1,N⁶-etheno deoxy and ribo adenosine and 3,N⁴-etheno deoxy and ribo cytidine phosphoramidites. Strongly fluorescent structures for selective introduction in defined sequence DNA and RNA molecules

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ABSTRACT

Synthesis of 1,N⁶-etheno-2'-deoxyadenosine, 3,N⁴-etheno-2'-deoxycytidine, and further chemistry on both deoxy and ribo series etheno nucleosides produces the corresponding phosphoramidites. These novel phosphoramidites are introduced selectively, quantitatively, and at specific positions at single or multiple sites into DNA or RNA sequences. The purification and chemistry involved in the synthesis of these products has been optimized to achieve the purity in excess of 99%. The resulting phosphoramidites were tested for their ability to couple and produce poly deoxy and ribonucleotides by solid phase chemistry. The coupling efficiency achieved was greater than 99% per step. Due to the instability of these etheno compounds in acidic and basic medium, various criteria to obtain pure oligomers have been established. The selective introduction of these fluorescent nucleosides into defined sequence DNA and RNA molecule will greatly facilitate the structure – function studies of various RNAs, protein – RNA structures, and DNA – RNA based diagnostics applications. The characteristic and high fluorescent intensity (detection below 1×10^{-9} M for adenosine sites and below 1×10^{-7} M for cytidine sites) is particularly suited for the biochemical and biological research and product development applications. The usefulness of these etheno containing modified sequences as sequencing and amplification primers is demonstrated by their full participation in polymerase chain reaction experiments.

INTRODUCTION

The application of fluorescent conjugates to study biochemical processes of macromolecules is well recognized and extremely useful. Recent advances in techniques and chemistry has provided many suitable compounds for application *in vivo*. Various fluorochromes [fluorescein (1–5), eosin (6), 7-chloro-4-nitro

benzo-2-oxadiazole (7), rhodamine dyes (1,2,8–9), and their reactive derivatives] are being used for such applications. The optical properties of a wide variety of fluorescent probes have been elegantly summarized (10). Fluorescent labelling of macromolecules, e.g. nucleic acids and synthetic oligonucleotides, are extremely useful in many areas of molecular biology. The automated DNA sequencing method utilizes rhodamine derivatives (11–13) and fluorescein derivatives (14–19). Other non-radioactive systems [fluorochromes (20–23), enzymes (24–27), biotin based (28–38) or hapten (39–42), digoxigenin (43–44)] are used to label oligonucleotide probes for diagnostic applications. Most of these methods involve covalent attachment of one or more of these reported groups through primary aliphatic amino group (38). With this approach remains the possibility of perturbation of discrete structure, especially in the neighborhood of the chromosphere. With the etheno nucleoside within the chain, this possibility is minimized significantly, as the small structural change can be tolerated to a certain degree. Furthermore, the fluorescence intensity and life time of these molecules, especially etheno adenosine, would allow development of useful probes for diagnostic, sequencing, and molecular structure recognition studies. The etheno substituted nucleosides, such as 1,N⁶-ethenoadenosine (IVa;eA), 3,N⁴-ethenocytidine (IVb;eC), corresponding 5'-monophosphates, 5'-diphosphates, 5'-triphosphates, 5',3'-cyclic phosphates, and also a number of coenzymes have previously been synthesized (45–51). Furthermore due to the strong fluorescent properties of the etheno modified nucleosides, a number of useful biological and biochemical applications of these modified nucleosides have been established (51).

The stepwise RNA primer synthesis incorporating the etheno modified nucleoside at a specific site in a sequence has been achieved in two stages; (a) enzymatic methods of chain elongation or fragment synthesis and (b) the etheno modification reaction on adenosine by reaction with chloroacetaldehyde (52). The fluorescent properties of the synthesized molecules, due to the presence of ethenonucleoside in the sequence, have been utilized in determination of the nature of protein – nucleic acid complexes. However, with the present state of art, it has not been possible

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to introduce the etheno modified ribonucleoside at specific positions of a defined sequence RNA molecule with any ease and convenience. One of the basic units, 1, N⁶-ethenodeoxyadenosine (Ia; edA), has been derivatized to introduce 5'-dimethoxytrityl group (IIa) (53) and the 3'-phosphoramidite group (IIIa) (54). However, due to the inherent instability of these nucleosides to acidic and basic reaction conditions (53,54), the synthesis and isolation of DNA and RNA incorporating them need to be addressed adequately. Similarly, purification techniques and full characterization of the phosphoramidite need to be established. This is extremely important in order to obtain good quality of oligomers reliably. Thus, when the synthesis of Ia and Ib, was carried out at the initial pH of 4.5, the pH fell quickly to strongly acidic during the course of the reaction (with negligible product formation). Henceforth, the readjustment of the pH periodically with aqueous sodium hydroxide is essential to the success of the reaction. Similarly, aqueous ammonia treatment of compounds IIIa and IIIb results in degradation products very quickly. The instability of this ring system is carried forward during DNA synthesis, in as much that the quality of the synthesized oligomer varies greatly with the deprotection chemistry utilized.

In order to overcome some of the instability properties of this ring system, various approaches have been adapted. Replacement of the commonly used dimethoxytrityl (DMT) group with p,p'-trimethoxytriphenyl (TMT) group (56,57) results in compounds which have favorable properties. Comparative studies indicated that during the solid phase DNA synthesis, the TMT-group could be satisfactorily removed with milder acid such as 3% monochloroacetic acid (MCA), as compared to the removal of DMT group, which necessitates either 2% trichloroacetic acid (TCA) or 3% dichloroacetic acid (DCA). Similarly, ammonia deprotection time course studies for the removal of base protecting groups and the solid support revealed the optimum conditions, which needs to be employed in order to obtain pure desired oligomers devoid of significant breakdown products.

