2 (d, $J = 7.1$, CH₂CN), 14.2 (2C, CO₂CH₂CH₃). ¹³C-NMR (100 MHz, CDCl₃, diastereomer B): 166.7 (2C, CONH₂), 164.7 (Pyr-C4), 158.6 (2C, MeOPh-C4), 150.2 (2C, Pyr-C2 & -C6), 144.6 (Ph-C1), 135.8 (MeOPh-C1), 135.8 (MeOPh-C1), 130.0 (4C, MeOPh-C2 & -C6), 128.1 (2C, Ph-C3 & -C5), 127.8 (2C, Ph-C2 & -C6), 126.9 (Ph-C4), 117.5 (CN), 114.3 (2C, Pyr-C3 & -C5), 113.1 (4C, MeOPh-C3 & -C5), 86.3 (Ar₃C), 71.2 (d, $J = 16.0$, CH₂CH(OP)CH₂), 69.2 (d, $J = 2.3$, PyrOCH₂), 63.0 (d, $J = 3.9$, DMTrOCH₂), 62.4 (2C, CO₂CH₂CH₃), 58.3 (d, $J = 18.8$, POCH₂), 55.2 (2C, OCH₃), 43.2 (d, $J = 12.5$, 2C, NCHCH₃), 24.7 (2C, NCHCH₃), 24.6 (2C, NCHCH₃), 20.3 (d, $J = 6.9$, CH₂CN), 14.2 (2C, CO₂CH₂CH₃). ³¹P-NMR (162 MHz, CDCl₃, diastereomer A): 149.9. ³¹P-NMR (162 MHz, CDCl₃, diastereomer B): 150.1. HR-ESI-MS: 838.3376 ([M + Na]⁺, C₄₄H₅₄N₃NaO₁₀P⁺; calc. 838.3439).

(*S*)-1-[(4,4'-Dimethoxytrityl)oxy]-3-[(2,6-dicarbamoylpyridin-4-yl)oxy]propan-2-yl (2-cyanoethyl) diisopropylphosphoramidite (**1**). Compound **4** (115 mg, 0.141 mmol) was dissolved in 7 M methanolic ammonia. The mixture was stirred at room temperature for 16 h, after which all volatiles were removed in vacuum. Yield 106 mg (near quantitative). ¹H-NMR (500 MHz, CDCl₃, diastereomer A): 7.87 (s, 2H, Pyr-H3 & -H5), 7.67 (d, $J = 3.4$ Hz, 2H, CONH), 7.45 (m, 2H, Ph-H2 & -H6), 7.33 (m, 4H, MeOPh-H2 & -H6), 7.28 (m, 2H, Ph-H3 & -H5), 7.22 (t, $J = 7.3$, 1H, Ph-H4), 6.85 (d, $J = 8.5$, 4H, MeOPh-H3 and & -H5), 5.94 (d, $J = 3.7$, 2H, CONH), 4.46 (m, 1H, CH₂CH(OP)CH₂), 4.33 (m,

2H, PyrOCH₂), 3.82 (s, 6H, OCH₃), 3.77 (m, 1H, POCH₂), 3.71 (m, 1H, POCH₂), 3.67-3.50 (m, 2H, NCHCH₃), 3.36 (dd, *J* = 9.9, 4.9, 1H, DMTrOCH₂), 3.29 (dd, *J* = 9.4, 5.9, 1H, DMTrOCH₂), 2.50 (ddd, *J* = 6.5, 6.3, 2.1, 2H, CH₂CN), 1.17 (d, *J* = 7.0, 6H, NCHCH₃), 1.08 (d, *J* = 6.7, 6H, NCHCH₃). ¹H NMR (500 MHz, CDCl₃, diastereomer B): 7.85 (s, 2H, Pyr-H3 & -H5), 7.66 (d, *J* = 3.7 Hz, 2H, CONH), 7.44 (m, 2H, Ph-H2 & -H6), 7.33 (m, 4H, MeOPh-H2 & -H6), 7.28 (m, 2H, Ph-H3 & -H5), 7.23 (t, *J* = 7.3, 1H, Ph-H4), 6.83 (d, *J* = 8.8, 4H, MeOPh-H3 and & -H5), 5.92 (d, *J* = 3.7, 2H, CONH), 4.32 (m, 2H, PyrOCH₂), 4.26 (m, 1H, CH₂CH(OP)CH₂), 3.84 (m, 1H, POCH₂), 3.81 (s, 6H, OCH₃), 3.78 (m, 1H, POCH₂), 3.67-3.50 (m, 2H, NCHCH₃), 3.43 (dd, *J* = 9.7, 4.4, 1H, DMTrOCH₂), 3.38 (dd, *J* = 10.5, 5.6, 1H, DMTrOCH₂), 2.65 (ddd, *J* = 6.6, 6.4, 0.7, 2H, CH₂CN), 1.19 (d, *J* = 7.1, 6H, NCHCH₃), 1.18 (d, *J* = 7.3, 6H, NCHCH₃). ¹³C-NMR (125 MHz, CDCl₃, diastereomer A): 167.6 (Pyr-C4), 165.7 (2C, CONH₂), 158.5 (2C, MeOPh-C4), 150.5 (2C, Pyr-C2 & -C6), 144.7 (Ph-C1), 135.8 (2C, MeOPh-C1), 130.0 (2C, MeOPh-C2 & -C6), 130.0 (2C, MeOPh-C2 & -C6), 128.1 (2C, Ph-C3 & -C5), 127.8 (2C, Ph-C2 & -C6), 126.8 (Ph-C4), 117.7 (CN), 113.1 (4C, MeOPh-C3 & -C5), 111.9 (2C, Pyr-C3 & -C5), 86.2 (Ar₃C), 71.1 (d, *J* = 16.1, CH₂CH(OP)CH₂), 69.3 (d, *J* = 2.2, PyrOCH₂), 63.1 (d, *J* = 3.8, DMTrOCH₂), 58.3 (d, *J* = 18.4, POCH₂), 55.2 (2C, OCH₃), 43.2 (d, *J* = 12.6, 2C, NCHCH₃), 24.6 (2C, NCHCH₃), 24.5 (2C, NCHCH₃), 20.3 (d, *J* = 6.9, CH₂CN). ¹³C-NMR (125 MHz, CDCl₃, diastereomer B): 167.7 (Pyr-C4), 165.6 (2C, CONH₂), 158.5 (2C, MeOPh-C4), 150.5 (2C, Pyr-C2 & -C6), 144.7 (Ph-C1), 135.9 (MeOPh-C1), 135.8 (MeOPh-C1), 130.1 (4C, MeOPh-C2 & -C6), 128.1 (2C, Ph-C3 & -C5), 127.9 (2C, Ph-C2 & -C6), 126.9 (Ph-C4), 117.5 (CN), 113.1 (4C, MeOPh-C3 & -C5), 111.8 (2C, Pyr-C3 & -C5), 86.3 (Ar₃C), 71.4 (d, *J* = 16.8, CH₂CH(OP)CH₂), 69.5 (d, *J* = 4.7, PyrOCH₂), 63.0 (d, *J* = 3.1, DMTrOCH₂), 58.3 (d, *J* = 18.9, POCH₂), 55.2 (2C, OCH₃), 43.2 (d, *J* = 12.5, 2C, NCHCH₃), 24.7 (2C, NCHCH₃), 24.6 (2C, NCHCH₃), 20.2 (d, *J* = 7.1, CH₂CN). ³¹P-NMR (202 MHz, CDCl₃, diastereomer A): 149.8. ³¹P-NMR (202 MHz, CDCl₃, diastereomer B): 150.1. HR-ESI-MS: 780.3130 ([M + Na]⁺, C₄₀H₄₈N₅NaO₈P⁺; calc. 780.3138).

