Synthesis of oligodeoxynucleotides containing 6-*N*-([^{13}C]methyl)adenine and 2-*N*-([^{13}C]methyl)guanine

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Oligonucleotides containing 6-*N*-([^{13}C]methyl)adenine and 2-*N*-([^{13}C]methyl)guanine have been prepared for NMR studies using the deprotection step to introduce the [^{13}C]methylamine group. For this purpose, the use of 2′-deoxy-6-*O*-(pentafluorophenyl)inosine 1 and 2′-deoxy-2-fluoro-6-*O*-[2-(4-nitrophenyl)-ethyl]inosine 2 as precursors of the *N*-methylated nucleosides is described. Preliminary NMR characterization of the ^{13}C-labelled oligonucleotides shows that the ^{13}C chemical shift of the methyl group in *N*-methylguanine is sensitive to duplex formation, making it a useful local probe.

Introduction

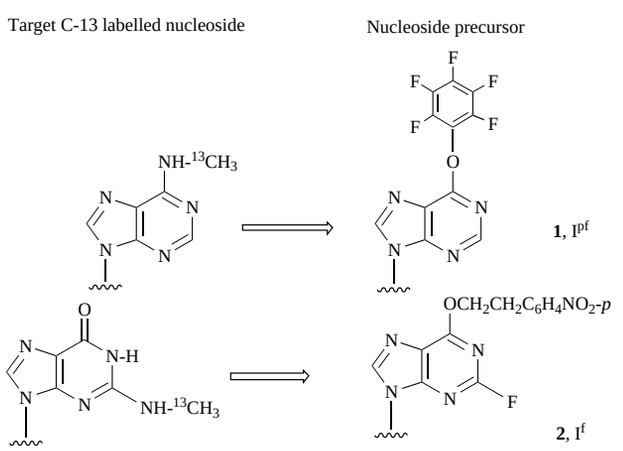
One of the problems found during the structural elucidation of DNA–protein interactions is the complexity of the spectra. The introduction of ^{15}N and ^{13}C in combination with special pulse sequences allows the selective observation of specific base pairs.[1] Although a large effort has been devoted to the preparation of oligodeoxynucleotides having ^{15}N labels,[1] there are few examples of ^{13}C-labelled oligodeoxynucleotides at specific sites. A thymidine phosphoramidite labelled at C-6 with ^{13}C has been prepared and incorporated into a synthetic hairpin oligonucleotide.[2] NMR relaxation measurements showed a higher internal motion in the loop region than in the duplex.[2,3] Also, oligonucleotides labelled with ^{13}C at the thymine methyl groups have been prepared to study hydrophobic contacts between DNA and the DNA-binding domain of the rat glucocorticoid receptor.[4] The analysis revealed a hydrophobic contact between a labelled thymine group and the methyl groups of a valine residue. Base-pairing properties of 2′-deoxy-5-(methoxymethyl)-uridine have also been studied by NMR spectroscopy using a heptanucleotide containing a ^{13}C label at the exocyclic position of this analogue.[5]

Recently, the development of nucleoside derivatives that can be transformed into different nucleoside analogues during deprotection has been described.[6–10] This method, named 'the convertible nucleoside approach,' has been used to prepare oligodeoxynucleotides containing ^{15}N labels at exocyclic positions.[10–12] In the present communication we describe the preparation of oligodeoxynucleotides containing *N*-methyladenine and *N*-methylguanine residues with a ^{13}C label at the methylamino exocyclic position. The methodology described here uses a modified deprotection protocol to incorporate the ^{13}C label and to our knowledge is the first time that ^{13}C labels have been incorporated in the amino groups involved in base-pairing.