MATERIALS AND METHODS

The ultraviolet spectra was obtained on a Shimadzu UV-1201 spectrophotometer. The fluorescence spectra was determined on a Hitachi F-4500 spectrophotometer. The ¹H and ³¹P nuclear magnetic resonance spectra were obtained on a Varian XL-300 MHz spectrometer equipped with a Varian 5 mm broad band probe. The ¹H-NMR spectra was referenced to the internal CHCl₃ signal and 1% TMS in the sample, 7.24 ppm and 0 ppm, respectively. ³¹P-NMR chemical shifts listed are downfield from 85% H₃PO₄, externally referenced. All the spectra was run in CDCl₃ (Cambridge Isotopes). CDCl₃ was also used as a lock reference in ³¹P-NMR analysis. DNA and RNA were synthesized on Milligen 6500 DNA-RNA synthesizer equipped with an on-line long wavelength detector (498nm) and on the Eppendorf Ecosyn D300-DNA-RNA synthesizer. The DNA sequences were synthesized with standard protocols for defined sequence DNA synthesis (0.5 micromole scale). The 0.5 μm scale RNA synthesis was similar to DNA, except with modification of the DNA synthesis protocol with increased coupling time to 9.5 minute for each natural RNA base, as well as the modified RNA base carrying the etheno functionality. All the phosphoramidites with classical base protecting group and fast alkali removable base protecting groups are produced by ChemGenes.

Thin layer chromatography (TLC) was carried out on Baker-Flex silica gel IB-F TLC plates (20×20 cm, and 5×20 cm). Column chromatography was carried out using silica gel 60 EM Science, particle size 0.04–0.063 mm (230–400 mesh), and particle size 0.063–0.200 mm (70–230 mesh). High pressure liquid chromatography (HPLC) was done on Rainin-Rabbit HPLC instrument with 254nm Altex UV detector and microsorb-MV(Rainin), C-18, 5μm 100A reverse phase column. For the determination of the detection limit and the establishment of purity of the ethenonucleosides (Ia, Ib, IIa, IIb, IIIa, IIIb, IVa, IVb, Va, Vb, VIa, VIb, VIIa, VIIb, VIIIa and VIIIb as depicted in scheme I), TLC and HPLC were used as a guide. When the TLC is carried out using high concentrations it was possible to detect distant R_f impurities to the extent of 0.25%–0.10%. Low concentration TLC and HPLC revealed closer impurities. For the phosphoramidites (IIIa, IIIb, VIIIa and VIIIb), an additional purity parameter was ³¹P-NMR analysis to determine phosphorus containing impurities. The desired phosphoramidite-associated peaks appear as sharp doublets. By taking up to three fold excess (as compared to the normal quantity of 16 mg) in CDCl₃ and increasing the data acquisition time to approximately 30 minutes, it was possible to detect the impurities up to the extent of 0.5%. This technique is especially useful for the ribonucleoside phosphoramidites which can have isomeric impurities. The purity was established from all data obtained from the analytical techniques such as TLC, HPLC, ¹H NMR and ³¹P-NMR wherever applicable.

The TLC were, chloroform/methanol/92/8 (system a), dichloromethane/hexane/acetone/55/20/25 (system b), dichloromethane/hexane/acetone/45/35/20 (system c), ethyl acetate/hexane/triethylamine/60/30/10 (system d), ethyl acetate/hexane/triethylamine/40/50/10 (system e), ethyl acetate/triethylamine/90/10 (system f), ethyl acetate/hexane/triethylamine/55/35/10 (system g). The HPLC were run using mobile phase of 0.1M triethyl ammonium bicarbonate (TEAB), pH 7.5 and acetonitrile at a flow rate of 1 ml/min. The systems consisted of gradient for 45 mins, 0–60% acetonitrile (system A); gradient for 45

Scheme 1. EthenoB: 1,N⁶Etheno adenyil (Ia, IIa, IIc, IIIa, IIIC); EthenoC: 3,N⁴Etheno cytosil (Ib, IIb, IIIb) (a) DMT-Cl/Pyridine, 5°C; (b) TMT-Cl/Pyridine, 5°C; (c) phosphorylating reagent 10°C.

mins, 0–65% acetonitrile (system B); isocratic, 80% acetonitrile (system C); isocratic, 90% acetonitrile (system D). The reaction time (R_t) is recorded for each compound.

The gel analysis (PAGE) was carried out on BRL Sequencing gel electrophoresis apparatus model S1 using 40×20cm gel plates. The electrophoresis power supply unit was from North Eastern Scientific Co. The gels used were 20% polyacrylamide with thickness of 1 mm. The oligomer purity was established from HPLC data in conjunction with the PAGE analysis. The melting temperature studies on the modified DNA sequences were carried out on Shimadzu UV-1201, UV-VIS spectrophotometer.

High levels of fluorescence were observed in these molecules. The fluorescence measurement of one of these compounds, 5'-DMT-etheno-2'-deoxyadenosine (IIa), is reported. At the 10 nm slit width, excitation at 230 nm wavelength, the fluorescence emission maxima was observed at a concentration of 1×10^{-9} M concentration. The fluorescence spectrophotometric measurements, single dimension, and three dimensional (3-D) spectral plots were also obtained. The fluorescence intensity and characteristics of various compounds and synthetic DNA and RNA molecules containing the etheno chromophore were determined as well. The spectrum for the compound, 5'-DMT-etheno/adenosine (Va) is detected at several concentrations. For synthetic DNA sequence #4 (the sequencing primer with the etheno-2'-deoxyadenosine), concentrations of 90 micromole and 90 picomole were detected with a characteristic pattern.

The polymerase chain reaction (PCR) was performed on a number of primers. An illustration of the amplification reaction and products is provided here on two modified sequences, #6 5'-OH- d(eAGTeAAAACGACGGCCAGT)-3'-OH and #7 5'-OH- (eAeAGTAAAACGACGGCCAGT)-3'-OH, where 'eA' represents 2'-deoxyetheno adenosine. These sequences are M13 sequencing primers. Sequencing primer #6 has the replacement of natural deoxyadenosine by two ethenoadenosine units. Sequencing primer #7 has been elongated at the 5'-terminal of sequencing primer #8 (no modification) by two units of 2'-deoxy ethenoadenosines. The M13 single strand universal sequencing primer #8 is the natural base, modified sequence. The PCR reaction conditions and the results are shown in figure (5).