(*R*)-4-(2,3-Dihydroxypropoxy)pyridine-2,6-dicarboxamide (**5**). Compound **2** (0.50 g, 1.41 mmol) was dissolved in a mixture of 37% aq. HCl (0.50 mL), MeCN (5.0 mL) and water (5.0 mL). The mixture was stirred at room temperature for 16 h, after which it was evaporated to dryness. The

residue was dissolved in 7 M methanolic ammonia and the mixture stirred at room temperature for 16 h, after which all volatiles were removed in vacuum. Yield 0.36 g (near quantitative). ^1H NMR (500 MHz, (D_6) DMSO): 8.85 (s, 2H, NH_2), 7.71 (s, 2H, NH_2), 7.67 (s, 2H, Pyr-H3 & -H5), 5.09 (s, 1H, $\text{CH}_2\text{CH}(\text{OH})\text{CH}_2$), 4.76 (s, 1H, CH_2OH), 4.25 (dd, 1H, $J = 10.2, 3.0$, PyrOCH_2), 4.09 (dd, 1H, $J = 9.0, 6.6$, PyrOCH_2), 3.84 (m, H, $\text{CH}_2\text{CH}(\text{OH})\text{CH}_2$), 3.47 (m, 2H, HOCH_2). ^{13}C NMR (125 MHz, (D_6) DMSO): 167.8 (Pyr-C4), 165.7 (2C, CONH_2), 151.6 (2C, Pyr-C2 & -C6), 110.7 (2C, Pyr-C3 & -C5), 70.9 (PyrOCH_2), 70.1 ($\text{CH}_2\text{CH}(\text{OH})\text{CH}_2$), 62.8 (HOCH_2). HR-ESI-MS: 278.0742 ($[M + \text{Na}]^+$, $\text{C}_{10}\text{H}_{13}\text{N}_3\text{NaO}_5^+$; calc. 278.0747).

General procedure for preparation of solid supports for the synthesis of GNA oligonucleotides. An appropriately protected GNA counterpart of a 2'-deoxynucleoside (0.15 mmol) was dissolved in anhydrous pyridine (8.0 mL). Succinic anhydride (0.60 mmol) and DMAP (cat.) were added and the resulting mixture stirred at room temperature for 16 h, after which it was evaporated to dryness. The residue was dissolved in CH_2Cl_2 (40 mL) and washed with saturated aq. NaHCO_3 (40 mL). The organic layer was dried with Na_2SO_4 and evaporated to dryness. An approximately 20 μmol portion of the crude product thus obtained was dissolved in anhydrous DMF (0.50 mL). HBTU (7.6 mg, 20 μmol) and DIPEA (7.0 μL , 41 μmol) were added and the resulting solution transferred to a microcentrifuge tube containing long chain alkylamine-CPG (100 mg). The tube was shaken at room temperature for 24 h, after which the CPG was transferred to an oligonucleotide synthesizer column and washed with anhydrous DMF (15 mL) and MeCN (15 mL). The unreacted amino groups of the CPG support were acetylated by treatment with a mixture of 2,6-lutidine (0.20 mL), Ac_2O (0.20 mL), *N*-methylimidazole (0.32 mL) and THF (3.28 mL) at room temperature for 3 min. Finally, the support was washed with anhydrous MeCN (15 mL) and CH_2Cl_2 (15 mL).

