(MH$^+$ – CO – NH$_3$) 124.07624, found 124.07713.

(5*R*,8*RS*)-8-Hydroxy-1,7-diazaspiro[4.4]nonane-2,6-dione (4) and (5*R*)-1,7-Diazaspiro[4.4]nonane-2,6-dione (3). Compound **5** (0.130 g, 0.770 mmol) was dissolved in H_2O (5 mL) and OsO_4 (39 μL of a 4% w/v solution in H_2O, 0.006 mmol) added. After 5 min only a very faint brown color had developed. $NaIO_4$ (0.363 g, 1.70 mmol) was then added and the solution stirred for 16 h. The solvent was then removed under reduced pressure by codistillation with *i*-PrOH and the solids extracted with *i*-PrOH. Filtration and removal of the solvent under reduced pressure gave 0.181 g of a hygroscopic solid whose NMR was consistant with the expected product, **4**. The spectral data for the impure product **4** were as follows: ^{1}H NMR (D_2O/300 MHz) δ 5.17 (dd, 1 H, *J* = 7, 6 Hz), 2.55 (dd, 1 H, *J* = 16, 7 Hz), 2.32 (t, 2 H, *J* = 10 Hz), 2.20–2.06 (m, 1 H), 2.05–1.94 (m, 1 H), 1.87 (dd, 1 H, *J* = 16, 6 Hz); MS (PCI) *m/e* (rel intensity) 153 (MH$^+$ – H_2O, 7), 112 (7), 100 (15), 59 (100); HRMS (EI) *m/e* calcd for $C_7H_8N_2O_2$ (M$^+$ – H_2O) 152.05858, found 152.05533.

To a mixture of the crude product **4** in $MeNO_2$ (5 mL) was added Et_3SiH (246 μL, 1.54 mmol) and TFA (593 μL, 7.70 mmol). After 18 h the solvent was removed under reduced pressure by codistillation with *i*-PrOH. The solid residue was dissolved in water, filtered, and chromatographed (Magnum-9 ODS-3, 100% H_2O, 2 mL/min). The fractions containing product were combined, evaporated to dryness under reduced pressure, dissolved in a minimum of water, filtered, and again evaporated to dryness to produce **3** as a white solid (0.179 g, 89% over the two steps): ^{1}H NMR (DMSO-d_6/300 MHz) δ 7.96 (s, 1 H), 7.86 (s, 1 H), 3.09–3.24 (m, 2 H), 1.88–2.31 (m, 6 H); ^{1}H NMR (D_2O/300 MHz) δ (std HOD 4.67 ppm) 3.24 (dd, *J* = 7.9, 5.2 Hz), 2.41–2.32 (m, 2 H), 2.26–2.20 (m, 4 H); ^{13}C NMR (DMSO-d_6/75 MHz) δ 176.7, 176.6, 62.3, 37.1, 34.0, 29.9, 29.7; ^{13}C NMR (D_2O/75 MHz) δ (unstandardized) 189.6, 187.4, 72.8, 46.3, 41.6, 38.0, 37.6; IR (KBr) 3242, 3145, 3068, 2907, 2870, 1716, 1683, 1653, 1360, 1306, 1246, 1054 cm^{-1}; MS (PCI) *m/e* (rel intensity) 155 (MH$^+$, 100); HRMS (EI) *m/e* calcd for $C_7H_{10}N_2O_2$ 154.07422, found 154.07240; CD λ_{max} = 227.5 nm ($\Delta\epsilon$ = 6.0 M^{-1} cm^{-1}).

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Supplementary Material Available: ^{1}H and ^{13}C NMR spectra for **3**, **5**, **7**, and **9**, as well as a ^{1}H NMR spectrum for **4** (10 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

A Convenient Method for the Direct Incorporation of 5-Fluoro-2′-deoxycytidine into Oligodeoxynucleotides

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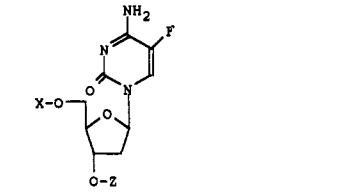
Introduction

To further elucidate the cellular functions governed by DNA methyltransferases we report the direct incorporation of 5-fluoro-2′-deoxycytidine into oligodeoxynucleotides using standard phosphoramidite chemistry. DNA methylation has become an area of great interest due to a demonstrated role in regulating gene expression.[1] In general, methylation of cytidine residues within a specific gene can inhibit transcription of that gene directly by inhibiting the binding of transcription factors or by affecting the surrounding chromatin structure.[2] Central to our interests, DNA methylation has been shown to affect the cellular processes of differentiation and carcinogenesis.[2] Oligodeoxynucleotides (ODNs) containing 5-fluoro-2′-deoxycytidine (FdC, **1A**) have been shown to inhibit bacterial methyltransferases (MTases).[3-5] The incorporation of FdC into ODNs is synthetically problematic and has prompted the development of specialized methodologies to circumvent these difficulties.[3,6,7] Although these different procedures ultimately incorporate FdC into ODNs, we sought a method to directly and simply introduce FdC into any desired ODN via an automated synthesizer.