Results and discussion

Preparation of oligonucleotides containing N^6-([^{13}C]methyl)adenine

Previous reports have shown that 6-chloropurine and 6-fluoropurine 2′-deoxyriboside react with amines to produce adenine adducts at nucleoside[13–17] and oligonucleotide[18,19] levels. On the other hand, 6-*O*-aryl-dI derivatives[8,9,12] have been described for the preparation of oligonucleotides containing modified adenines. Recently, use of 6-(methylthio)purine has been described for the preparation of 6-substituted purines including 6-*N*-methyladenine.[20] We have selected 2′-deoxy-6-*O*-(pentafluorophenyl)inosine **1** (Scheme 1) as precursor of the



Scheme 1 Nucleoside derivatives used in this work for the preparation of oligonucleotides containing 6-*N*-([^{13}C]methyl)adenine and 2-*N*-([^{13}C]methyl)guanine

N-methyladenine residue because previous studies have shown that this derivative is stable to oligonucleotide synthesis conditions and reacts readily with amines even in the presence of water without detectable hydrolysis.[12]

2′-Deoxy-6-*O*-(pentafluorophenyl)inosine [12] **1** was prepared following previously described protocols. Overnight treatment of 2′-deoxy-6-*O*-(pentafluorophenyl)inosine with aq. methylamine (prepared by addition of triethylamine to aq. CH_3NH_2·HCl) at 60 °C yielded the desired 2′-deoxy-*N*-methyladenosine as the only nucleoside product that was isolated and characterized.

When amines are used for the deprotection of synthetic oligonucleotides, approximately 10–20% of the *N*-benzoylcytidine residues react with the amine, yielding the corresponding 4-*N*-alkylcytidine[6] derivatives. This side reaction can be minimized by use of other protected derivatives of cytidine.[21,22] Among them, *tert*-butylphenoxyacetyl[23] (Expedite™) protective groups were selected. A short oligonucleotide containing 3 cytosines

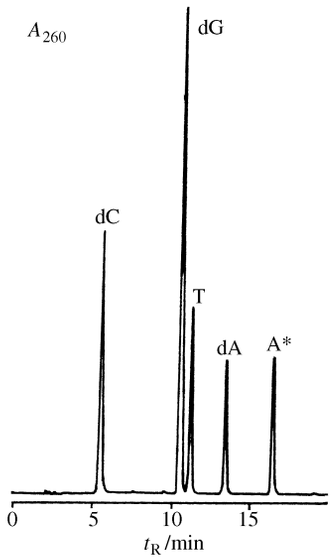


Fig. 1 Enzymic digestion of purified 6-*N*-([^{13}C]methyl)adenine dodecamer {5′-GCGAA*TTCGCGC-3′ where A* is 6-*N*-([^{13}C]-methyl)adenine}

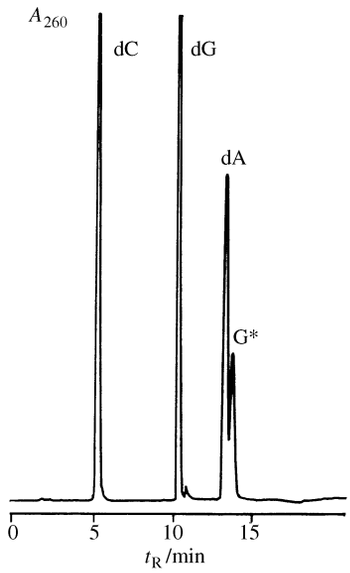


Fig. 2 Enzymic digestion of purified 2-*N*-([^{13}C]methyl)guanine dodecamer {5′-CACCG*ACGGCGC-3′ where G* is 2-*N*-([^{13}C]-methyl)guanine}

(5′-CCCAT-3′) was prepared using *tert*-butylphenoxyacetyl phosphoramidites. At the end of the synthesis, the support was treated overnight at 60 °C with aq. methylamine (prepared by addition of triethylamine to an aq. CH_3NH_2·HCl). The resulting oligonucleotide was purified by HPLC and analysed by enzyme digestion. Only dC, dA and T were found. The product resulting from the attack of methylamine on protected cytidine, 4-*N*-methyl-dC, was not detected. An authentic sample of 4-*N*-methyl-dC was prepared from 4-*O*-ethyl-dU to determine the position at which 4-*N*-methyl-dC elutes.

The dodecamer having the recognition sequence of *E. coli* methylase (5′-GCGAI^{pf}TTCGCGC-3′ with I^{pf} being 6-O-pentafluorophenyl-dI) was prepared using *tert*-butylphenoxyacetyl and 6-*O*-pentafluorophenyl-dI [12] phosphoramidites. Treatment of the support with aq. [^{13}C]methylamine overnight at 60 °C yielded a main product that was purified by HPLC using standard protocols. We used 250 mg of [^{13}C]methylamine hydrochloride per 2 μmols of oligonucleotide, which is approximately 90 times the amount needed to remove the protecting groups and perform the substitution. Enzyme digestion showed the presence of the four natural nucleosides together with 6-*N*-methyl-dA in the expected proportions (see Fig. 1).