For quantification of its loading, 5.0 mg of the CPG support was placed in a microcentrifuge tube and 3% solution of dichloroacetic acid in CH_2Cl_2 (1.0 mL) was added. The resulting orange solution was separated and the above treatment repeated until the acidic solution remained colourless. The aliquots were then combined and diluted to 25.0 mL with CH_2Cl_2 . The absorbance of

this solution was then measured at 503 nm and the loading calculated based on the known molar absorptivity of $0.076 \text{ L } \mu\text{mol}^{-1} \text{ cm}^{-1}$. The loadings obtained by this method were 46.1, 48.6, 41.9 and $38.9 \mu\text{mol g}^{-1}$ for A, C, G and T, respectively.

Oligonucleotide synthesis. The oligonucleotides were assembled by an automated synthesizer on CPG supports prepared as described above. Conventional phosphoramidite strategy with 5-benzylthio-1H-tetrazole as the activator and 300 s coupling time was used throughout the sequences. Based on the trityl response, the coupling yields for the modified building block **1** and the canonical building blocks were 44% and more than 98%, respectively. Oligonucleotides **ON1** and **ON2** were released from the supports and the phosphate and base protections removed by a 35 min treatment with 16% aq. ammonia at 55 °C. In the case of **ON3**, 20% aq. methylamine was used instead. To prevent nucleophilic attack of 3'-OH on the vicinal phosphate during aminolysis, this step was carried out with the 3'-O-DMTr protection in place. This protecting group was finally removed by a 60 min treatment with 80% aq. acetic acid at room temperature.

The crude oligonucleotides were purified by RP-HPLC on a Thermo Scientific Aquasil C18 column ($150 \times 4 \text{ mm}$, $5 \mu\text{m}$), eluting with a mixture of acetonitrile (linear gradient of 0 to 30% over 30 min) and 0.10 M triethylammonium acetate. The flow rate was 1.0 mL min^{-1} and the detection wavelength 260 nm. The purified oligonucleotides were characterized by electrospray ionization mass spectrometry (ESI-MS, mass spectra presented as supporting information) and their concentrations estimated UV-spectrophotometrically based on absorbance at 260 nm. The molar absorptivities were calculated by an implementation of the nearest-neighbors method assuming the hypochromicities of GNA and DNA oligonucleotides to be equal.[24, 25]

*Preparation of the Pd²⁺-carrying oligonucleotide **ON1**₂:Pd₂.* A 215 μM aq. solution of oligonucleotide **ON1** (300 μL , 64.5 nmol) was lyophilized and the residue dissolved in 2.0 mM aq. solution of K_2PdCl_4 (48.4 μL , 96.8 nmol). After vigorous vortexing, the mixture was incubated at room temperature for 16 h. Phosphate buffer (48.4 μL , 120 mM, pH 7.2) was added and the mixture

incubated at room temperature for another 12 h. The product mixture was fractionated by RP HPLC on a Thermo Scientific Aquasil C18 column (150 × 4 mm, 5 μm), eluting with a mixture of acetonitrile (linear gradient of 0 to 30% over 30 min) and 60 mM phosphate buffer (pH 7.2). The flow rate was 1.0 mL min⁻¹ and the detection wavelength 260 nm. The main fraction, eluting at 12.5 min, was collected and desalted by RP HPLC on the same column, eluting with a mixture of acetonitrile (0% for the first 6 min, followed by a linear gradient to 60% over 0.5 min and isocratic elution at 60% for 9 min) and water. Formation of the desired structure **ON1**₂:Pd₂ was verified by ESI-MS (mass spectrum presented as supporting information).

*Preparation of the Pd²⁺-carrying oligonucleotide **ON2**:Pd:**ON3**.* A 399 μM aq. solution of oligonucleotide **ON2** (300 μL, 119.7 nmol) was lyophilized and the residue dissolved in 2.0 mM aq. solution of K₂PdCl₄ (65.8 μL, 131.6 nmol). After vigorous vortexing, the mixture was incubated at room temperature for 16 h and then lyophilized. The residue was dissolved in a 280 μM aq. solution of oligonucleotide **ON3** (427.5 μL, 119.0 nmol) and the resulting solution diluted with phosphate buffer (427.5 μL, 120 mM, pH 7.2). After incubation for 1 h at room temperature, the product mixture was fractionated by RP HPLC under the same conditions as described above for **ON1**₂:Pd₂. The main fraction, eluting at 10.4 min, was collected and used as such in the hybridization studies. Attempts to desalt **ON2**:Pd:**ON3** were unsuccessful. For characterization, a small sample was treated as described above for **ON1**₂:Pd₂ and analyzed by ESI-MS (data presented as supporting information). Oligonucleotides **ON2**, **ON3** and **ON2**:Pd were detected, consistent with formation of the desired structure **ON2**:Pd:**ON3**.

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ACCEPTED MANUSCRIPT

Table 1 Structures of the GNA oligonucleotides used in this study.

Scheme 1 Synthesis of the modified GNA phosphoramidite building block **1**. Reagents and conditions: (a) K_2CO_3 , DMF; (b) HCl, MeCN, H_2O ; (c) DMTrCl, pyridine; (d) 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite, Et_3N , CH_2Cl_2 ; (e) NH_3 , MeOH.

Scheme 2 Preparation of solid supports for the synthesis of GNA oligonucleotides. Reagents and conditions: (a) succinic anhydride, DMAP, pyridine; (b) LCAA-CPG, HBTU, DIPEA, DMF.

Scheme 3 Synthesis of the unprotected monomer **5**. Reagents and conditions: (a) HCl, MeCN, H_2O ; (b) NH_3 , MeOH.

