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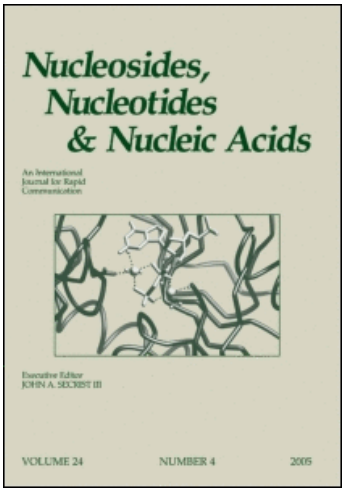

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
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Synthesis and Applications of Oligoribonucleotides Containing N^4-Methylcytidine

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To cite this Article Grasby, Jane A. , Singh, Mohinder , Karn, Jonathan and Gait, Michael J.(1995) 'Synthesis and Applications of Oligoribonucleotides Containing N^4-Methylcytidine', Nucleosides, Nucleotides and Nucleic Acids, 14: 3, 1129 — 1132
To link to this Article: DOI: 10.1080/15257779508012548
URL: http://dx.doi.org/10.1080/15257779508012548

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NUCLEOSIDES & NUCLEOTIDES, 14(3-5), 1129-1132 (1995)

SYNTHESIS AND APPLICATIONS OF OLIGORIBONUCLEOTIDES CONTAINING N^4-METHYLCYTIDINE

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Abstract. The modified nucleoside N^4-methylcytidine was incorporated in place of individual C residues in synthetic TAR and RRE RNA duplexes representing the binding sites for the HIV-1 tat and rev proteins respectively. In no case was cognate protein binding disrupted showing that the exocyclic amino groups of C residues are not sites of protein recognition.

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Introduction

We report a method for the synthesis of a phosphoramidite synthon of the modified nucleoside N^4-methylcytidine (N^4Me-C). When N^4Me-C is incorporated into RNA duplexes in place of C, normal Watson-Crick base-pairing may be maintained but hydrogen-bond donation by the exocyclic amino group of C to proteins binding in the major groove is eliminated. We have utilized N^4Me-C substitution in our studies of the interaction of the Human Immunodeficiency Virus (HIV) TAR and RRE RNA elements with their corresponding regulatory proteins tat and rev[1].

Results

A 5'-*O*-DMT- 2'-*O*-tBDMS-N^4Me-C 3'-*O*-(2-cyanoethyl-*N*,*N*-diisopropylamino)phosphite synthon was prepared from 2',3',5'-tri-*O*-benzoyluridine in 6 steps. The route to N^4Me-C followed a similar procedure to that previously used for the corresponding 2'-deoxy derivative which involves displacement of an intermediate 4-triazolo derivative by methylamine[2]. The desired phosphoramidite was prepared from N^4Me-C by dimethoxytritylation, *t*-butyldimethylsilylation and phosphitylation by

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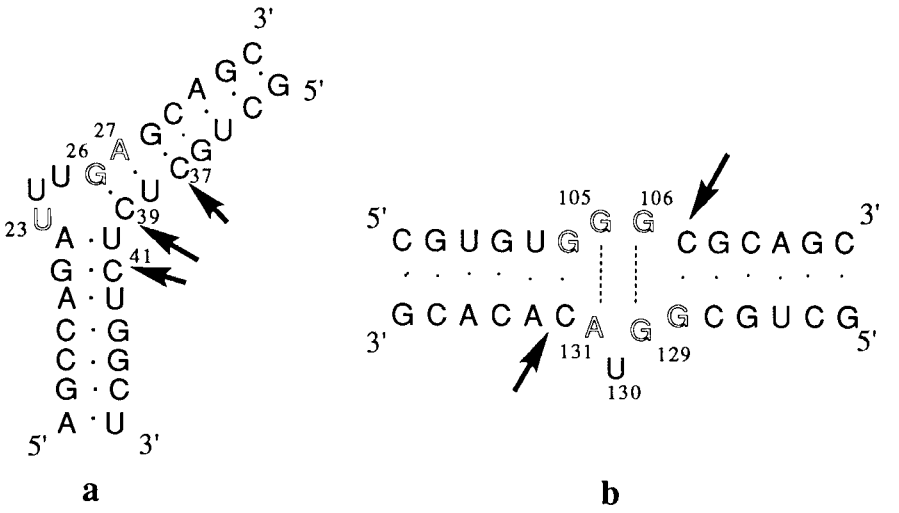

FIG. 1. (a) A synthetic model TAR RNA duplex (tat recognition) and (b) a synthetic model RRE duplex (rev recognition). Bases implicated in protein binding are outlined. Arrows indicate individual N^4Me-C substituted residues.

