Uncharged stereoregular nucleic acid analogs: 2. Morpholino nucleoside oligomers with carbamate internucleoside linkages

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ABSTRACT

A novel oligonucleotide analog has been prepared from ribonucleoside derived morpholine subunits linked by carbamate groups. Oxidative cleavage of the 2′,3′ vicinal diol of cytidine followed by reductive amination of the resulting dialdehyde afforded the morpholine subunit. Coupling of the subunits are through carbamate moieties and the oligomers were characterized by ^{1}H NMR and FAB MS. Evidence for interaction of the hexamer *19* with p(dG_6) was found, but an atypical interaction of *19* with a RNA target was observed.

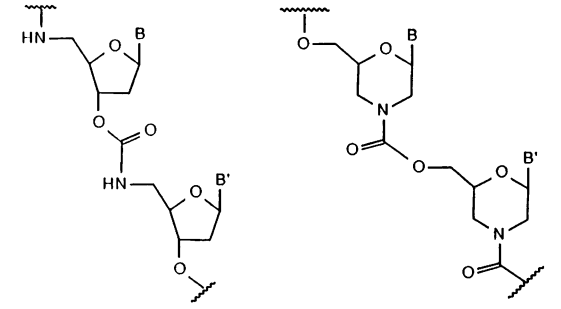
INTRODUCTION

Oligonucleotide analogs, containing wholly or substantially nonionic backbones, are potential agents for the treatment of viral diseases and cancers, and for the study of genetic mechanisms[1]. Several structural criteria have been postulated as necessary for effective use of oligonucleotide analogs in these applications[1a], including proper spacing and orientation of the bases to allow hybridization, resistance to enzymatic degradation[2], and possibly a stereoregular backbone for homogeneous binding of the analog to a complementary nucleic acid sequence[3]. Additionally, the avidity of binding of the analog to its target must be sufficient for effecting the desired biological action.

Examples of nonionic nucleic acid analogs have been reported[4-9]. In most cases, these are obtained by simple replacement of the nucleic acid phosphodiester linkage by an uncharged phosphorus species; the most studied examples are the methanephosphonates and phosphoramidates. We have pursued a program of developing novel oligonucleotide analogs wherein both the sugar portion and the intersubunit linkages of the backbone have been modified. Representative of this approach are oligomers of carbamate linked, 5′-amino-2′,5′-dideoxyribonucleosides (*1*). The morpholine nucleoside[10] *2* is an even more highly modified nucleoside analog. Molecular modeling suggested that carbamate oligomers of this species would be capable of binding nucleic acid complements. The use of *2* is attractive in that the preparation of the subunit is rapid and inexpensive while coupling of the subunits with a carbamate group would follow our established protocol[9b]. We therefore examined the synthesis and physical properties of an oligomer of *2* to validate the accuracy of this analysis and report our findings in this paper.

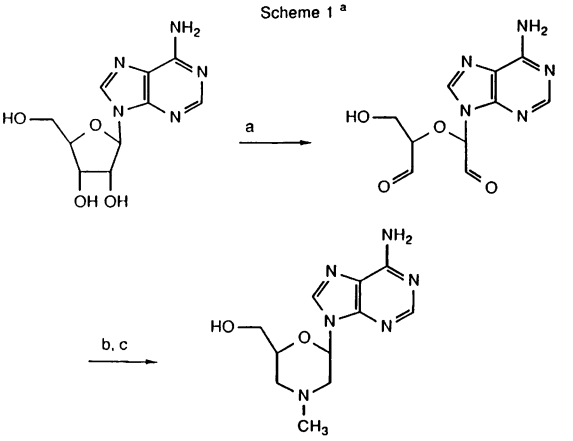
RESULTS AND DISCUSSION

Our initial goal was the efficient preparation of the morpholine monomer of cytosine. Khym[11a], and later Read and Brown[11b], developed a two step procedure for the preparation of morpholine nucleosides based upon ribonucleoside cleavage with sodium



1 2

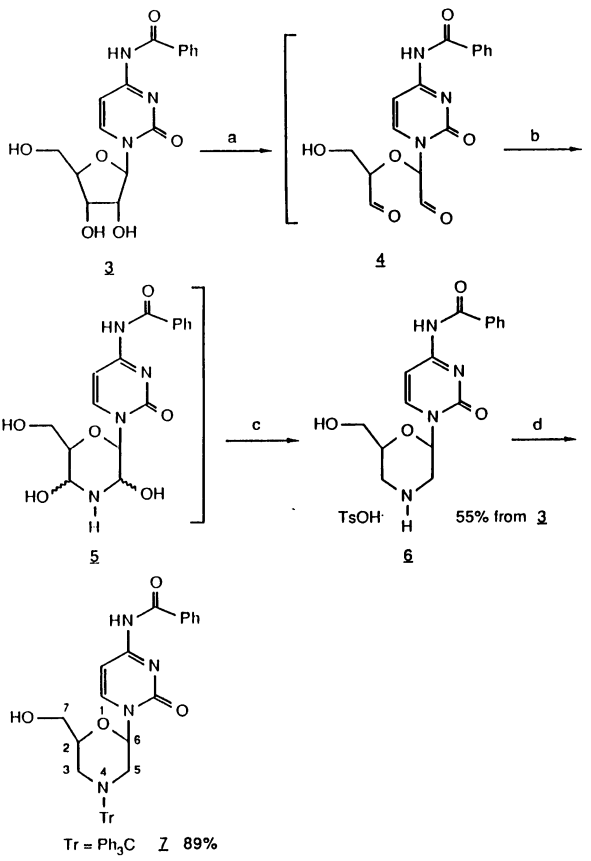
Scheme 1 [a]