Figure 1 HPLC traces for (A) purified **ON1** and (B) product mixture of the reaction of **ON1** with 1.5 eq. of K_2PdCl_4 ; Thermo Scientific Aquasil C18 column (150×4 mm, $5 \mu m$); flow rate = 1.0 mL min^{-1} ; linear gradient (0 to 30% over 30 min) of MeCN in 60 mM phosphate buffer (pH = 7.2).

Figure 2 (A) Proposed structure of **ON1**₂:Pd₂ and (B) calculated and observed mass spectra for its tetraanion.

Figure 3 HPLC traces for (A) purified **ON2**, (B) purified **ON3**, (C) product mixture of the reaction of **ON2** and 1.1 eq. of K_2PdCl_4 , followed by 1.0 eq. of **ON3** and (D) reinjection of the faster-eluting peak

of chromatogram C; Thermo Scientific Aquasil C18 column (150 × 4 mm, 5 μm); flow rate = 1.0 mL min⁻¹; linear gradient (0 to 30% over 30 min) of MeCN in 60 mM phosphate buffer (pH = 7.2).

Figure 4 UV melting profiles for (A) **ON1₂** (●), **ON1₂:Pd₂** (□) and **ON1₂** with 2.0 eq. of K₂PdCl₄ added *in situ* (△) and (B) **ON2:ON3** (●) and **ON2:Pd:ON3** (□); [ON1] = 6.0 μM; [ON2] = [ON3] = 3.0 μM; [Pd²⁺] = 0 / 3.0 / 6.0 μM; I(NaClO₄) = 0.10 M; pH = 7.4.

Figure 5 CD spectra of oligonucleotide duplexes (A) **ON1₂** and (B) **ON1₂:Pd₂**, recorded at 10 °C intervals between 10 and 90 °C; [ON1₂] = [ON1₂:Pd₂] = 3.0 μM; pH = 7.4 (20 mM cacodylate buffer); I(NaClO₄) = 0.10 M.

Figure 6 CD spectra of oligonucleotide duplexes (A) **ON2:ON3** and (B) **ON2:Pd:ON3**, recorded at 10 °C intervals between 10 and 90 °C; [ON2:ON3] = [ON2:Pd:ON3] = 3.0 μM; pH = 7.2 (20 mM phosphate buffer); I(NaClO₄) = 0.10 M.