Preparation of oligonucleotides containing 2-*N*-([^{13}C]methyl)guanine

For the introduction of ^{13}C label at position 2 of guanine, 2′-deoxy-2-fluoroinosine was selected. This nucleoside and its protected derivatives have been used as precursors of 2-amino substituted guanine derivatives at the nucleoside and oligonucleotide level.[13,14,19,24–27] 2′-Deoxy-2-fluoro-6-*O*-[2-(4-nitrophenyl)ethyl (NPE)]inosine [12] **2** (Scheme 1) was prepared as previously described. Treatment of compound **2** with aq. methylamine at 60 °C gave the desired 2-*N*-methyl-dG as the major product. Small amounts (less than 10%) of NPE-protected 2-*N*-methyl-dG were also observed, but the hydrolysis product (dG) was not detected. When treatment with methylamine was performed at room temperature for 16 h, two products were obtained: NPE-protected 2-*N*-methyl-dG and 2-*N*-methyl-dG, together with small amounts of starting material (data not shown). This indicates that the displacement of fluoride by methylamine is easier than the removal of the NPE group.

The dodecamer 5′-CACCI^{f}ACGGCGC-3′ (R5) [28] with I^{f} = 2-fluoro-6-*O*-NPE-dI, was prepared using *tert*-butylphenoxyacetyl and 2-fluoro-6-*O*-NPE-dI [12] phosphoramidites. After the assembly of the sequence, the support was treated with a 0.5 M solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) solution in acetonitrile at room temperature for 15 min to remove the NPE group. Under these conditions loss of oligonucleotide by cleavage of the succinyl bond is negligible [12,29] and the majority of the NPE groups at position 6 in the guanine are removed. Treatment of the support with aq. [^{13}C]methylamine overnight at 60 °C yielded a main product that was purified by HPLC using standard protocols. Enzyme digestion showed the presence of dC, dG and dA together with 2-*N*-methyl-dG in the expected proportions (Fig. 2). Moreover, mass spectral analysis of the purified product by matrix-isolated laser-desorption time-of-flight mass spectrometry (MALDI-TOF) gave the expected molecular mass (Found: M^+, 3631.2, expected *M*, 3631). In this oligonucleotide we used 63 mg of [^{13}C]methylamine hydrochloride per 4 μmols of oligonucleotide, which is 10 times the amount needed for removal of the protecting groups. The cost of the ^{13}C label in this preparation is lower than the cost of the chemicals needed to produce the oligonucleotide. The 1D proton spectrum at 600 MHz of the dodecamer revealed a homogeneous product with sharp signals. To confirm the labelled methyl group was on the guanine moiety a 2D heteronuclear multiple quantum filtered coherence (HMQC) [30] spectrum was recorded in which the carbon signals are indirectly detected *via* the proton resonances of the coupled spins. This inverse spectrum of the oligonucleotide showed very clearly two antiphase satellites only at a centred resonance frequency of δ[^{1}H] 2.58 split by a coupling constant of 137 Hz for the C–H coupling (data not shown).

A proton-decoupled carbon spectrum of R5 showed a carbon signal at δ[^{13}C] 34.37, which was assigned to the $^{13}CH_3$ group of 2-*N*-methyl-G (see Fig. 3), also confirmed by a gated decoupled spectrum (quadruplet at the same frequency). The change of the chemical shift of the $^{13}CH_3$ group of 2-*N*-methyl-G on comparing the spectrum of R5 dodecamer with the spectrum of the two annealed complementary oligonucleotides (δ[$^{13}CH_3$] = 32.84, $\Delta\delta$ 1.53 ppm) reveals a hybridization of the two strands.