1A(FdC) where X and Z = H
1B where X = 4,4′-dimethoxytrityl and
Z = P(OCH$_2$CH$_2$CN)(N[CH(CH$_3$)$_2$]$_2$)

2= CTATATGXGACCTG where X = FdC
3= CTATATGCGACCTG

Figure 1. Overview of structures.

Results and Discussion

Initial studies explored the acid stability of aliphatic amides of the N4 amino group of FdC as a prerequisite for their use as protecting groups during automated ODN synthesis. The isobutyryl protecting group appeared to be stable in a 2% trichloroacetic acid solution (by volume in CH_2Cl_2) for up to 40 min, as evidenced by thin-layer chromatography. However, repeated efforts to amidate the N4 amino group of FdC were only partially successful and clearly indicated that this amine was relatively nonreactive. From these observations, we reasoned correctly that the exocyclic amino group of FdC is sufficiently deactivated by the presence of the 5-fluoro group so that protection of the amino group would not be necessary. Thus, we report the synthesis of ODNs containing FdC which was introduced using standard phosphoramidite chemistry.

Preparation of the desired phosphoramidite of FdC and its incorporation into ODNs was accomplished in the following manner. The 5′-hydroxyl group of FdC was first converted to the trityl ether with 4,4′-dimethoxytrityl chloride in pyridine.[8,9] This intermediate was converted

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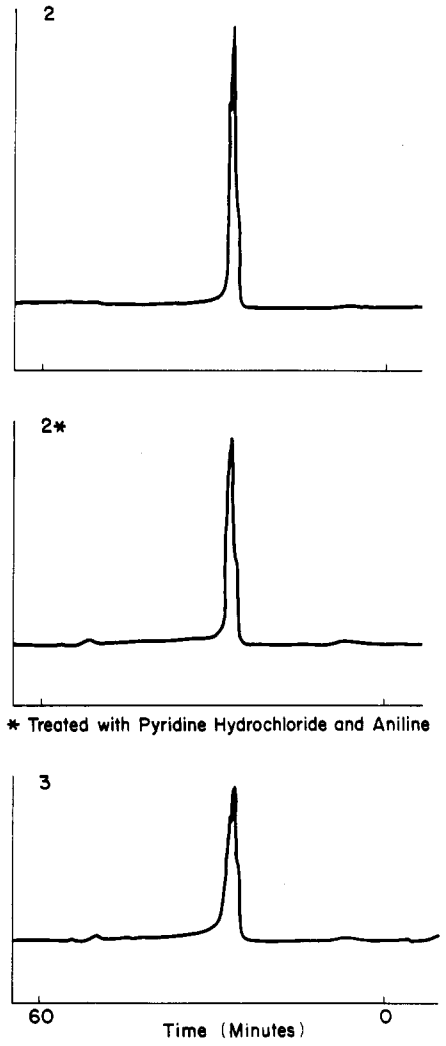

Figure 2. HPLC profiles of DNA analogues 2 and 3.

to the corresponding phosphoramidite (1B) with 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite and the diisopropylammonium salt of tetrazole.[10,11] This analogue was purified by silica gel column chromatography, dried under vacuum, taken up in an appropriate amount of anhydrous CH_3CN to give a 0.1 M solution, and used directly on an ABI 381A synthesizer with commercially available reagents and phosphoramidites. The DNA analogue 2 was synthesized as the prototypical ODN, and its unsubstituted parent, 3, was also synthesized. The ODNs were synthesized via the manufacturer's recommended coupling cycles, except for the introduction of FdC phosphoramidite which required two coupling cycles. The ODNs were cleaved from the support resin with concentrated NH_4OH and then deprotected at 55 °C for 18 h. The ODNs were purified by HPLC,[12] and the major UV-containing fractions, as indicated by thin-layer chromatography, were pooled.[13] The HPLC profiles of the pooled fractions are shown in Figure 2. HPLC indicated that the presence of the unprotected N4 amino group on FdC did not cause extensive side branching off the amine or the formation of complex side products as evidenced by one major peak with the same retention time as that of the unsubstituted parent.