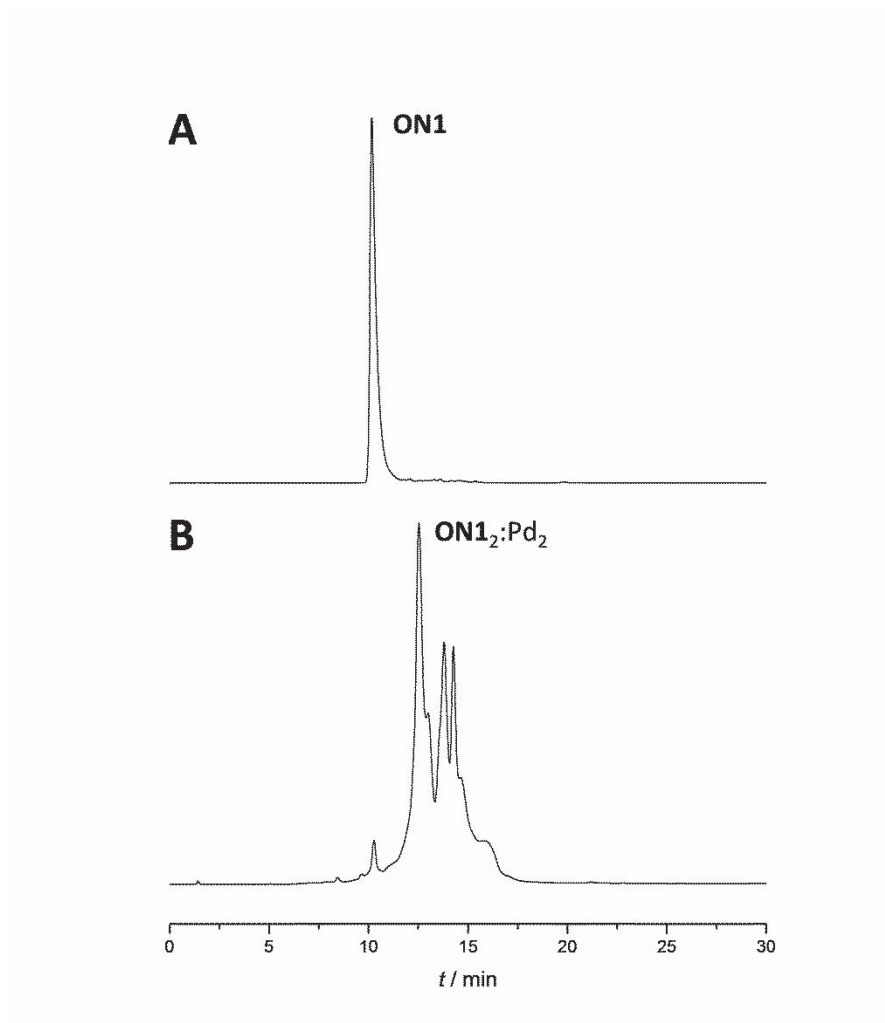


Figure 1

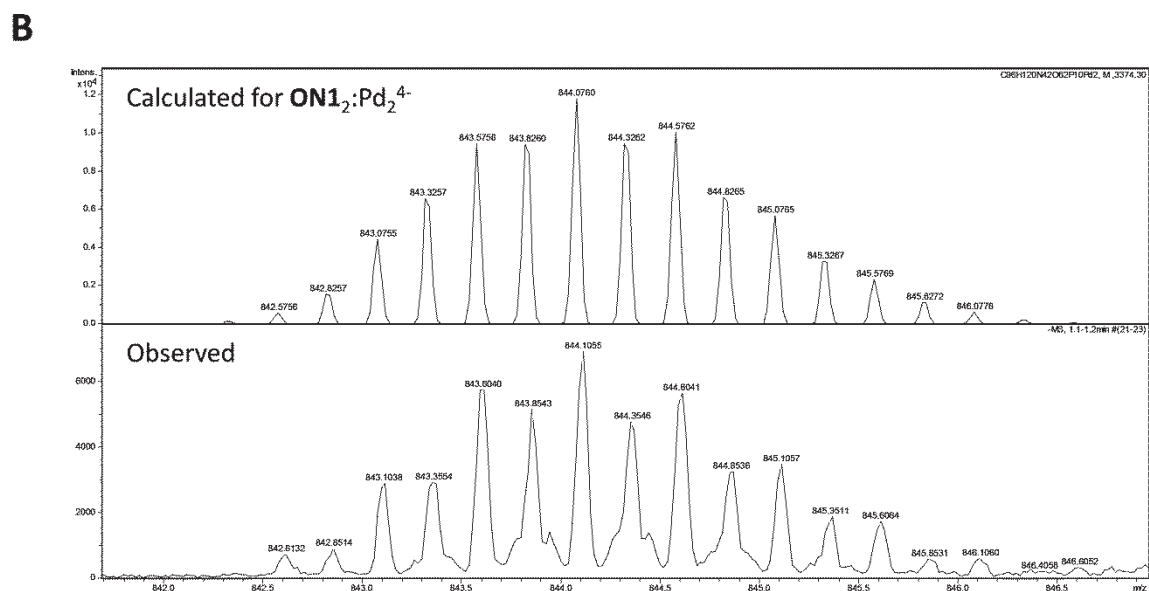
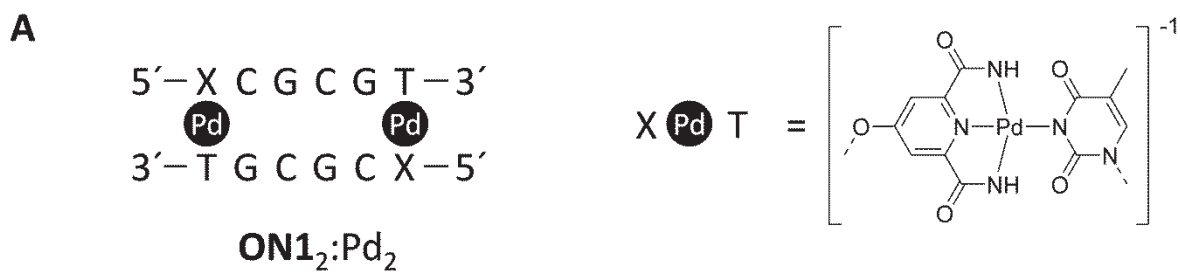