[a]a) $NaIO_4$; b) CH_3NH_2; c) Ra-Ni, H_2.

periodate and treatment of the resulting 2',3'-secodialdehyde with methylamine under reducing conditions to afford the N4'-methylmorpholine derivative (See scheme 1). Our synthesis of the monomer followed the same outline with the exception that an unsubstituted morpholine nitrogen was required and led to the approach shown in scheme 2. In one pot, N4-benzoylcytidine *3* was treated with sodium periodate and ammonium biborate to afford the postulated intermediate *5*[12]. The resulting salts were removed by filtration and the solution of *5* was added to sodium cyanoborohydride to give a crude preparation of the desired morpholine nucleoside *6* in 55% yield. The crude morpholine nucleoside was isolated as the p-toluenesulfonic acid salt which precipitated from the reaction mixture. The amine of the morpholine ring of *6* was protected with trityl chloride to give the readily purified derivative *7* (49% yield from *3*). Ammonium biborate was originally employed as the nitrogen source for the morpholine ring because of the buffering capability of the biborate salt and the concern for epimerization of the dialdehyde *4*. However, use of either ammonium carbonate or ammonium bicarbonate as the nitrogen source also afforded a single diastereomer of the morpholine nucleoside in good yield. The trityl derivative was stable to silica gel chromatography and subsequent reaction conditions but allowed rapid liberation of the amine of *7* in 80% acetic acid in methanol. The monomethoxytrityl

Scheme 2[a]

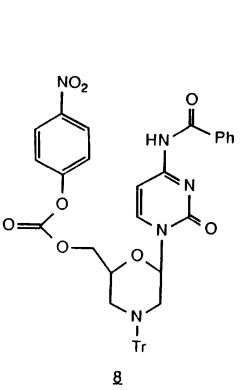


[a]a) $NaIO_4$; b) $(NH_4)_2B_4O_7$; c) $NaCNBH_3$, TsOH; d) TrCl, Et_3N, DMF

derivative as was used for the simple amino derivatives for the oligomers *1*, proved too labile for use with the morpholine nucleosides.

Coupling of two morpholine nucleosides was secured by the reaction of an activated carbonate derivative with an N4′-unprotected morpholine nucleoside. Preparation of the active carbonate *8* was achieved by treatment of *7* with bis(p-nitrophenyl)carbonate employing triethylamine as the catalyst. The trityl group of *7* was removed with 80% acetic acid in methanol and isolated as the p-toluenesulfonic acid salt. That *7* was used as the source of the free amine for the coupling reaction was due to the ease of purification of *7* relative to *6* derived directly from reductive amination. The crude salt was treated with *8* in the presence of diisopropylethylamine at room temperature. The coupling occurred regiospecifically at the N4′-amine to give *9* in 90% yield; no undesired product of attack on the active carbonate *8* by the 5′-hydroxyl of *6* was detected.

Dimer *9*, our basic block for the preparation of the hexamer *14*, was activated in the same manner as for *7* to afford *10*. Detritylation of *9* gave *11* and coupling of *11* to *10*



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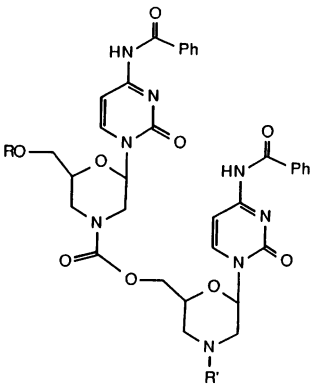


9 R = H, R' = Tr

10 R = CO_2pNitrophenyl, R' = Tr

11 R = R' = H

produced the tetramer *12*. Repetition of this sequence with tetramer *12* undergoing detritylation and the resulting amine *13* coupling to *10* furnished hexamer *14*. The yields of these steps were uniformly high.