The melting temperature studies were performed on DNA sequence #4 (M13 single strand universal sequencing primer with the replacement of six deoxyadenosine units with six ethenoadenosine units), as well as on sequence #6 and #7. The complementary sequence, with natural unmodified bases sequence #9, was hybridized with the natural complementary sequence #8, as well as with the etheno modified sequence #4, #6 and #7.

EXPERIMENTAL

Synthesis of 3B-D-2'-deoxyribofuranosylimidazo [2,1-i] purine {1,N⁶-etheno-2'-deoxyadenosine} (Ia) and 5,6-dihydro-5-oxa-6B-D-2'-deoxyribofuranosyl imidazo [1,2-c] pyrimidine {3,N⁴-etheno-2'- deoxycytidine} (Ib). A solution of 2'-deoxyadenosine (5.02 g; 20 mmole) and 2'-deoxycytidine (4.54 g; 20 mmole) was taken in 2M aqueous chloroacetaldehyde (Aldrich Chemical Co., Milwaukee, WI, 30% w/v, diluted appropriately with distilled water) and stirred at 30°C for a period of 12 hours. The pH of the solution was checked every 30 minutes and maintained at 4.5 by dropwise addition of 4M aqueous sodium hydroxide solution. The solution was then evaporated under high vacuum to dryness. The residue was shaken and washed with a mixture

of ether and ethylacetate and filtered to yield colorless flakes. Using thin layer chromatography, the compounds indicated a purity of approximately 90% and 95% for deoxyadenosine and deoxycytidine, respectively. Further purity check was done on HPLC, and compared with the reference sample obtained from Sigma Chemical Co., St Louis, MO. These products were converted directly to their corresponding 5'-O-dimethoxytrityl derivatives without further attempts at purification.

General procedure for the synthesis of 5'-O-DMT-etheno-2'-deoxy and ribo nucleosides and 5'-O-TMT-etheno deoxy and ribo nucleosides: 5'-O-DMT-3B-D-2'-deoxyribofuranosylimidazo [2,1-i] purine (IIa); 5'-O-DMT-5,6-dihydro-5-oxa-6B-D-2'-deoxyribofuranosylimidazo [1,2-c] pyrimidine (IIb); 5'-O-TMT-3B-D-2'-deoxyribofuranosylimidazo[2,1-i]purine (IIc); 5'-O-(IIc); 5'-O-DMT-3B-D- ribofuranosylimidazo [2,1-i] purine (Va); 5'-O-DMT-5, 6-dihydro-5-oxa- 6B-D-ribofuranosylimidazo [1,2-c] pyrimidine (Vb); 5'-O-TMT-3B-D-ribofuranosylimidazo[2, 1-i] purine (Vc). The appropriate ethenodeoxyribonucleoside (Ia or Ib) prepared as describe above and ethenoribonucleoside (48) (IVa or IVb), was taken (10 mmole) in dry pyridine and evaporated several times under high vacuum. The residual gum was taken in dry pyridine and to the solution was added DMT-Cl (11 mmole, for the synthesis of 5'-O-DMT derivatives) or TMT-Cl (11mmole, for the synthesis of 5'-O-TMT derivative) at 5°C. The reaction mixture was stirred for 4–5 hours at 5°C. The mixture was then quenched at 0°C with 20% aqueous pyridine. The total mixture was dried under vacuum. The gum was extracted with chloroform, washed with saturated aqueous sodium bicarbonate once, followed by saturated aqueous sodium chloride solution, and evaporation of the solvent on a rotary evaporator. The residue was purified on a silica gel column using chloroform and a gradient system consisting of 2–6% methanol in chloroform. The pure product was pooled and dried. The yields were 82–87%. TLC R_f for compound IIa, 0.32 (system a); IIb, 0.57 (systemd d); IIC, 0.30 (system a); Va, 0.27 (system a); Vb, 0.52 (system a); Vc 0.25 (system a). HPLC analysis shows sharp single peak in all the cases, compound IIa, R_t , 4' (system C), IIB 225sec (system C), IIC, R_t , 220sec (system C), Va, R_t 240sec (system C), Vb, 4' (system C), Vc, 240sec (system C). UV (methanol) λ max (nm) IIa, 250 (ϵ 10264), 260 (ϵ 7842) ratio 250/260; 1.31; 260/280; 1.24; 280/300; 1.74, IIb, 280 nm, (ϵ 13102) ratio 250/260; 0.94, 260/280; 0.84 280/300; 12.24.

¹H NMR(CDCl₃) IIa, δ 8.67 (s,1,H₅), 8.07 (s,1,H₂), 7.61–7.62 (d,1,H₇), 7.64–7.65 (d,1,H₈), 6.7–7.4 (m,13,Ar), 6.50–6.54 (t,1,H'₁), 4.16–4.27 (m,1,H'₃), 4.74–4.85 (m,1,H'₄), 3.77 (m,6,OCH₃), 3.34–3.49 (2qt,2,H'₅ and H''₅), 2.54–2.64 and 2.80–3.04 (m,2,H'₂ and H''₂). IIb, δ 7.70–7.71 (d,1,H₃), 7.40–7.41 (d,1,H₂), 7.62–7.65 (d,1,H₇), 6.33–6.35 (d,1,H₈), 6.80–7.40 (m,13,Ar), 6.57–6.61 (t,1,H'₁), 4.60–4.67 (m,1,H'₃), 4.12–4.08 (qt,1,H'₄), 3.79 (s,6,OCH₃), 3.45–3.58 (2qt,2,H'₅ and H''₅), 2.30–2.41 and 2.48–2.58 (m,2,H'₂ and H''₂). IIC, δ 8.67 (s,1,H₅), 8.09 (s,1,H₂), 7.61–7.62 (d,1,H₇), 7.64–7.65 (d,1,H₈), 6.74–7.31 (m,12,Ar), 6.50–6.54 (t,1,H'₁), 4.16–4.23 (m,1,H'₃), 4.74–4.81 (m,1,H'₄), 3.74 (s,9,OCH₃), 3.34–3.49 (m,2,H'₅ and H''₅), 2.54–2.64 and 2.80–2.90 (m,2,H'₂ and H''₂). Va, δ 8.39 (s,1,H₅), 8.00 (s,1,H₂), 7.486–7.491 (d,1,H₇), 7.505–7.510 (d,1,H₈), 6.7–7.4 (m,13,Ar), 6.14–6.16 (d,1,H'₁), 4.50–4.55 (m,1,H'₂), 4.37–4.39 (m,1,H'₃), 4.96–5.02 (m,1,H'₄), 3.33–3.58 (bm,2,H'₅ and H''₅), 3.69 (s,6,OCH₃). Vb, δ 7.66–7.67 (d,1,H₃), 7.39–7.40 (d,1,H₂),