Figure 2

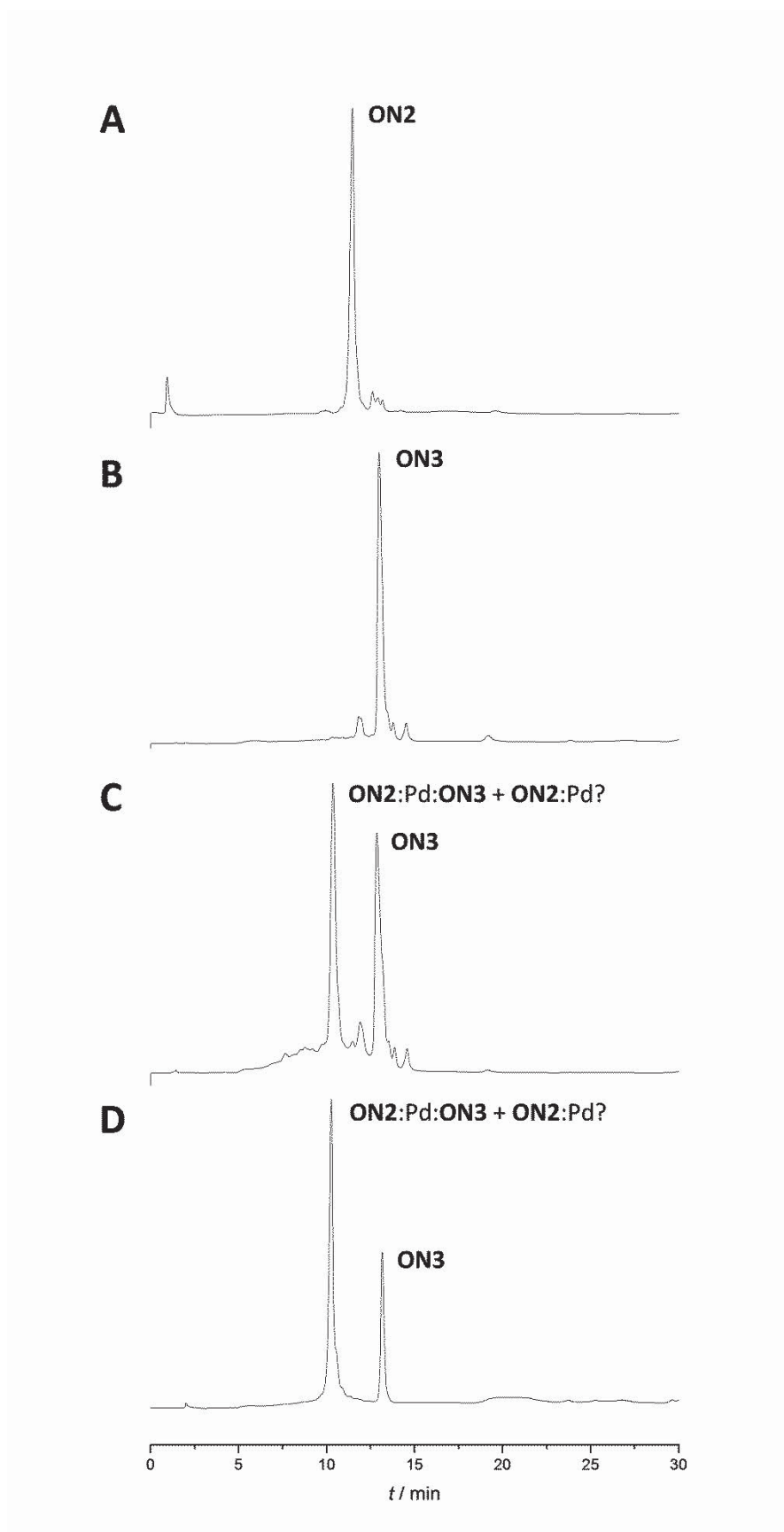


Figure 3

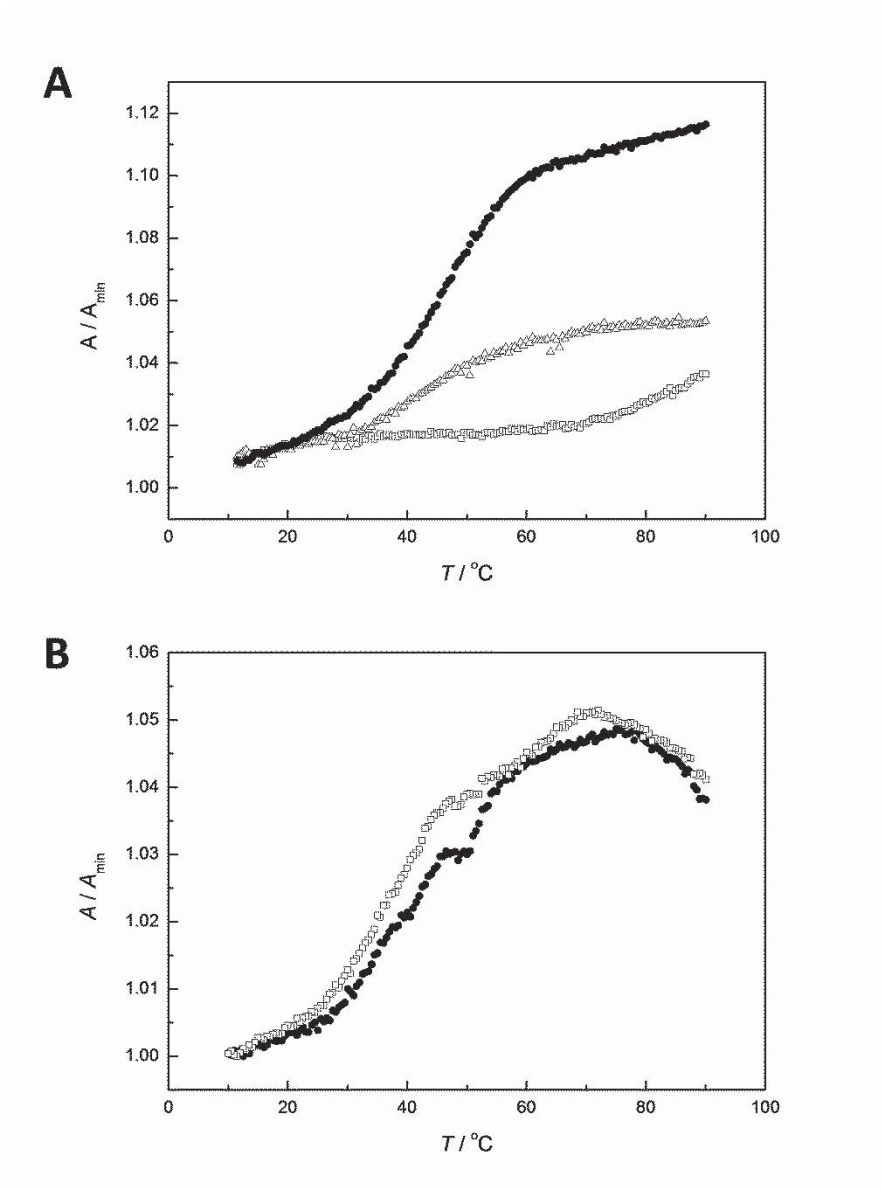


Figure 4

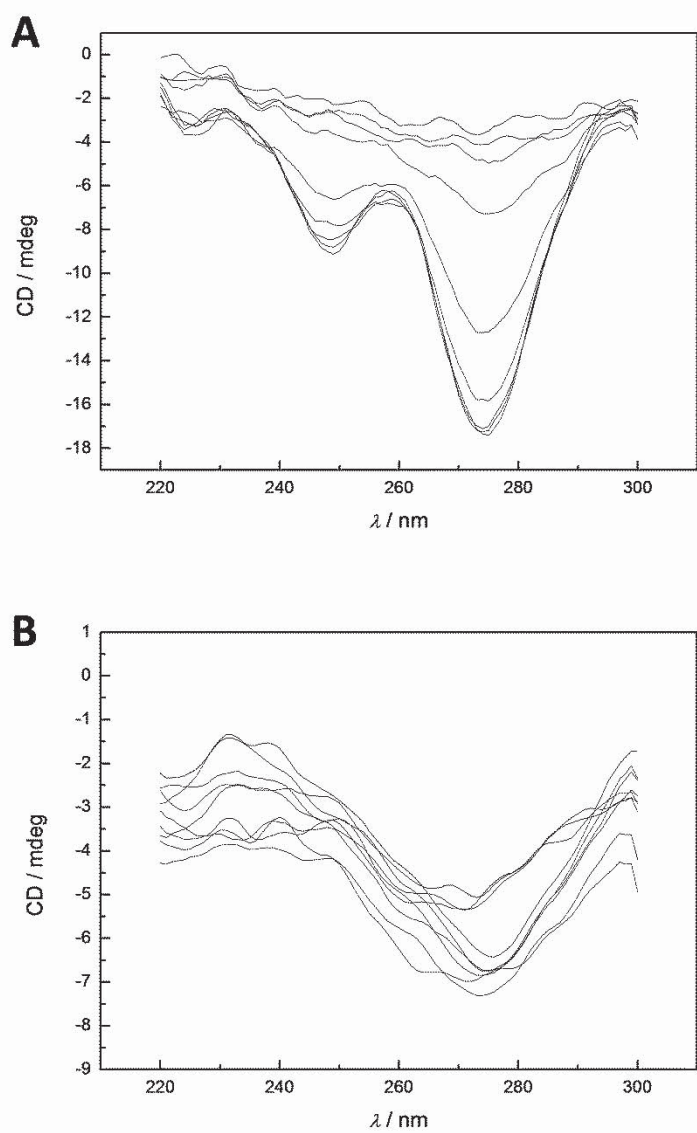