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standard methods and essentially as described previously for N^2-acetyl-O^6-methylguanosine[3]. The synthon was incorporated into oligoribonucleotides by solid-phase phosphoramidite synthesis with good coupling yields (ca. 98%).

Oligoribonucleotides containing single N^4Me-C substitutions were synthesized corresponding to one of the strands of a model duplex for either the *trans*-activation response element (TAR) (3 oligonucleotides) or the high affinity site of the rev-response element (RRE) (2 oligonucleotides)(Figure 1). After annealing, each RNA duplex was tested in an *in vitro* competitition gel retardation binding assay to the corresponding HIV regulatory protein, tat or rev respectively [4-6]. In none of the 5 cases (3 tat-TAR, 2 rev-RRE) was protein binding found to be affected significantly by N^4Me-C substitution compared to the unmodified RNA duplex.

Discussion

Both HIV tat and rev proteins have been proposed to bind to the major groove of their respective RNA recognition elements (TAR and RRE) at regions of duplex distortion, and in each case binding is disrupted by N^7-deaza-G substitution at G:C pairs flanking the distortion (G26 in TAR[4], G104

and G128 in RRE[5,6]). However, the lack of effect of N^4-methylation on protein binding of any of the corresponding C residue partners (C39 in TAR, C132 or C107 in RRE) suggests that none of the exocyclic amino groups are recognized, a surprising result bearing in mind the proximity of N^7 and NH_2 groups in the duplex major groove.

We have found that the T_m of N^4-Me-C substituted RRE duplex at position 132 is unaltered compared to the unmodified duplex (53°C), although the co-operativity of melting is reduced. We therefore conclude that N^4Me-C substitution does not significantly perturb RNA duplex formation and that correct Watson-Crick pairing of G: N^4Me-C takes place. Note that N^4Me-C substitution in DNA duplexes has been reported to cause considerable duplex destabilization[2,7] but crystals of d(CGCGm4CG) in duplex Z-form show good Watson-Crick G:N^4Me-C pairs with the methyl group *trans* to the N^3 [8].

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Methods

4-Triazolo-2',3',5'-tri-*O*-benzoyluridine (1). To a solution of 1,2,4-triazole (10.4g, 15mmol) in dry acetonitrile (250ml) was added phosphorusoxychloride (2.7ml, 30mmol) dropwise under anhydrous conditions. The resulting solution was stirred at 0° for 10min. Triethylamine (25ml, 18mmol) was added dropwise and the resultant slurry was stirred for a further 30min at 0°. A solution of 2,3,5-tri-*O*-benzoyluridine (5.56g, 10mmol) in acetonitrile (150ml) was added dropwise and the mixture allowed to warm slowly to room temperature. After 3h, the reaction was complete (tlc: CH_2Cl_2 90: Et_2O 10) resulting in the formation of a fluorescent product. Acetonitrile was removed by evaporation and the resultant oil was dissolved in chloroform (250ml), extracted with 5% $NaHCO_3$ (2x250ml) and with saturated NaCl solution (250ml), dried with Na_2SO_4 and evaporated to yield a yellow foam. The product was used without further purification.

2',3',5'-Tri-*O*-benzoyl-N^4-methylcytidine (2) To a solution of crude (1) in dry pyridine (150ml) methylamine hydrochloride (2.03g, 30mmol) and DBU (1.57ml, 10.5mmol) were added and the reaction mixture was stirred at room temperature. After 2h tlc (CH_2Cl_2 95: MeOH 5), pyridine was removed by evaporation, the resultant oil was dissolved in chloroform (250ml) and extracted with 5% $NaHCO_3$ solution (2x250ml) and with saturated NaCl solution (250ml). The organic layer was dried with Na_2SO_4 and concentrated to an oil. Repeated co-evaporation with toluene (4x50ml) removed any residual pyridine. The crude product was dissolved in methylene chloride and applied to flash chromatography silica column which was eluted with methylene chloride (500ml) and then with a gradient of methanol up to 4%. Product-containing fractions were combined and evaporated to yield 5.26g (92%). ^{1}H-NMR (250MHz, d6DMSO): δ 2.76 (CH_3, 3H, d), 4.6 (H4',H3',H2', 3H,m), 5.74 (H5, 1H, d), 5.91 (H5', 2H, d), 6.11 (H1', 1H,d),7.4-8.0 (aromatic, N-H, H6, 17H, 4m); m/z 570.4 [$M^+(H^+)$, 32%].