7.62–7.65 (d,1,H₇), 6.33–6.36 (d,1,H₈), 6.70–7.40 (m,13,Ar), 6.12 (d,1,H'₁), 4.38–4.43 (d,1,H'₂), 4.48–4.43 (d,1,H'₃), 4.27–4.29 (m,1,H'₄), 3.42–3.56 (q,2,H'₅ and H''₅), 3.77 (s,6,OCH₃). Vc, δ 8.40 (s,1,H₅), 8.02 (s,1,H₂), 7.44–7.46 (d,1,H₇), 7.49–7.50 (d,1,H₈), 6.69–7.30 (m,12,Ar), 6.15–6.17 (d,1,H'₁), 4.51–4.56 (m,1,H'₂), 4.38–4.40 (m,1,H'₃), 4.96–5.02 (m,1,H'₄), 3.43–3.50 (bm,2,H'₅ and H''₅), 3.69 (s,9,OCH₃).

General procedure for the synthesis of 5'-O-DMT-etheno-2'-and 3'-tert-butylidimethylsilyl-ribonucleosides

This procedure was used to make the following compounds: 5'-O-DMT-3B-D-2'-tert-butylidimethylsilyl-ribofuranosylimidazo [2,1-i] purine (VIa), 5'-O-DMT-5,6-dihydro-5-oxa-6B-D-2'-ribofuranosylimidazo [1,2-c]pyrimidine (VIb), 5'-DMT-3BD-2'-tert-butylidimethylsilyl-ribofuranosylimidazo [2,i-c] purine (VIIa), and 5'-DMT-5,6-dihydro-5-oxa-6B-D-3'-tert-butylidimethylsilyl-ribofuranosylimidazo [1,2-c] pyrimidine (VIIb).

A solution of 5'-O-DMT-ethenoribonucleosides (Va, Vb, 5 mmole) in THF (40ml) was added to a slurry of AgNO₃ (6 mmole) in dry pyridine (18.5 mmole). The total mixture was stirred under dry atmosphere for 15 minutes, to this mixture was added solid tert-butylidimethylsilylchloride (6.5 mmole), all at once. The mixture was stirred for 5 hours at room temperature in the dark. The mixture was filtered to remove the solid. To the filtrate was added 5% aqueous KHCO₃ (40 ml) and the combined mixture was extracted into chloroform (3×80 ml). The total organic layer was washed with water to neutral (3×20 ml), followed by drying over anhydrous sodium sulfate. The dried organic layer was concentrated under vacuum. The residual gummy mass was dried with dry toluene (2×20 ml). The residual gum was purified by medium pressure column chromatography. Compounds VIa and VIIa were formed in a ratio of 65:35. The column chromatography systems were as follows: for compounds VIa and VIIa, dichloromethane, hexane, and acetone in the ratio of 55:20:25. Compounds VIb and VIIb were obtained in a ratio of 88:12. The column chromatography system for structures VIb and VIIb was dichloromethane, hexane, and acetone in a ratio of 45:35:20. TLC R_f for compound VIa, 0.38 (system b); VIb, 0.42 (system c), VIIa, 0.19 (system b); VIIb, 0.33 (system c). The HPLC analysis showed sharp single peak in all the cases, VIa, R_t, 360sec (system C), VIb, 500sec (system C). Elemental analysis: VIa, found; C, 65.38; H, 6.64; N, 9.62; calculated, C, 66.19; H, 6.36; N, 9.84; VIb, found; C, 65.73; H, 6.85; N, 5.90; calculated, C, 66.76; H, 6.59; N, 6.15.

¹H NMR(CDCl₃) VIa, δ 8.63 (s,1,H₅), 8.16 (s,1,H₂), 7.66 (s,2,H₇ and H₈), 6.68–7.51 (m,13,Ar), 6.09–6.11 (d,1,H'₁), 4.35–4.41 (m,1,H'₂), 4.28–4.31 (m,1,H'₃), 5.04–5.07 (m,1,H'₄), 3.32–3.41 and 3.49–3.58 (qt,2,H'₅ and H''₅), 3.77 (s,6,OCH₃), 0.00 (s,6,Si-Me), 0.84 (m,9,t-Bu-Si). VIb, δ 7.73–7.74 (d,1,H₃), 7.43–7.44 (d,1,H₂), 7.75–7.77 (d,1,H₇), 6.23–6.26 (d,1,H₈), 6.80–7.40 (m,13,Ar), 6.23–6.26 (d,1,H'₁), 4.37–4.45 (m,2,H'₂ and H''₃), 4.15–4.18 (m,1,H'₄), 3.46–3.68 (qt,2,H'₅ and H''₅), 3.79 (s,6,OCH₃), 0.00–0.12 (s,6,Si-Me), 0.91–0.93 (s,9,t-Bu-Si).