Figure 5

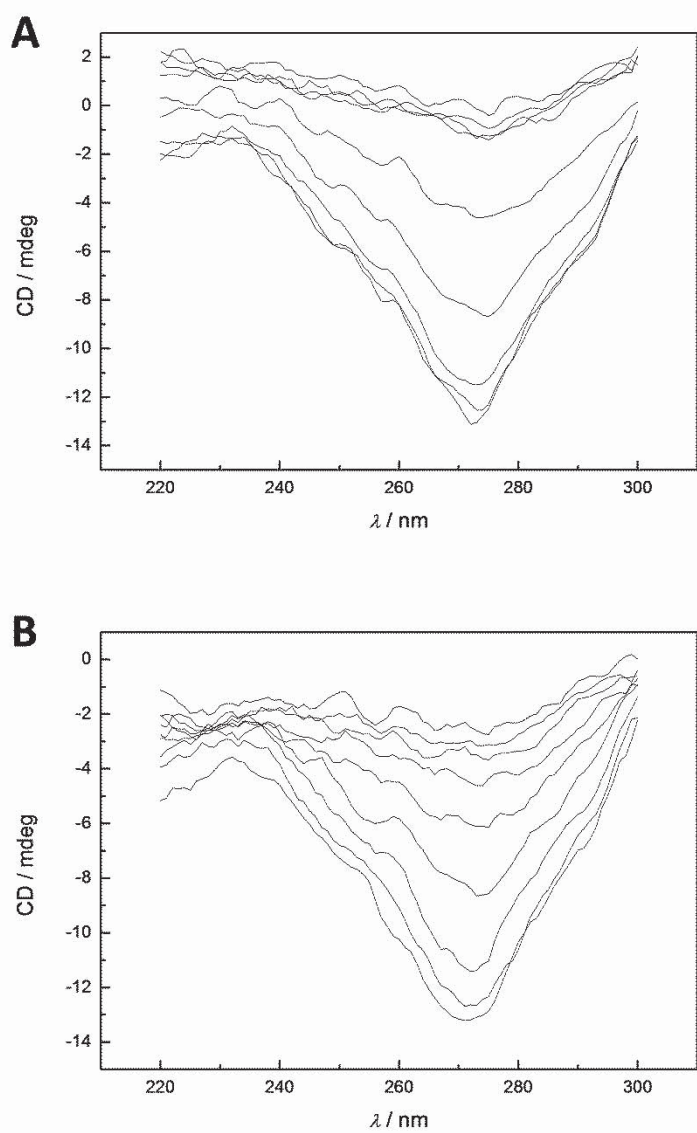
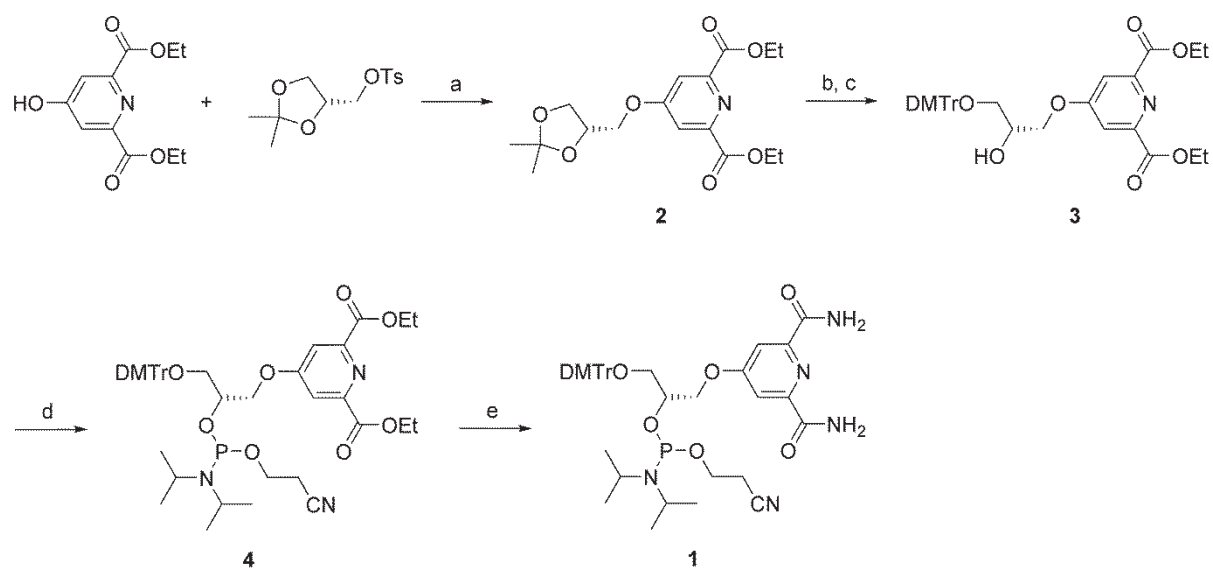
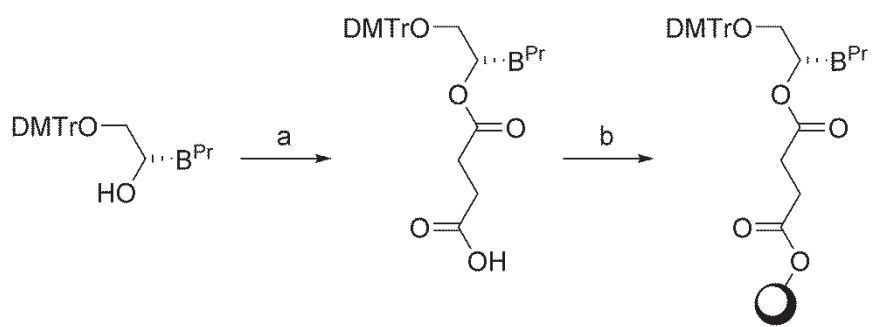