Characterization of the intermediates and the full length oligomer was achieved by proton NMR and negative ion FAB mass spectroscopy. Mass spectrometry of carbamate linked nucleic acid analogs *1*, where the amide portion of the molecule was secondary[12], afforded regular fragmentation patterns which allows for determination of both composition and sequence of these oligomers. With the tertiary carbamates of the morpholine oligomers the fragmentation of the oligomers was greatly suppressed so that little sequence information was available. However, the parent ion signals were quite strong and allowed confirmation of the composition of the morpholine oligomers[14]. High resolution mass spectrometry of the oligomers *7*, *9*, *12* and *14* provided satisfactory analytical data.

Several features of the proton spectrum of the oligomers were interesting as well as informative of the oligomer composition. The coupling constants for the 2H and 6H protons are indicative of an axial orientation for these protons, with the 2-hydroxymethyl and the

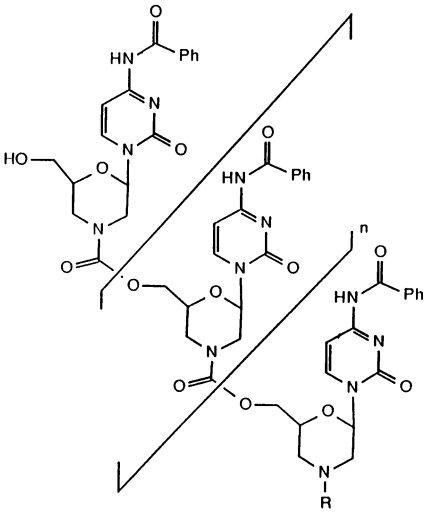
Table 1 Proton chemical shifts of dimer *9* and monomer *7*

	PROTON(a)						
COMPOUND/ SUBUNIT(b)	7H	6H	5aH	5eH	3aH	3eH	2H
DIMER *9* / 7	3.51 (2H)	5.62	4.00	3.34	3.87	3.07	3.65
DIMER *9* / 4H	4.19, 4.05	6.17	1.30	2.80	1.48	2.80	4.48
MONOMER *7*	3.42	6.11	1.23	3.37	3.18	3.10	4.22

NOTES:

(a). The numbering refers to the 2-hydoxymethylmorpholine ring numbering system where oxygen is 1, the anomeric carbon is 6, etc.

(b). Subunit refers to, in *9*, the 4N-tritylated subunit as 4 and the N4 acylated subunit as 7. The 7 terminus is a free hydroxyl.



12 n = 2, R = Tr

13 n = 2, R = H

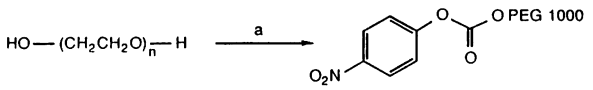
14 n = 4, R = Tr

6-pyrimidinyl substituents in the equatorial orientation. The signals for the 3H and 5H axial protons of the monomer *7* were upfield of the 3H and 5H equatorial protons by 2.2 and 1.7 ppm, respectively. The cause of this effect was attributed to the trityl group on N4 shielding the axial protons. This effect is also seen in the spectrum of the dimer *9* (see table 1), where the terminal subunit of dimer *9*, which is acylated at N4, showed signals for the 3H and 5H protons downfield from those of the monomer *7*. This trend was continued through the series of oligomers. The length of the oligomers could be ascertained by comparing the integration of the various signals. For example in dimer *9* the two 2H protons were separated by .5 ppm and gave a 1.02/1 ratio of integrations. For the hexamer *14*, this ratio was 4.65/1 against the expected value of 5/1.

The bases of *14* were deprotected by treatment with concentrated ammonia for 24 h. The 4'-terminal amine was liberated by treatment of the crude oligomer with 1% acetic acid in trifluoroethanol. In order to assess the stability of these molecules under these

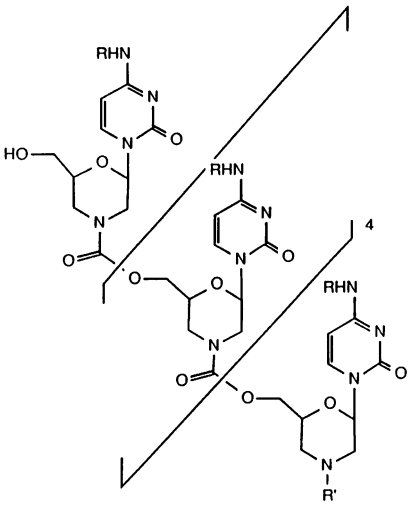
Table 2 Proton asssignments of signals of interest for *15*

SIGNAL	PROTONS
8.04 ppm (1H)	6-proton of Base of amine terminus
7.65 ppm (5H)	6-protons of remaining bases
6.78 ppm (1H)	5-proton of Base of amine terminus
5.85 ppm (5H)	5-protons of remaining bases
4.35−4.15 ppm (10H)	7H-protons of acylated subunits
4.14−3.90 ppm (17H)	2H-protons of acylated subunit (5) 3H-equatorial protons of all subunits (6) 5H-equatorial protons of all subunits (6)
3.70 ppm (1H)	2H-proton of hydroxyl terminus



PEG 1000 16

a) bis-p-(nitrophenyl) carbonate, Et_3N



15 R = R' = H

17 R = COPh, R' = H

18 R = COPh, R' = CO_2PEG 1000

19 R = H, R' = CO_2PEG 1000

conditions, dimer *9* was treated with concentrated amonia for 60 h; no cleavage of the intersubunit linkage was observed. Under all conditions used to date no cleavage of the carbamate linkage under acidic conditions has occurred.