Gryaznov et al.[14] have recently developed methodology that eliminates the need to protect amino groups of conventional nucleosides for use in ODN synthesis. This entails treatment after each coupling with pyridine hydrochloride and aniline to eliminate the formation of undesired side products.[14] Therefore, ODN, 2, was also synthesized using the protocol of Gryaznov et al.[14] solely for the incorporation of FdC, whereas the remaining nucleotides were incorporated as their fully protected phosphoramidites. We determined that it was not necessary to treat this ODN with pyridine hydrochloride and aniline to obtain the desired analogue. Gel electrophoresis of the FdC-containing ODN, the FdC-containing ODN which underwent phosphatidyl transfer, and their unsubstituted parent confirmed that these ODNs were identical.

This method allows for the direct incorporation of FdC into ODNs utilizing traditional phosphoramidite methodology. This will also facilitate the routine automated preparation of a wide range of FdC-containing ODNs of biological interest.

Experimental Section

5-Fluoro-5'-(dimethoxytrityl)-2'-deoxycytidine. To a solution of 5-fluoro-2'-deoxycytidine (0.101 g, 0.41 mmol) in 12.5 mL of anhydrous pyridine was added 4,4'-dimethoxytrityl chloride (0.184 g, 0.56 mmol). The reaction mixture was stirred continuously at room temperature under a positive pressure of argon for 72 h. The reaction mixture was concentrated in vacuo and the oil taken up in 75 mL of CH_2Cl_2 with 1% triethylamine. The organic layer was washed with 75 mL of brine solution and 75 mL of water, dried over magnesium sulfate, filtered, and concentrated. Purification by silica gel flash chromatography (discontinuous gradient of ethyl acetate/methanol (1:0–5:1) with 1% triethylamine by volume as eluant) afforded pure product (48%) as a white solid. Attempts to improve the yield by using (dimethylamino)pyridine and triethylamine in the reaction mixture were unsuccessful. ^{1}H NMR (DMSO-d_6) δ 2.18 (m, 2 H, 2'-CH_2) 3.15 (m, 2 H, 5'-CH_2), 3.75 [s, 6 H, $(OCH_3)_2$ of trityl], 3.93 (m, 1 H, 3'-CH), 4.30 (m, 1 H, 4'-CH), 5.30 (d, 1 H, OH), 6.15 (m, 1 H, 1'-CH), 6.90–7.40 [m, 13 H, (C_6H_5), $(C_6H_4)_2$], 7.53 (m, 2 H, 4-NH_2), 7.83 (d, 1 H, 6-CH).

5-Fluoro-5'-(dimethoxytrityl)-2'-deoxycytid-3'-yl 2-Cyanoethyl *N,N*-Diisopropylphosphoramidite. To a solution of tritylated FdC (0.101 g, 0.18 mmol) in 15 mL of CH_2Cl_2 was added diisopropylammonium–tetrazole salt (0.033 g, 0.19 mmol) and 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (0.136 g, 0.45 mmol). The reaction mixture was stirred overnight at room temperature under a positive pressure of argon. The reaction mixture was diluted to 50 mL with CH_2Cl_2, washed with 50 mL of brine, 50 mL of water, dried over magnesium sulfate, filtered, and concentrated in vacuo. Purification by silica gel flash chromatography (discontinuous gradient of ethyl acetate/methanol (1:0–5:1) with 1% triethylamine by volume as eluant) afforded pure product (71%): ^{1}H NMR (DMSO-d_6) δ 1.25 [d, 12 H (CH-$(CH_3)_2)_2$], 2.15–3.60 [m, 10 H, 2' and 5'-CH_2 OCH_2CH_2CN, CH-$(CH_3)_2$], 3.70 [s, 6 H, $(OCH_3)_2$ of trityl], 3.80–4.20 (m, 2 H, 3' and 4'-CH), 6.15 (m, 1 H, 1'-CH), 6.85–7.35 [m, 13 H, (C_6H_5), $(C_6H_4)_2$], 7.80 (d, 1 H 6-CH).

(8) Purification by silica gel flash chromatography (discontinuous gradient of ethyl acetate/methanol (1:0–5:1) with 1% triethylamine by volume as eluant) afforded pure product as determined by proton NMR.