General procedure for the synthesis of 5'-O-DMT-etheno-2'-deoxy/5'-O-TMT-etheno-2'-deoxy/2'-tert-butylidimethylsilyl-3'-cyanoethylphosphoramidite-pyrimidine and purine nucleosides (IIIa, IIIb, IIIc, VIIIa, and VIIIb)

Compound IIa or IIb (1 mmole) was thoroughly dried with dry pyridine and taken up in dry chloroform in a flask equipped with

an argon gas inlet and outlet. To the stirred solution was added N,N-diisopropyl-ethylamine (5 mmole), the solution maintained at 5°C. To this solution was added dropwise N,N-diisopropyl-amino cyanoethyl phosphonamidic chloride (2.5 mmole), followed by further reaction at 20°C for additional 2 hours. The reaction mixture was diluted with ethylacetate and washed with saturated aqueous sodium bicarbonate once, followed by saturated aqueous sodium chloride once. The organic layer was dried over anhydrous sodium sulfate, followed by evaporation under vacuum. The residue was purified by column chromatography. The solvent system for compound IIIa was ethylacetate and triethylamine in the ratio of 90:10. The solvent system for compound IIIb was ethylacetate, hexane, and triethylamine mixture in a ratio of 55:35:10. The TLC R_f of compound IIIa, 0.40 and 0.46 (system f); IIIb, 0.46 and 0.56 (system g); IIIc, 0.44 and 0.51 (system f). HPLC analysis showed sharp doublets, IIIa, R_t 390sec (system C); IIIb, 515sec and 600sec (system C); IIIc, 360sec (system C).

Products VIIIa and VIIIb were synthesized from compounds VIa and VIb. The dried compounds, VIa or VIb (1 mmole), were taken in dry tetrahydrofuran (approximately 15 ml) and connected with argon gas inlet and outlet adapter. To this solution was added collidine (7.5 mmole) and 1-methylimidazole (1 mmole), the solution maintained at 5°C. To this solution, N,N-diisopropylamino- cyanoethyl phosphonamidic chloride (2.5 hours) was added dropwise. The reaction mixture was then diluted with ethylacetate to ten times the solution volume and washed once with saturated aqueous sodium bicarbonate, followed by washing once with saturated aqueous sodium chloride. The organic layer was dried with anhydrous sodium sulfate. The residue was evaporated under high vacuum. The crude product was purified by column chromatography. The solvent system for compound VIIIa was ethylacetate, hexane, and triethylamine in a ratio of 60:30:10. The solvent system for compound VIIIb was ethylacetate, hexane, and triethylamine in a ratio of 40:50:10. TLC R_f VIIIa, 0.51 (system d); VIIIb, 0.56 (system e). HPLC analysis showed sharp doublet. R_t, VIIIa, 490sec (system D), VIIIb, 630sec (system D). Elemental analysis: IIIa, found, C, 64.26; H, 6.46; N, 12.30; calculated, C, 64.86; H, 6.18; N, 12.61; IIIb, found, C, 67.65; H, 6.76; N, 9.19; calculated, C, 65.34; H, 6.37; N, 9.30; Ic, found, C, 62.16; H, 6.62; N, 11.49; C, 63.94; H, 6.20; N, 12.14; VIIIa, found, C, 62.92; H, 6.94; N, 10.52; calculated, C, 63.5; H, 6.8; N, 10.8; VIIIb, found, C, 64.19; H, 7.29; N, 7.74; calculated, C, 63.87; H, 7.02; N, 7.93.

¹H NMR(CDCl₃) IIIa, δ 8.68–8.70 (as,1,H₅), 8.10–8.14 (ts,1,H₂), 7.64–7.65 (as,1,H₇), 7.66–7.67 (ss,1,H₈), 6.7–7.4 (m,13,Ar), 6.49–6.53 (bt,1,H'₁), 2.88–2.99 and 2.66–2.76 (tm,2,H'₂ and H''₂), 4.31–4.38 (m,1,H'₃), 4.73–4.83 (m,1,H'₄), 3.57–3.75 (tm,2,H'₅ and H''₅), 3.75–3.76 (ts,6,OCH₃), 3.34–3.52 (m,2,POCH₂), 2.46–2.52 and 2.58–2.66 (tt,2,CH₂CN), 1.22–1.32 (m,2,pylmethine), 1.12–1.27 (Octet,12,pylmethyls). IIIb, δ 7.71–7.72 (m,1,H₃), 7.41 (m,1,H₂), 7.60–7.70 (td,1,H₇), 6.29–6.32 (q,1,H₈), 6.80–7.40 (m,13,Ar), 6.57–6.63 (q,1,H'₁), 2.61–2.65 (m,2,H'₂ and H''₂), 4.70–4.75 (m,1,H'₃), 4.17–4.22 (m,1,H'₄), 3.66–3.90 (m,2,H'₅ and H''₅), 3.79 (d,6,OCH₃), 3.39–3.66 (t,2,POCH₂), 2.43–2.45 (t,2,CH₂CN), 1.20–1.38 (m,2,pylmethine), 1.08–1.19 (tq,12,pylmethyls). IIIc, δ 8.68–8.70 (as,1,H₅), 8.10–8.14 (ts,1,H₂), 7.64–7.65 (as,1,H₇), 7.66–7.67 (as,1,H₈), 6.70–7.40 (m,13,Ar), 6.49–6.53 (bt,1,H'₁), 2.89–2.99 and 2.67–2.77 (tm,2,H'₂

and H₂), 4.32–4.38 (m, 1, H₃), 4.73–4.84 (m, 1, H₄), 3.57–3.76 (tm, 2, H₅ and H₅'), 3.34–3.52 (m, 2, POCH₂) 2.46–2.52 and 2.57–2.65 (tt, 2, CH₂CN), 1.21–1.32 (m, 2, pylmethine), 1.11–1.28 (Octet, 12, pylmethylyls). VIIIa, δ8.60–8.63 (d, 1, H₅), 8.16–8.19 (d, 1, H₂), 7.64–7.65 (as, 1, H₇ and H₈), 6.78–7.70 (m, 13, Ar), 6.03–6.05 and 6.12–6.14 (td, 1, H₁'), 4.32–4.42 (m, 1, H₂'), 3.81–4.02 (m, 1, H₃'), 5.03–5.13 (m, 1, H₄'), 3.58 and 3.59–3.69 (bm, 2, H₅ and H₅'), 3.77 (ts, 6, OCH₃) 3.25–3.30 and 3.48–3.61 (tt, 2, POCH₂), 2.24–2.31 and 2.64–2.71 (tt, 2, CH₂CN), 1.15–1.30 (m, 2, pylmethine), 1.00–1.23 (m, 12, pylmethylyls), 0.00 (d, 6, Si-Me), 0.76 (m, 9, t-Bu-Si). VIIIb, δ7.65–7.69 (m, 1, H₅), 7.37–7.41 (m, 1, H₂), 7.75–7.78 (d, 1, H₇), 6.20–6.23 (d, 1, H₈), 6.75–7.40 (m, 13, Ar), 6.12–6.19 (tm, 1, H₁'), 4.23–4.31 (m, 1, H₂'), 4.32–4.48 (m, 1, H₃'), 4.18–4.24 (m, 1, H₄'), 3.54–3.64 (m, 2, H₅ and H₅'), 3.72–3.73 (ts, 6, OCH₃) 3.29–3.49 (tm, 2, POCH₂), 2.27–2.34 (tm, 2, CH₂CN), 1.07–1.24 (m, 2, pylmethine), 0.92–1.16 (m, 12, pylmethylyls), 0.02 (bm, 6, Si-Me), 0.78–0.79 (s, 9, t-Bu-Si). ³¹PNMR (CDCl₃) IIIa, δ143.518, 143.624, Δ0.106 H₂; IIIb, δ143.524, 144.026, Δ0.502 H₂; IIIc, δ143.130, 143.232, Δ0.102 H₂; VIIIa, δ144.680, 146.680, Δ2.00 H₂; VIIIb, δ144.326, 145.047, Δ0.721 H₂.