Oligomer *15* was taken up in pH 2.1 buffer and purified by cation exchange chromatography on S-Sepharose Fast Flow[13] eluting with potassium chloride gradients. The chromatograms of *15* showed one major peak comprising over 95% of the cytosine containing materials in the mixture and confirms little or no cleavage of the oligomer occurs in the deprotection of the bases and the morpholine amine. After neutralization oligomer *15* was desalted on a polypropylene column eluting with water-acetonitrile gradients.

The purified hexamer was analyzed by ^{1}H NMR. The assignment of the protons signals of *15* was made on the basis of a COSY plot (see Table 2 for assignments). One cytosine base has signals that were found downfield relative to the other bases (8.04 to 7.65 and 6.78 to 5.85 ppm). The relative integrations of these peaks confirmed that the hexamer was deprotected and has been purified intact. The 7H protons were assigned to the signal at 4.35−4.15 downfield of the 2H protons signal at 4.14−3.90ppm. These chemical shifts run against the trend identified in the protected oligomers where the 2H proton of the same base(s) was always downfield of the 7H protons of the same base(s) (see table 1 for an

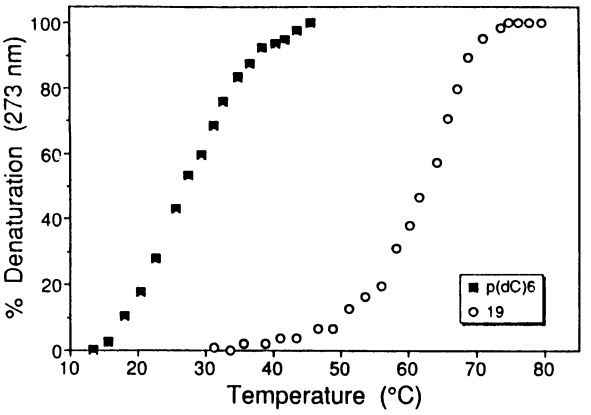


Figure 1. Thermal denaturations of *19* and $p(dC)_6$ with $p(dG)_6$.

example). Apparently the benzoyl groups in the protected oligomers play a role in shaping the environment of the 2H and 7H protons.

The solubility of hexamer *15* was found to be 4 μM in pH 7.5 buffer[15]. In order to increase water solubility, methodology to attach a polyethylene glycol (PEG) tail to the oligomers was developed. PEG 1000[16] was treated with one equivalent of bis(p-nitrophenyl)carbonate to give monoactivated PEG *16*. Detritylation of *13* with 80% acetic acid in methanol afforded the amine *17*. Treatment of *17* with active carbonate *16* under standard coupling conditions resulted in attachment of the PEG tail to the hexamer. The bases were deprotected by treatment of *18* with concentrated ammonia for 24 h. Oligomer *19* was taken up in pH 2.1 buffer and purified by cation exchange chromatography on S-Sepharose Fast Flow[13] eluting with potassium chloride gradients. After neutralization *19* was desalted on a polypropylene column eluting with water-acetonitrile gradients. Analog *19* was found to be freely soluble in pH 7.5 buffer in concentrations up to 50 μM.

The characterization of *19* by the ^{1}H NMR methods employed above was not possible. In the spectrum of the tailed hexamer *19* there was no differentiation between the signals of the base protons, precluding the assessment of the oligomer length as was done with *15*. Additionally, the envelope containing the PEG tail obscured the majority of the signals of the morpholine rings. However, the ion exchange chromatography of *19* gave one major peak indicating little cleavage of the oligomer during deprotection. The pattern of the chromatogram of *19* was the same as found for *15* except that the tailed material elutes faster than the untailed oligomer. Despite the difficulty in fully characterizing the tailed unprotected oligomer, sufficient supporting evidence was in hand to assign the structure of *19* as drawn and to continue on with the physical testing of *19*.

The stability of complexs of *19* with complementary nucleic acids was investigated by thermal denaturation experiments. Difference spectra between mixed and unmixed samples of *19* and the selected phosphodiester complement were obtained from 10°C to 75°C and over a range of 320 to 260 nm(see experimental section for details). As a control, the duplex of $p(dC)_6$ with $p(dG)_6$ was thermally denatured and found to have a Tm of 26.5°C.