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(12) HPLC was performed on a Waters 600E Chromatographic system equipped with an AQUAPORE RP-300 C8 column (100 × 4.6 mm) using a gradient of 100% A (4% acetonitrile by volume in water with 50 mM triethylamine acetate) to 100% B (80% acetonitrile by volume in water with 50 mM triethylamine acetate) in a 1-h period with a flow rate of 0.5 mL/min.

(13) Thin-layer chromatography was performed with ANALTECH Silica Gel GF plates (2.5 × 10 cm, thickness 250 μm) using *n*-propanol-/water/concd NH_4OH (5.5:3.5:1) as eluant.

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Registry No. 1A, 10356-76-0; 1B, 143774-48-5; 1B Z=H, 143774-47-4; 4,4'-dimethoxytrityl chloride, 40615-36-9; 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite, 102691-36-1.

Simultaneous Determination of Conformation and Configuration Using Distance Geometry

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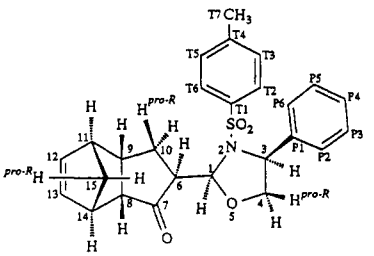
Introduction

We have recently shown that for a small, complex oxazolidine the conformation and relative configuration of the stereogenic centers could be solved simultaneously with the use of distance restraints from NOESY data in molecular dynamics (MD) simulations.[1] With the use of an extremely large force constant for the NOE constraints, the energy of the NOE term is many times larger than the energetic component of the force field set to maintain the configuration, and therefore, the stereogenic centers can switch producing the stereochemistry consistant with the experimental data. This procedure was illustrated with the title compound 1,[1] which has been synthesized in the course of examining asymmetric Diels–Alder reactions with chiral oxazolidines.[2]



The induced stereo configuration at the bridgehead positions C8 and C9 as well as the stereo configuration at C6 was determined from NOEs and restrained MD (rMD) simulations as described above. Starting with the (1*S*,3*S*,6*S*,8*R*,9*R*,11*R*,14*S*) (exo) structure, the chirality at the bridgehead positions quickly reversed, forming the endo structure.[1] In contrast, beginning with the endo structure the NOEs were completely fulfilled.

During this study we ran into the problem that there were no parameters for some of the bonds, bond angles, and dihedrals in the potential energy force field used. This is a commonly occurring situation; the parameters to describe specific moieties or groups in naturally occurring or synthetic molecules are not defined. Most of the programs widely available today have been designed specifically for peptides and proteins[3-6] or DNA.[7] Although, there is a large body of work concentrating on a variety of functional groups (e.g., the MM2 program[8] and later versions[9]), transferring parameters from one program to the one in use is often problematic; the question arises of maintaining a self-consistent force field.

However, it is often the case that the geometry of the moiety or functionality, for which no parameters exist, is either known or can be approximated (without introduction of too great of error) from X-ray structures of related molecules. The problem therefore is not the equilibrium values of the bond lengths, bond angles, or torsions, but the force constants that should be utilized as a penalty for moving away from the equilibrium value (not to mention the partial atomic charges or Lennard–Jones parameters for the nonbonded terms of the force field).

Small synthetic or natural molecules have several properties that are quite favorable for conformational analysis that should allow one to overcome the aforementioned drawbacks. In solution, these small molecules are rapidly tumbling (i.e., small correlation time) which at the field strengths used in modern NMRs lead to sizeable, positive NOEs. In addition, it is commonly the case that the spectral resolution of the proton resonances is good, easing the assignment and accurate integration of NOEs. Of course, as previously discussed by us[1] and others,[10] great care must be used in the interpretation of the NOEs to ensure that external relaxation (leakage) is negligible and that the molecule is conformationally homogeneous (absence of a rapid conformational equilibrium). We know of no sound procedure for addressing the latter problem. It is common to work under the assumption of one conformation (or family of closely related conformations) and examine the reproduction (back-calculation) of the experimental observations. If there is a rapid equilibration between conformations (which differ significantly) then inconsistencies between the experimental and back-calculated data will appear.[11] Agreement between the experimental and theoretical data can then only be obtained with the assumption of two or more conformations.

Considering the large number of NOEs (large relative to molecular weight) and the well-defined geometry (bond lengths and angles) it seemed reasonable that conformations of such molecules could be generated using distance geometry (DG) techniques. Here, we describe the use of DG in the conformational examination of oxazolidine 1. In addition, DG calculations were carried out to examine the possibility of determining the relative configuration,

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