N^4-Methylcytidine (3). A solution of (2) (4.83g, 8.5mmol) in saturated methanolic ammonia (200ml) was stirred overnight at room temperature in a sealed reaction vessel (tlc : chloroform 80: methanol 20). The methanolic ammonia was removed by evaporation and the resultant solid dissolved in water (150ml). The aqueous layer was extracted with ethyl acetate (3x150ml), concentrated by evaporation and then dried *in vacuo* at 60° to yield 2.11g (96%). ^{1}H-NMR: δ 2.75 (CH_3, 3H, d), 3.55 (H3', H2', 2H, m), 3.81, (H4', 1H, m), 3.92 (H5', 2H, m), 4.97 (3'-OH, 1H, d), 5.03 (5'-OH, 1H, t), 5.29 (2'-OH, 1H, d), 5.69 (h5, 1H, d), 5.76 (H1', 1H, d), 7.65 (N-H, 1H, d), 7.78 (H6, 1H, d); m/z 2.58.2 [$M^+(H^+)$, 100%].

5'-*O*-Dimethoxytrityl-N^4-methylcytidine (4) : Yield: 3.02g (79%) from 1.8 g of (3), flash chromatography elution with chloroform/methanol (gradient to 5%) containing 0.5% triethylamine. ^{1}H-NMR: δ 2.74 (CH_3, 3H, d), 3.74 (CH_3O, 6H, s), 5.03 (3'-OH, 1H, d), 5.45 (2'-OH, 1H, d), 5.49 (H5, 1H, d), 5.76 (H1', 1H, d), 6.87-7.41 (aromatic, 13H, m), 7.68 (N-H, 1H, d), 7.71 (H6, 1H, d); m/z 560.4 [$M^+(H^+)$, 45%].

5'-*O*-Dimethoxytrityl-2'-*O*-*t*-butyldimethylsilyl-N^4-methylcytidine (5): Yield: 1.28g, (50%) from 2.1g of (4), flash chromatography elution with dichloromethane 40: hexane 40: ethylacetate 20 and then with a gradient of ethyl acetate up to 60%. Fractions containing the 3'-isomer were pooled, evaporated and dissolved in methanol to which was added triethylamine. Upon isomer equilibration as analysed by tlc (ethyl acetate) the reaction mixture was evaporated and then applied to a further flash chromatography column. ^{1}H-NMR: δ 0.05, 0.07 (CH_3-Si, 2x3H, 2s), 0.86 ($(CH_3)_3C$, 9H, s), 2.74 (CH_3, 3H, d), 3.74 (CH_3O, 6H, s), 5.01 (3'-OH, 1H, s), 5.49 (H5, 1H, s), 5.75 (H1', 1H, d), 6.88-7.41 (aromatic, 13H, m), 7.68 (N-H, 1H, d), 7.74 (H6, 1H, d).

5'-*O*-(4,4-Dimethoxytrityl)-2'-*O*-(*t*-butyldimethylsilyl)-N^4-methylcytidine 3'-*O*-(2-cyano ethyl-*N*,*N*-diisopropylaminophosphite (6) : Yield: 0.63g (65%) from 740mg of (5), flash chromatography : ethylacetate/ 0.5% triethylamine. ^{31}P-NMR ($CDCl_3$): δ 149.4, 150.1.

Preparation of Oligoribonucleotides. Oligoribonucleotides were synthesized on a 1μM scale utilizing 2'-*O*-(*t*-butyldimethylsilyl)-3'-*O*-(2-cyanoethyl-*N*,*N*-diisopropyl) phosphoroamidite monomers with phenoxyacetyl amino group protection for A and G and benzoyl protection for C (Glen Research-Cambio) as described previously[9]. Oligomers were purified by anion exchange chromatography [9] and characterized by total enzymatic digestion and reversed phase hplc analysis as previously described[3]. Elution time of N^4MeC: 10.9min, cf. C: 7.2min, U: 9.4min.

Competition Binding Assays. Binding reactions of RNA duplexes to HIV-1 tat protein or HIV-1 rev protein were carried out as previously described[4-6].

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