In summary, we have shown that oligonucleotides carrying 6-*N*-([^{13}C]methyl)adenine and 2-*N*-([^{13}C]methyl)guanine bases can be easily prepared using the phosphoramidites of 6-*O*-pentafluorophenyl-dI and 2-fluoro-6-*O*-NPE-dI together with a special deprotection protocol using [^{13}C]methylamine. Under these conditions, oligonucleotides are deprotected and released from the solid support and the modified nucleoside is converted into the desired 6-*N*-([^{13}C]methyl)adenine and 2-*N*-([^{13}C]methyl)-

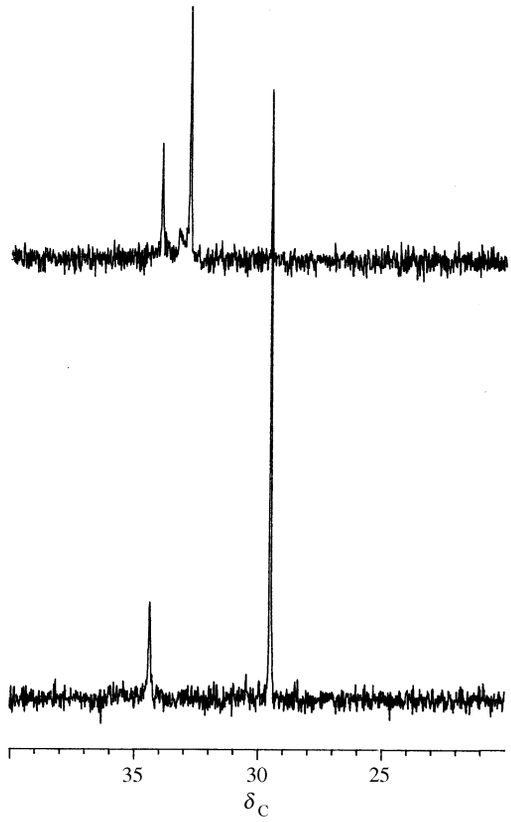


Fig. 3 ^{13}C NMR spectra of (a) dodecamer 5′-CACCG*ACGGCGC-3′ where G* is 2-*N*-([^{13}C]methyl)guanine and (b) duplex formed by 5′-CACCG*ACGGCGC-3′ and 3′-GTGGCTGCCGCG-5′. The peak at δ_C 29.5 is the methyl carbon of acetate used as a reference.

guanine residues. Modification of C residues described[6] for 4-*N*-benzoyl dC is avoided by using *tert*-butylphenoxyacetyl-protected phosphoramidites.[23] The method is simple and efficient and the introduction of the label is at the end of the synthetic process. The introduction of the methyl group does not disturb the formation of the duplex as shown in previous reports.[31,32] Preliminary NMR characterization showed that the $^{13}CH_3$ group in 2-*N*-([^{13}C]methyl)guanine moved upfield upon duplex formation indicating that this type of labelling could be used to monitor duplex formation as well as protein–DNA interactions.

Experimental

Abbreviations: ACN: acetonitrile, DCM: dichloromethane, DMT: 4,4′-dimethoxytrityl, dI^{f}: 2′-deoxy-2-fluoroinosine, dI^{pf}: 2′-deoxy-6-*O*-(pentafluorophenyl)inosine, pyr: pyridine. 2′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)-6-*O*-(pentafluorophenyl)-inosine 3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite,[12] 2′-deoxy-5′-O-(4,4′-dimethoxytrityl)-2-fluoro-6-*O*-[2-(4-nitrophenyl)ethoxycarbonyl]inosine 3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite[12] and 5′-*O*-(4,4-dimethoxytrityl)-4-O-ethylthymidine[33] were prepared as described. Expedite™ (*tert*-butylphenoxyacetyl) phosphoramidites were from PerSeptive Biosystems. 99% [^{13}C]Methylamine hydrochloride was purchased from Cambridge Isotope Laboratories, Inc.

HPLC conditions

In all cases solvent A was 20 mM triethylammonium acetate (pH 7.8) and solvent B was a 1:1 mixture of water and acetonitrile. For analytical runs the following conditions were used. Columns: Nucleosil 120C18, 250 × 4 mm, flow rate: 1 ml min^{-1} and Radial Pak C_{18}, flow rate 2 ml min^{-1}. (A) 5–95% B linear gradient in 40 min. (B) 5–50% B linear gradient in 20 min. For semipreparative runs the following conditions were used: Columns: Nucleosil 120C18, 250 × 10 mm and C_4-Vydac, 250 × 10 mm. Flow rate: 3 ml min^{-1}. A 20–80% B linear gradient in 30 min for oligonucleotides containing the DMT group and a 5–50% B linear gradient in 30 min for oligonucleotides without the DMT group.