The difference UV spectrum of *19* with $p(dG)_6$ was similar to that of the control DNA duplex of $p(dC)_6$ and $p(dG)_6$, except that the amount of hypochromicity before denaturation of the *19*-$p(dG)_6$ duplex was much greater than that of the control. Th

thermal denaturation of *19*-p$(dG)_6$ gave a Tm value of 62.5°C (see figure 1 for plot). A preliminary mixing curve indicated a multi-strand complex is formed, rather than a simple Watson-Crick duplex, and this accounts for the greater thermal stability of the morpholine-p$(dG)_6$ relative to the DNA-DNA control.

The difference spectrum of the interaction of poly G with the morpholine hexamer *19* did not show the classical difference spectrum seen with the controls. The data did not show a break point in the thermal denaturation of this complex and suggests atypical interactions between *19* and poly G.

We suggest the following hypothesis to explain these results. The carbamate linkage of *1* is calculated to be shorter than a phosphodiester linkage by 0.32 angstroms. Despite this shorter length, at least two examples of binding of a simple carbamate linked oligonucleotide analog have been found[9b,17]. However, a carbamate linked morpholine oligomer, with the amine of the backbone being a part of a ring, would have a shorter effective backbone length than a carbamate oligomer of *1* by an estimated 0.5 to 0.6 angstroms. For DNA in the B conformation the C1′-C1′ distance along the backbone is 5.03 angstroms. For RNA, which generally adopts an A conformation due the constraints imposed by the 2′ hydroxyl substituent of the ribose, the C1′-C1′ distance is 5.55 angstroms. The shorter length requirement of DNA allows binding of *19* where the greater length of RNA targets may compromise pairing or result in a duplex structure significantly different from that found in A form duplexes.

Further studies of this interesting structure are in progress.

EXPERIMENTAL

General.

UV spectra were obtained using a 100−80A Hitachi UV-Vis Spectrophotometer. Optical rotations were obtained at ambient temperature using a Perkin Elmer Model 243 Polarimeter. ^1H proton NMR spectra were obtained with a Bruker 400 MHz NMR. All samples were d_6-DMSO solutions containing a few drops of D_2O. Chemical shifts are reported (ppm) relative to protio-DMSO (2.49ppm). Negative ion mass spectra were obtained with a Kratos MS-50TC high resolution Mass Spectrometer. Except where noted, the sample matrix consisted of the oligonucleoside dissolved in 2/1 thioglycerol/glycerol. The primary xenon atom, with 8 keV kinetic energy, was directed at a target sample of 5 mm^2. Calibration was achieved with dry cesium iodide. Analytical analysis of *7*, *9*, *12*, and *14* were high resolution spectra using the system described above.

Column chromatography was carried out using Silica Gel, Merck, grade 60, 230−400 mesh with all eluting solvents containing 0.1% dimethylaniline (DMA). Triethylamine and dimethylformamide (DMF) were distilled from CaH_2 under N_2 and stored over activated sieves. Pyridine was distilled from calcium hydride and stored over activated sieves. Tetrahydrofuran (THF) was distilled from lithium aluminum hydride.

The physical studies of the synthetic oligomer were performed on a double beam 100−80A Hitachi UV-Vis Spectrophotometer. The temperature was regulated by circulating water from a Lauda RM3 constant temperature water bath through the cell holder and monitored by a probe immersed in the cell holder block. Temperature increments were manually performed.

Preparation of 6-(N4-benzoylcytosin-1-yl)-2-hydroxymethyl-4-tritylmorpholine. 7

N4-benzoylcytidine (10.065 g, 29.0 mmol) and ammonium biborate (8.780 g, 33.3 mmol) were suspended in methanol (500 ml) and treated with sodium periodate (6.303 g, 29.5

mmol) for 2.25 h. The solution was filtered and the solid washed with a small amount of methanol. The filtrate and wash were combined and added to a flask containing sodium cyanoborohydride (2.497 g, 39.7 mmol). After 20 min, an additional 0.62 g (9.87 mmol) of the hydride reagent was added and the solution stirred for 15 min longer. p-Toluenesulfonic acid (5.962 g) was added to the reaction vessel and, after the evolution of gas had ceased, an additional amount p-toluenesulfonic acid (5.883 g) was added to the flask. Precipitation of the product began as the second aliquot of acid was added. A third portion of p-toluenesulfonic acid (3.0 g) was added to the reaction vessel. After stirring overnight the solution was filtered and the solid washed with water, then methanol, and last with ether. The solid was dried under vacuum and afforded 8.022 g (55 % yield) of the desired morpholine salt.