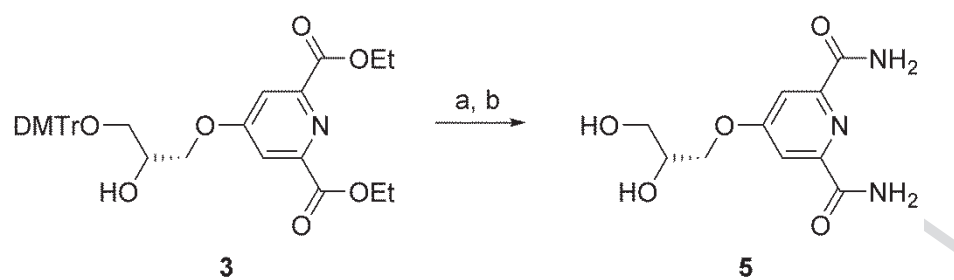
Figure 6



Scheme 1

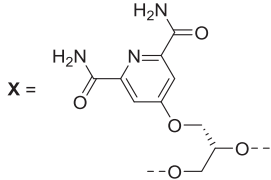


Scheme 2

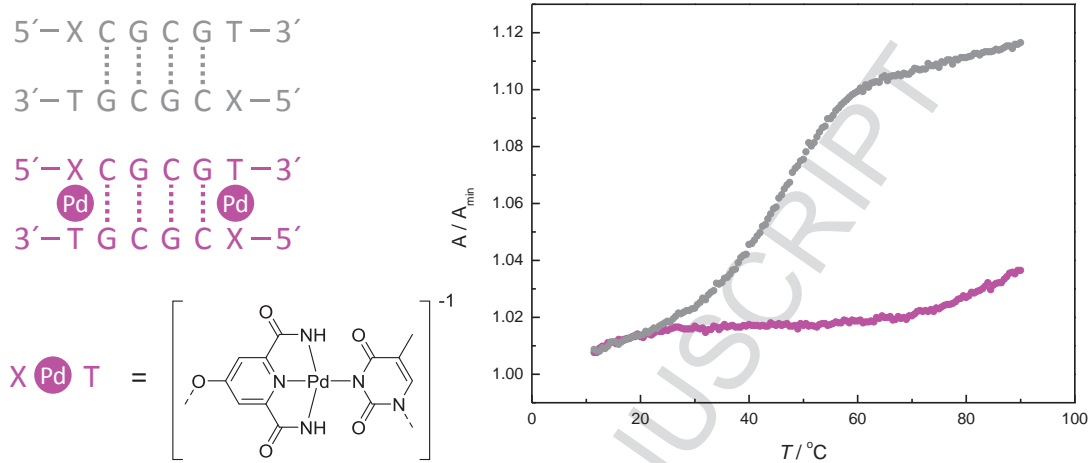


Scheme 3

Table 1

| | Sequence | |
|------------|---------------|---|
| ON1 | 5'-XCGCGT-3' |  |
| ON2 | 5'-CGCXGGC-3' | |
| ON3 | 5'-GCCTGCG-5' | |

Graphical abstract



Terminal Pd^{2+} -mediated base pairs between pyridine-2,6-dicarboxamide and thymine greatly enhance the thermal stability of short GNA oligonucleotides relative to their metal-free counterparts. The effect was only observed with metallated oligonucleotides isolated prior to the hybridization studies, not when Pd^{2+} salt was added to the oligonucleotide solution *in situ*.

HIGHLIGHTS

- Pd²⁺-mediated base-pairing within oligonucleotides has been demonstrated.
- At the termini of a duplex, Pd²⁺-mediated base-pairing is highly stabilizing.
- Mixing dilute oligonucleotide and Pd²⁺ solutions may not afford the desired structure.

ACCEPTED MANUSCRIPT