Synthesis of 5'-O-DMT-etheno-2'-deoxy adenosine/5'-O-TMT-etheno-2'-deoxy adenosine/3'-hemisuccinate (XI) and attachment to CPG

Compound IIa was converted to the corresponding hemisuccinate (XI) as outlined in scheme 2, under standard conditions and purified by column chromatography. The purified hemisuccinate was converted to its N-hydroxy succinimide and treated with aminopropyl CPG in DMF. The derivatized CPG was capped with acetic anhydride/pyridine to yield the final CPG (XII). Compound IIc was similarly attached to the CPG and a loading of approximately 37 μm/gm was obtained. The TLC R_f for XI, 0.32 (system a). HPLC analysis of XI showed sharp single peak, R_t, 255sec (system B).

DNA and RNA synthesis with etheno deoxy and ribonucleoside phosphoramidites (IIIa, IIIb, VIIIa, and VIIIb)

The following defined sequence DNA and RNA molecules containing etheno modified deoxy and ribo nucleosides were synthesized in order to test the coupling efficacy, as well as to produce certain sequences which are known to possess specific biological properties. The coupling obtained at each step was greater than 98.5% per step, for the etheno deoxy ribo nucleoside, as well as etheno ribonucleoside phosphoramidites. The synthesis was carried out using standard protocols, which are used for protected deoxy and ribo-nucleoside phosphoramidites. The capping step involved the cap A solution (acetic anhydride/2,6-lutidine/CH₃CN; 1:1:8) and the cap B solution (1-methylimidazole/THF; 16% w/v), for a period of 15 seconds.

Representative DNA Sequences: (1) 5-mer DNA sequences: 5'-OH-d(eA-eA-eA-eA)-dC-3'-OH. (2) 7-mer DNA sequences: 5'-OH-d(eA-eA-eA-eA-eA-eA)-dC-3'-OH. (3) 11-mer DNA sequence: 5'-OH-d(eC-eC-eC-eC-eC-eC-eC-eC-eC-eC)-dC-3'-OH. (4) 17-mer universal sequencing primer with ethenodeoxy adenosine substitution in place of natural deoxyadenosine 5'-OH-d(G-T-eA-eA-eA-eA-C-G-eA-C-G-G-C-C-eA-G-T)-3'-OH. (5) 17-mer universal sequencing primer with ethenodeoxy cytidine substitution in place of natural deoxycytidine

5'-OH-d(G-T-A-A-A-A-eC-G-A-eC-G-G-eC-A-G-T)-3'-OH. (6) 18-mer universal sequencing primer with one internal ethenoadenosine, and another external ethenoadenosine (at the 5'-end) 5'-OH-d(eA-G-T-eA-A-A-A-C-G-A-C-G-G-C-C-A-G-T)-3'-OH. (7) 19-mer universal sequencing primer with two external ethenoadenosine (at the 5'-end) 5'-OH-d(eA-eA-G-T-A-A-A-A-C-G-A-C-G-G-C-C-A-G-T)-3'-OH. (8) M13 single strand universal sequencing primer 5'-OH-d(G-T-A-A-A-A-C-G-A-C-G-G-C-C-A-G-T)-3'-OH. (9) M13 universal sequencing primer complementary strand 5'-OH-d(A-C-T-G-G-C-C-G-T-C-G-T-T-T-T-A-C)-3'-OH.

Representative RNA sequences: (1) 7-mer RNA sequence: 5'-OH-r(eC-eC-eC-eC-eC-eC)-C (2) 7-mer RNA sequence: 5'-OH-r(eA-eA-eA-eA-eA-eA)-C. The HPLC profile of representative sequences is shown in figure (2 and 3). The UV absorbance of the DNA sequence #4, ratio 250/260; 1.0, 260/280; 1.40, 280/300; 4.72.

Purity and stability studies of the ethenodeoxynucleosides and the synthetic oligodeoxynucleotides

In order to establish conditions for obtaining essentially pure oligomers, various reaction parameters were studied. Since aqueous ammonia, (28%) treatment is one of the key steps for deprotection of base protecting groups and detachment from the solid support, its effect on the ethenonucleosides was noted. The most convenient compound for this study was 5'-O-DMT-2'-deoxy etheno adenosine (IIa). The breakdown of this compound in contact with aqueous ammonia was easily monitored on thin layer chromatography. As seen in fig (1), this compound is quite stable in aqueous ammonia upto 2 hrs. During prolonged treatment there is progressively increasing amount of a major breakdown product. The nature of the breakdown products has been discussed previously (53). In the DNA synthesis, we therefore utilized our own commercially available phosphoramidites of natural DNA bases, which have been protected with fast deprotecting groups (viz. 5'-O-DMT-N⁶-phenoxyacetyl-2'-deoxy adenosine-3'-phosphoramidite, 5'-O-DMT-N⁴-isobutyryl-2'-deoxy cytidine-3'-phosphoramidite, 5'-O-DMT-N²-phenoxyacetyl-2'-deoxy guanosine-3'-phosphoramidite).