A portion of the solid isolated above (3.996 g, 7.96 mmol) was dried by evaporation from DMF. The dried solid was dissolved in DMF (30 ml) and triethylamine (3.03 ml, 21.7 mmol) and to this solution was added trityl chloride (2.762 g, 9.93 mmol). After 30 min the solution was diluted with chloroform and washed with 5% aqueous sodium bicarbonate. The organic solution was dried over sodium sulfate then evaporated to dryness. The residue was chromatographed on silica gel eluting with 2% methanol/chloroform/0.1% N,N-dimethylaniline followed by 4% methanol/chloroform/0.1% N,N-dimethylaniline. The fractions containing the desired nucleoside were combined and evaporated to dryness. The solid was dissolved in $CHCl_3$ and washed once with 0.1 N NaOH, then dried (Na_2SO_4). The solvent was evaporated and the residue was taken up in a minimum volume of chloroform. Addition of the $CHCl_3$ solution to a large excess of hexanes afforded a solid. The solid was collected by filtration and the residue dried under vacuum to afford 4.069 g (89%) of the morpholine nucleoside *7*. *7*: UV λmax = 257 nm^{-1} (ϵ = 34700), 301 (ϵ = 16000). $[\alpha]_D$ +192° (c 0.52, $CHCl_3$). ^1H NMR δ = 11.2 (1H,bs exchangeable with D_2O); 7.97 (2H, d, J = 7.61Hz); 7.62 (1H, t, J = 7.37Hz); 7.60−7.10 (9H, m); 6.11 (1H, d, J = 8.28Hz); 4.22 (1H, m); 4.78 (1H, t, J = 5.85Hz, exchangeable with D_2O); 3.50−3.35 (2H, m), overlapped with 3.40−3.32 (1H, m); 3.10 (1H, bd, J = 11.5Hz); 1.38 (1H, t, J = 11.5Hz); 1.23 (1H, t, J = 10.2Hz). Mass spectrum M−1, 571.3 (100), 329 (10), 214.1 (40).

Anal. For $C_{35}H_{32}N_4O_4$ calculated: 572.2423. Found: 572.2427.

Preparation of 6-(N4-benzoylcytosin-1-yl)-2-(p-nitrophenoxycarbonyloxy-methyl-4-tritylmorpholine. 8

The base protected morpholine nucleoside *7* (789 mg, 1.38 mmol) was evaporated once from DMF. Bis-(p-nitrophenyl)carbonate (730 mg, 2.37 mmol) was added to the reaction vessel and the mixture was evaporated from DMF once. The residue was dissolved in DMF (8 ml) and to this solution triethylamine (0.65 ml, 5.5 mmol) was added slowly. The solution was stirred at room temperature for 1.3 h and then evaporated to dryness. The residue was applied to a column of silica gel (35 g) and the product was eluted from the column using a 2% MeOH/$CHCl_3$/0.1% DMA solution. Fractions containing the product were pooled and washed 4 times with 0.01 N aqueous NaOH, once with water and the resulting solution dried over Na_2SO_4. The solvent was evaporated and the residue was dissolved in a minimum volume of chloroform. Addition of the chloroform solution to a large volume of hexanes and collection of the resulting precipitate afforded, after thorough drying under vacuum, 919 mg (90%) of the activated morpholine nucleoside *8*. *8*: UV (MeOH) λmax. = 257 (ϵ = 38000), 301 (ϵ = 17000). $[\alpha]_D$ +122° (c 0.56, $CHCl_3$). ^1H NMR δ = 8.31 (1H, d, J = 2Hz), 8.06 (2H, m), 7.75−7.10 (23H, m), 6.18 (1H, dd, J = 9, 2Hz), 4.63 (1H, m,), 4.34 (1H, dd, J = 12, 3Hz), 4.26 (1H, dd,

J = 12, 6Hz), 3.34 (1H, bd, J = 11Hz), 3.10 (1H, bd, J = 11Hz), 1.44 (1H, bt, J = 11Hz), 1.29 (1H, bt, J = 10Hz). Mass Spectrum M−1 = 736 (50, also a very strong M− peak of 737 (60)), 571.2 (40), 214 (40), 138 (100).

Preparation of Morpholine Dimer 9.

The morpholine monomer *7* (667 mg, 1.17 mmol) was taken up in 25 ml of 80/20 acetic acid/methanol. After standing for 1.5 h, p-toluenesulfonic acid (222 mg, 1.17 mmol) in methanol (4 ml) was added to the solution. The solvent was evaporated and the resulting residue was twice evaporated from DMF. The solid was dissolved in DMF (7 ml) and to this solution the activated monomer *8* (862 mg, 1.17 mmol) was added. The solids were dissolved and triethylamine (0.4 ml, 2.89 mmol) was added to the reaction vessel. The solution was stirred at room temperature overnight. The solvent was evaporated and the residue chromatographed on silica gel. The product was eluted with 4% MeOH/$CHCl_3$. The fractions containing the product were pooled, evaporated and redissolved in $CHCl_3$. The chloroform solution was added to a large excess of hexanes. The product was collected by filtration and dried to yield 972 mg (90%) of the desired dimer *9*. *9*: UV λmax = 258 (ε = 40300), 302 (ε = 18500). $[\alpha]_D$ +122° (c 0.62, $CHCl_3$). ^1H NMR δ = 11.4 (1H, bs lost with D_2O), 8.30−7.80 (6H, m), 7.70−7.10 (23H, m), 6.17 (1H, d, J = 8.96Hz), 5.62 (1H, d, J = 7.57Hz), 4.95 (1H, m exchangeable with D_2O), 4.48 (H, m), 4.19 (1H, bd), 4.05 (1H, dd, J = 12, 4Hz), 4.00 (1H, bm), 3.87 (1H, bd), 3.65 (1H, bs), 3.51 (2H, bs), 3.34 (1H, bd), 3.07 (1H, d, J = 11.8Hz), 2.80 (H, t, J = 12Hz), 1.48 (1H, t, J = 11.2Hz), 1.30 (1H, t, J = 10.2Hz). Mass spectrum M−1 927.2 (80), 823.2 (45), 685.1 (15), 571.1 (25), 329.1 (15), 214.1 (100).