NMR measurements

The R5 sample contained 4.6 mM (60 O.D. units at 260 nm) of the purifed product in 0.6 ml of D_2O at pH 6.8. The duplex sample consisted of 1.2 mM (16 O.D. units) of each dodecamer (R5 and its complementary) in 0.6 ml of D_2O at pH 6.8.

The 1D-proton and the HMQC spectra were recorded on a Bruker 600 MHz spectrometer, the chemical shifts were referenced to D_2O at δ 4.75. Carbon spectra were recorded on a Bruker AM 250 MHz spectrometer with proton decoupling. The spectra were recorded at 298 K, TD = 16K, with a spectral width of 14 000 Hz corresponding to 220 ppm and an acquisition time of 0.59 sec, zero filling to 64K points after Gaussian multiplication. The methyl carbon of the acetate at δ_C 29.5 ppm was used as internal reference in both samples.

Conversion of 2′-deoxy-6-*O*-(pentafluorophenyl)inosine into 2′-deoxy-6-*N*-methyladenosine

2′-Deoxy-6-*O*-(pentafluorophenyl)inosine[12] (0.37 mmol) was dissolved in 2 ml of water and methylamine hydrochloride (7.4 mmol) and triethylamine (7.4 mmol) were added. The solution was heated at 60 °C overnight. The resulting solution was concentrated to dryness and the residue was purified on silica gel column chromatography with a 5–10% MeOH gradient in DCM. Yield of product 0.2 g (52%). UV spectrum as previously described.[34] TLC (10% MeOH–DCM) R_f 0.26; HPLC (conditions A): one peak at t_R 16.2 min. The product migrated together with commercially available 2′-deoxy-6-*N*-methyladenosine (Sigma); δ_H($CDCl_3$; 200 MHz) 8.3 (1 H, s, H-2), 7.9 (1 H, s, H-8), 6.4 (1 H, m, H-1′), 6.3 (1 H, m, OH-3′), 5.1 (1 H, m, OH-5′), 4.4 (1 H, m, H-3′), 4.3 (1 H, m, H-4′), 3.9 (2 H, m, H_2-5′), 3.1 (3 H, s, NCH_3), 2.9 (1 H, m, H-2′) and 2.5 (1 H, m, H-2′); δ_C($CDCl_3$; 75 MHz) 155.3, 152.0, 146.0, 139.2, 122.1, 121.7, 89.3, 87.2, 72.1, 63.0, 40.4 and 13.3 (NCH_3).

Preparation of 2′-deoxy-4-*N*-methylcytidine

2′-Deoxy-5′-DMT-4-*O*-ethyluridine[33] (40 mg, 0.07 mmol) was treated with a solution of methylamine hydrochloride (3.6 mmol) and triethylamine (3.6 mmol) in water (2 ml). ACN was added until a clear solution was obtained. The solution was heated at 55 °C overnight and concentrated to dryness. The residue was treated with aq. 80% acetic acid for 15 min at room temperature. The solution was concentrated to dryness and the residue was purified on silica gel and eluted with a 10–30% MeOH gradient in DCM. Yield of product 17 mg (90%). Physical data and UV spectra as previously described.[31,35] TLC (20% MeOH–DCM) R_f 0.13; HPLC (conditions A): one peak at t_R 9.7 min; δ_H(CD_3OD; 200 MHz) 7.8 (1 H, d, *J* 6.3, H-6), 6.3 (1 H, t, *J* 6.4, H-1′), 6.0 (1 H, d, *J* 6.3, H-5), 4.4 (1 H, m, H-3′), 4.0 (1 H, m, H-4′), 3.8 (2 H, m, H_2-5′), 2.3 (2 H, m, H_2-2′) and 2.9 (3 H, s, NCH_3).