Figure 1. Aqueous ammonia, (28%) time course study on 5'-O-DMT-2'-deoxy etheno adenosine (IIa): Lanes (1); 2 hours aqueous ammonia, (2); 4 hours aqueous ammonia, (3); 7 hours aqueous ammonia, (4); 24 hours aqueous, (5); 31 hours aqueous ammonia, (6); 48 hours aqueous ammonia, (7); 72 hours aqueous ammonia.

Scheme 2. (a) Succinic anhydride/Pyridine, 37°C (b) Amino CPG; (c) Acetic anhydride/Pyridine (capping) (d) aq ammonia; (e) H⁺Rn on solid support using 3% MCA, 3% DCA, 2% TCA.

Etheno B: 1,N⁶Etheno adeny1 (IVa, Va, Vc, VIa, VIIb, VIIIb); Etheno B: 3,N⁴Etheno cytosil (IVb, Vb, VIb, VIIb, VIIIb); (a) DMT-Cl/Pyridine, 5°C; (b) BDSi-Cl; (c) Phosphorylating reagent, 10°C; (d) TMT-Cl/Pyridine, 5°C.

Scheme 3.

Figure 2. (i), DNA seq # 2; crude after aq ammonia, (2hr); (ii), DNA seq # 7, crude aq ammonia, (2hr) utilizing fast deprotecting nucleoside bases.

This enabled us to carry out the total deprotection step within 2 hr. The effect of acid was similarly studied on 5'-O-DMT-2'-deoxy etheno adenosine (IIa). This was done in a simple way by extended contact (from 30secs up to 5 mins) with 3% MCA, 3% DCA and 2% TCA solution in dichloromethane (DCM) on CPG solid support (Scheme 2). The columns were packed with derivatized CPG (XII), loading of 0.2 μ m. Each column was separately brought in contact with the acids for different time periods (30 sec, 60sec, 120sec, 240sec and 300sec) on the DNA synthesizer. At the end of the time period the columns were washed with DCM cycle ($\times$ 3). The CPG was then treated with aqueous ammonia for 1hr at room temp. After the removal of ammonia, the products were analyzed on TLC and HPLC (fig 2). The listed deoxy oligonucleotide sequences were synthesized using the etheno phosphoramidites (IIIa and IIIb), in conjunction with the natural DNA phosphoramidites with conventional base protecting groups (viz., 5'-O-DMT-Nbz-dA-CNEt phosphoramidite, 5'-O-DMT-Nbz-dC-CNEt phosphoramidite, 5'-O-DMT-Nibu-dG-CNEt phosphoramidite, 5'-O-DMT-T-CNEt phosphoramidite). Sequences #2 and #7 were also synthesized from 5'-TMT-etheno-dA phosphoramidite (IIIc) in conjunction with the natural DNA phosphoramidites with fast base deprotecting groups (viz., 5'-O-DMT-N⁶-phenoxy-acetyl-2'-dA-CNEt phosphoramidite, 5'-O-DMT-N⁴-ibu-2'-dC-CNEt phosphoramidite, and 5'-O-DMT-N²-phenoxy-acetyl-2'-dG-CNEt phosphoramidite).

RESULTS

The chemistry has been developed to produce etheno modification on deoxynucleosides, viz., 2'-deoxyadenosine and 2'-deoxycytidine. These compounds (Ia) and (Ib) are then protected at the 5'- position with dimethoxytrityl group (DMT) to yield compounds IIa and IIb, in 82–87% yield. These products are then converted to cyanoethyl phosphoramidites (IIIa) and (IIIb) in high yields. Similarly the 1,N⁶ etheno adenosine (IVa) and 3,N⁴ etheno cytidine (IVb) were selectively converted to their

Figure 3. HPLC profile of etheno dA (Ia, peak A) generated after 3% DCA time course study on 5'-O-DMT-deoxy ethenoadenosine attached to CPG (structure XII) followed by 1hr aq ammonia treatment DCA contact time, (i); 30 secs, (ii); 300 secs, peak B increased almost twice during the 5 min. acid contact.

5'-DMT derivatives (Va) and (Vb) in high yields. Sugar protection of these RNA intermediates was carried out by t-butyl dimethyl silyl chloride, by modification of the general procedures (58). The 2'- and 3'- Silyl-isomers (VIa, VIb; VIIa and VIIb) were obtained with purity in excess of 99.95%. The selective 3'-phosphorylation of the 2'-tert butyl silyl protected products (VIa, VIb) was carried out with N,N-disopropylamino-cyanoethyl phosphonamidic chloride, by modification of the literature procedure (59). The pure products (VIIIa and VIIIb) were obtained in 100% isomeric purity and above 99.2% overall purity.

The solid phase DNA synthesis utilizing the intermediates (IIIa and IIIb), when carried out utilizing the standard protocol for deoxynucleoside phosphoramidites; the coupling time of 180 secs, proceeded smoothly to greater than 99% per step. When the standard deprotection conditions (16–18 hours aq ammonia @ 37°C) were utilized, the reversed phase HPLC analysis of the crude oligomer revealed one major peak and several additional smaller peaks. However reducing the aq ammonia treatment time to less than 2 hrs, had significant effect on the HPLC profile. Only a single peak was observed. This modification of deprotection condition was done on sequences #2 and #7, and these sequences were synthesized utilizing the deoxy nucleoside phosphoramidites protected with fast base deprotecting groups (figs 2). The effect of stronger acids (3% DCA and 2% TCA in DCM) was studied (Scheme 2) in a simple way. The contact with deoxy etheno adenosine derivatized CPG (structure XII) at various time intervals, followed by 1hr aq ammonia treatment to detach the CPG bound etheno dA, showed increasing amount of peak B (fig 3). Contact with 3% MCA did not generate peak B. These data suggest that replacement of 5'-O-DMT-group with 5'-O-TMT would minimize the breakdown caused by the conventional detritylating reagent during the DNA synthesis. The solid phase RNA synthesis; utilizing the intermediates (VIIIa and

1 2 3 4 5 6 7

Figure 4. PAGE analysis of the etheno deoxy oligomers. Lanes (1); DNA seq # 8, (2); a 21-mer natural DNA sequence, (3); Blank (4) 18-mer seq # 6, crude using standard protecting groups, 16 hr aq ammonia at 37°C, (5); 19-mer etheno seq # 7, using fast deprotecting groups, 2hr aq ammonia at room temp, (6); 19-mer etheno seq # 7, as in (5), HPLC purified, (7); 18-mer seq # 6, aq ammonia, 12hr at room temp.