Anal. For $C_{52}H_{48}N_8O_9$ calculated: 928.3544 Found: 928.3555.

Activation of Dimer 10.

The dimer *9* (491 mg, 529 μmol) was treated with bis-(4-nitrophenyl)carbonate (277 mg, 898 μmol) and triethylamine (0.25 ml, 1.81 mmol) in the same manner as for the preparation of *8*. Purification of activated dimer *10* was carried out employing 3% MeOH/$CHCl_3$ as the eluting solvent. The fractions containing the product were pooled and washed with dilute NaOH in the same manner as for *7*. Isolation by precipitation afforded 420 mg (71%) of the activated dimer *10*. *10*: UV λ max = 261 (ε = 53600), 302 (ε = 23000). $[\alpha]_D$ +102° (c 0.41, $CHCl_3$). ^1H NMR δ = 8.35−7.78 (8H, m), 7.70−7.05 (25H, m), 6.17 (1H, bd, J = 9Hz), 5.74 (1H, bd, J = 10Hz), 4.53−4.35 (3H, m), 4.18 (1H, m), 4.09 (1H, m), 4.20−3.85 (3H, m), 3.34 (1H, bd, J = 11Hz), 3.09 (1H, bd, J = 10Hz), 2.91 (2H, bt, J = 10Hz), 1.52 (1H, t, J = 10Hz), 1.31 (1H, t, J = 10 Hz). Mass Spectrum M−1 = 1092 (70) (also a large M− peak 1093 (85), 927 (25), 571 (5), 214 (40), 183 (100).

Preparation of Tetramer 12.

De-tritylation of dimer *9* (148 mg, 159 μmol) was carried out in the identical manner to the monomer *7*. Activated dimer *10* (175 mg, 160 μmol) was used as described above to couple to the amino dimer p-toluenesulfonic acid salt *11* to afford 138 mg(52%) of the tetramer *12*. *12*: UV λ max = 260 (ε = 87800), 302 (ε = 37200), with NaOH λmax shifts to 315 nm^{-1} (ε = 90000). ^1H NMR δ = 8.25−7.15 (43H, m), 6.18 (1H, d, J = 8.9Hz), 5.78 (1H, bd), 5.70 (H, bd), 4.49 (1H, m), 4.40−3.80 (15H, m), 3.53 (2H, bs), 3.40−2.85 (8H, m), 1.49 (1H, t, J = 10 Hz), 1.28 (1H, t, J = 10Hz), 1.28 (1H, t, J = 10Hz). Mass Spectrum (3-nitrobenzyl alcohol matrix) M−1 = 1639.4 (8), 459.1 (25), 306.1 (100).

Anal. For $C_{86}H_{80}N_{16}O_{19}$ calculated: 1640.5785. Found: 1640.5803.

Preparation of Hexamer 14.

Preparation of hexamer *14* from the tetramer *12* (97 mg, 59 μmol) and the activated dimer

10 (65.2 mg, 59 μmol) was carried out in the same manner as for preparation of the dimer *8*. This procedure afforded 82 mg (59%) of morpholino hexamer *14*. *14*: UV λmax = 260 nm^{-1} (ε = 126000), 303 nm^{-1} (ε = 53300). ^{1}H NMR δ = 8.25−7.90 (18H, m), 7.65−7.05 (39H, m), 6.16 (1H, bd), 5.77 (4H, m), 5.69 (1H, bd), 4.46 (1H, m), 4.35−3.80 (25H, m), 3.56 (2H, m), 3.25−2.75 (12H, m), 1.47 (1H, m), 1.24 (1H, m). Mass Spectrum (3-nitrobenzyl alcohol matrix) M−1 = 2352.6 (2), 459.2 (30), 306.2 (100).

Anal. For $C_{120}H_{112}N_{24}O_{29}$ calculated: 2352.8026. Found: 2352.8197.

Preparation of Deprotected Hexamer 15.