Conversion of 2′-deoxy-2-fluoro-6-*O*-[2-(4-nitrophenyl)ethyl]-inosine into 2′-deoxy-2-*N*-methylguanosine

2′-Deoxy-2-fluoro-6-*O*-[2-(4-nitrophenyl)ethyl]inosine[12] (150 mg, 0.35 mmol) was dissolved in 1 ml of 20% methylamine (5 mmol) in water. The solution was incubated at 60 °C overnight. The resulting solution was evaporated to dryness. The residue was dissolved in 5% MeOH in DCM containing 1% triethylamine and was purified on a silica gel column eluted with a 10–60% MeOH gradient in DCM containing 1% triethylamine. Fractions containing 2′-deoxy-2-*N*-methylguanosine (eluted between 40 and 60% MeOH) were pooled and evaporated to dryness to yield the title compound (40 mg, 40%). Physical and chromatographic data as described previously.[36] TLC (30%

MeOH in DCM) R_f 0.41; HPLC (conditions A): one peak at t_R 13.6 min; δ_C[$(CD_3)_2SO$; 250 MHz] 156.9 (C-6), 153.2 (C-2), 150.5 (C-4), 135.7 (C-8), 116.6 (C-5), 87.5 (C-4′), 82.6 (C-1′), 70.6 (C-3′), 61.8 (C-5′), 39.5 (C-2′) and 27.4 (CH_3).

Oligonucleotide syntheses

The sequences A: 5′-CCCAT-3′, B: 5′-GCGAI^{pf}TTCGCGC-3′ and C: 5′-CACCIfACGGCGC-3′ were prepared on an Applied Biosystems automatic DNA synthesizer using *tert*-butylphenoxyacetyl 2-cyanoethyl phosphoramidites and the modified phosphoramidite of 2′-deoxy-5′-*O*-DMT-6-*O*-(pentafluorophenyl)inosine. Sequence A was prepared on a 1 μmol scale without the DMT at the end, sequence B on a 2 μmol scale without DMT, and sequence C on a 4 μmol scale with the last DMT group.

Oligonucleotide supports were deprotected with aq. methylamine overnight at 60 °C. Cold methylamine hydrochloride was used for sequence A and [^{13}C]methylamine hydrochloride was used for sequences B and C.

For sequences A and B the following protocol was used: Approx. 50–100 mol equiv. of methylamine hydrochloride were dissolved in water (50 mg of CH_3NH_2·HCl in 0.5 ml for sequence A and 250 mg of [^{13}C]CH_3NH_2·HCl in 1 ml for sequence B) and the solution was added to the support followed by an equimolar amount of triethylamine with respect the amount of CH_3NH_2·HCl (75 mg for sequence A and 374 mg for sequence B). The reaction mixtures were kept overnight in a screw-cap vial tightly closed inside the oven at 60 °C.

For sequence C the following protocol was used: The DMT-oligonucleotide support was treated with 0.5 M DBU in ACN at room temperature for 15 min. Afterwards, the support was washed successively with ACN, 1% Et_3N solution in ACN (to remove DBU salts) and again with acetonitrile and was dried under vacuum. 10 Mol equiv. of [^{13}C]CH_3NH_2·HCl (0.92 mmol, 63 mg) were dissolved in 0.5 ml of water, and 0.13 ml of Et_3N (0.92 mmol) were added. This solution was added to the oligonucleotide support and the mixture was treated at 60 °C for 16 h. After the treatment, the solutions were concentrated to dryness and the products were purified by reversed-phase HPLC (see conditions above). All syntheses presented a major peak that was collected and analysed by snake venom phosphodiesterase and alkaline phosphatase digestion followed by HPLC analysis of the nucleosides (conditions B, see above; and Figs. 1 and 2). Yield (O.D. units at 260 nm) : Pentamer A (1 μmol synthesis): 35 O.D.; dodecamer B (2 μmol): 60 O.D.; dodecamer C (4 μmol) : 170 O.D. Mass determination of the HPLC-purified product using MALDI showed a molecular peak at M^+, 3631.18 as expected for the labelled oligonucleotide (expected mass 3631 amu).

The complementary strand of R5 (5′-GCGCCGTCGGTG-3′) was synthesized on a 1 μmol scale using standard protocols and the last DMT group was left on during deprotection with ammonia. This oligonucleotide group was purified from truncated sequences using COP™ (Cruachem Ltd., Scotland) cartridges.

In order to avoid the triethylammonium signals in the NMR spectrum purified oligonucleotides were passed through a Dowex 50W (Na^+-form) column. Remaining acetate ions were used as NMR reference. An amount of 16 O.D. units of each strand were annealed at 65 °C and cooled slowly to form the duplex before the carbon spectrum was recorded.

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