VIIIb), when carried out utilizing the standard protocols for 2'-tert butyl silyl protected-ribo nucleoside phosphoramidites, with the coupling time of 570 seconds (9.5 minutes), proceeded smoothly to greater than 99% per step. The coupling efficiency is maintained consistent throughout the multiple addition of the fluorescent unit.

The fluorescent characteristics of various intermediates and the synthetic DNA were determined and three dimensional contour plots were obtained. The compounds DMT ribo-etheno A (at 200 mM conc), showed strong emission at 445 nm, with the excitation at 350 nm. The 200ppm solution of this compound has a slight shift of emission maxima at 455, with the excitation at 340 nm. The synthetic DNA primers (sequence # 4), at concentration of 1.5 μ M in deionized water, showed emission maxima at 410 nm, with excitation at 270 nm. The same primer was observed to possess emission at 410 nm at a concentration of 15 pM adjusted for excitation at 300 nm. A number of secondary emission bands were observed in the 3-D spectrum of the above DNA sample (the data is available from the authors).

The PCR amplification was tested on a number of DNA sequences. The initial amplification was attempted on the sequence # 4 (containing all edA replacement in place of dA units), the 17-mer universal sequencing primers, and the sequence # 5 (containing all edC replacement in place of dC units), in the 17-mer universal sequencing primer and using sequence # 8, the 17-mer natural universal sequencing primer as a control. The results indicated that the primers 4 and 5 did not undergo amplification. Failure of this test indicated that such extensive modification would not be tolerated either by the taq polymerase

1 2 3 4 5 6 7 8 9 10 11

Figure 5. PCR* amplification of the modified primers, Agarose 1% Composigel in TAE with ETBR. Reaction (Rcn) conditions: Rcn A: Denaturation time (t); 20 secs; temp; 95°C, annealing t; 30 secs; temp 55°C, elongation t; 60 secs; temp; 72°C, # of repetitions; 35. Rcn B: Denaturation time (t); 20 secs; temp; 95°C, annealing t; 30 secs; temp 40°C, elongation t; 60 secs; temp; 72°C, # of repetitions, 35. Lanes (1); Phi-X/HaeIII marker, (2); DNA seq # 6 Rcn A, (3); DNA seq # 7 Rcn A, (4); DNA sequencing primer # 6 Rcn A, (5); DNA seq # 6 Rcn A, (6); DNA seq # 7 Rcn A, (7); DNA seq primer # 8 Rcn A (8); DNA seq # 6 Rcn B, (9); DNA seq # 7 Rcn B, (10); DNA seq primer # 8 Rcn B.

or for the lack of substantial hybridization. Incorporation of the edA units at the 5'- end of the primer; as depicted in DNA sequence # 6, or by extension of the sequencing primer by a few units at the 5'- end, as depicted in DNA sequence # 7, resulted in primers which are now fully capable of participation in the amplification and chain extension to size matching with the control 17-mer natural universal sequencing primer (sequence # 8, fig 5).

DISCUSSION

For the purpose of introduction of selective single or multiple units of 1,N⁶-ethenodeoxy adenosine; 3,N⁴-etheno deoxy cytidine; 1,N⁶-ethenoadenosine and 3,N⁴ etheno cytidine units into defined sequence DNA and RNA, various intermediates (Ia, Ib, IIa, IIb, IIc, Va, Vb, Vc, VIa, VIb, VIIa, VIIb), (XI and XII), and the 3'-phosphoramidite (IIIa, IIIb, IIIc, VIIIa, VIIIb) have been developed. The phosphoramidite purity has been achieved to a level, which yields consistently higher levels of incorporation of these units into DNA and RNA sequence. The fluorescence properties and characteristics have been determined in these intermediates and they are maintained into the defined sequence DNA and RNA molecules. Furthermore, since the coupling efficiency is maintained greater than 99% per step during the DNA/RNA synthesis, it indicates that the phosphoramidites can be successfully utilized for multiple addition. Deprotection of the synthetic oligonucleotides requires many modifications of the standard DNA synthesis protocol. Fast base deprotecting groups and a short treatment with ammonia is essential to ensure that no degradation of the etheno moiety takes place. This can be achieved if the total contact time with aq ammonia is less than 2 hrs. We also observed that the conventional capping step during DNA synthesis does not cause modification or the breakdown of deoxyetheno adenosine. However continued contact with the conventional detritylating reagents generates additional peak, presumably depurination (fig 3). The protection of 5'-hydroxyl with the TMT group and the use of 3% MCA minimizes this

side reaction. With these modifications, the quality of crude oligomers is consistently very high. We did not encounter any unusual stability problems with the synthesis of 5'-TMT protected nucleosides (IIc) or the corresponding phosphorimidites (IIIc) (56,57).

The PCR amplification on selective test primers, which were modified with etheno deoxy Adenosine and etheno deoxy Cytidine, revealed that multiple incorporation (replacement) of these units in the sequence towards the 3'- end or in the center of the primer results in loss of amplification capability. Incorporation or replacement of these units towards the 5'- end of the molecule do not cause loss of amplification capability. Instead the later modifications have PCR amplification capability, which is comparable to the natural primer.

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ABBREVIATIONS

s:singlet; d:doublet; q:quartet; m:multiplier; sd:sharp doublet; ss:single singlet; td: two doublets; tq:two quartets; Ar:areomatic; dq:doublet of quartet; tm:two multiplets; ts:two sharp singlets; as:apparent single peak; bt:broad triplet; tt: two triplets; bm:broad multiplet; t-Bu-Si:tertbutylsilyl; pylmethine: isopropyl methine; Si-Me:methylsilyl; isopylmethine:isopropylmethine.

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