The hexamer *14* (20 mg, 6.1 mmol) was taken up in 2 ml of 1/1 (v/v) conc. NH_3/DMSO and allowed to sit for 30 h at 30°C. The solvent was evaporated and the residue was taken up in trifluoroethanol containing 1% acetic acid. After 5 min the solvent was evaporated and the residue was purified by chromatography on S-Sepharose[13] eluting with KCl gradients in pH 2.1 phosphate buffer. The fractions containing the hexamer *15* were pooled and evaporated nearly to dryness. The solution was de-salted by chromatography of *15* on polypropylene (Pharmacia) eluting first with water and then with water/CH_3CN gradients. The fractions containing the hexamer *15* were combined and evaporated to dryness. The residue was taken up in trifluoroethanol and was added to an excess of ether. Solid *15* was collected by centrifugation and dried under vacuum. *15*: UV λmax = 267nm^{-1} at pH 7.5; 267 at pH 13 (0.1 N NaOH); 274 at pH 1 (0.1 N HCl) (ε = 77100 (calculated value)). ^{1}H NMR δ = 8.04 (1H, m), 7.65 (5H, m), 6.78 (1H, m), 5.85 (5H, m), 5.70 (6H, m), 4.34−4.15 (10H, m) 4.14−3.90 (17H, m), 3.70 (1H, m), 3.50 (2H, m), 3.02−2.80 (12H, m).

Tailing of Hexamer. 19

Polyethylene glycol 1000 (15 g, 15 mmol) was dried by evaporation from dry DMF and then was treated with bis-(4-nitrophenyl)carbonate (2.28g, 7.5 mmol) and triethylamine (4.2 ml, 30 mmol) in DMF (100 ml) for 24 h at 30°C to give a solution of *16* in DMF. This solution was used directly in the following step.

Hexamer *14* (50 mg, 15.2 μmol) was treated with 0.1 ml acetic acid in 4 ml trifluoroethanol for 3 min at room temperature. Ether (25 ml) and diisopropylethylamine (0.3 ml) was added to the sample. The precipitate was collected by centrifugation and the solution was decanted. The pellet was treated with the stock solution of the PEG 1000 p-nitrophenylcarbonate *17* (10 ml) overnight at 30°C. To this solution was added conc NH_3 (10 ml) and the resulting mixture was allowed to stand at 30°C for 24 h. The solution was evaporated to dryness and the residue was taken up in methanol, added to an excess of ether and the precipitate collected by centrifugation. Solid *19* was taken up in pH 2 buffer and chromatographed on S-Sepharose[13] eluting with KCl gradients in pH 2.1 buffer. The fractions containing the hexamer *19* were pooled and evaporated to near dryness. The solution was desalted by chromatography of *19* on polypropylene eluting first with water and then with water/CH_3CN gradients. The fractions containing the hexamer *19* were combined and evaporated to dryness. The residue was taken up in trifluoroethanol and was added to an excess of ether. Solid *19* was collected by centrifuge and was dried under vacuum. *19*: UV λmax = 267.1 nm^{-1} (ε = 42800, pH 7.5); 1 λmax = 275.7 at pH (ε = 77100 (calculated value)). ^{1} H NMR δ = 7.74 (6H, broad d), 5.97 (6H, broad d), 5.65 (6H, broad d), 4.30−4.05 (12H, m) 4.04−3.80 (18H, m), a large envelop containing the PEG protons, and several signals of the oligomer, 2.99−2.80 (12H, m).

Thermal Denaturation Studies of 19.

The DNA target $p(dG)_6$ (four 5 A_{260} unit packages, purchased from Pharmacia LKB and used without purification) was dissolved in deionized water (50 μl) and this solution was

diluted with 200 μl DMSO. The oligomer *19* (1.8 mg) was dissolved in DMSO (360 μl).

These stock solutions were assayed for the actual concentration of solute by UV spectroscopy. The absorbance of the stock solution of *19* (15 μl) was measured in 0.1N HCl and the absorbance of the $p(dG)_6$ stock solution was measured in 0.1N NaOH. The concentration of the stock solutions were calculated using molar absorption coefficients of 7.7×10^4 for *19* at pH 1 and 6.5×10^4 $p(dG)_6$ at pH 13[18].

Samples for melt assays were prepared by diluting the stock solutions of *19* and $p(dG)_6$ with 0.05N phosphate buffer containing 0.001 N EDTA; the final sodium ion concentration was 0.05 N while the concentration of *19* and $p(dG)_6$ was 10 μM each. The reference beam held the two components in the unpaired state while the sample beam held the components mixed. The samples were warmed to 60°C and allowed to cool over several hours. The difference spectrum of the paired versus the unpaired components was measured at ca 15°C and the wavelength of maximum hypochromicity was identified as 273 nm. Absorbance difference at 273 nm versus temperature profiles were obtained by manually raising the temperature in ca 2°C increments up to 75°C. The absorbance difference data from a run was normalized to % denaturation[8a] and plotted against temperature to obtain the melt curve. The Tm was determined as the midpoint of the plot between the two states of the system and the value for *19* with $p(dG)_6$ was 62°C (see figure 1).

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