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Synthesis and base-pairing properties of the nuclease-resistant α-anomeric dodecaribonucleotide α-[r(UCUUAACCCACA)]

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ABSTRACT

The non natural oligoribonucleotide α-[r(UCUUAACCCACA)] consisting exclusively of α-anomeric ribonucleoside units was synthesized according to the phosphoramidite methodology and the solid support technology. For this purpose, the base-protected α-ribonucleosides were synthesized and converted into their O-methylphosphoramidites. Assembling was carried out on a DNA synthesizer with an average efficiency of 97% per step. Base composition of this nuclease-resistant α-RNA strand was ascertained after chemical and enzymatic hydrolysis and HPLC analysis of the hydrolysate. Whereas no spectroscopic evidence of base pairing was found above 0°C between α-[r(UCUUAACCCACA)] and β-[d(TGTGGGTTAAGA)], a clear UV absorbance transition (Tm 25.5°C) was observed during the hybridization of the same α-RNA strand with β-[d(AGAATTGGGTGT)]. In this latter case, the mixing curve titration suggests formation at low temperature of a triplex involving two α-RNA and one β-DNA strands. Moreover, this α-decaribonucleotide complementary in parallel orientation of the splice receptor of HIV-1 tat mRNA was found to inhibit (10 μM < ED_{50} < 20 μM), with apparent lack of sequence specificity, the *de novo* HIV-1 infection in cultured cells.

INTRODUCTION

In the last twelve years the use of synthetic oligonucleotides binding specifically to complementary sequences of RNA or DNA through hydrogen bonding of base pairs has attracted much interest as modulators of gene expression (1–5). Potential chemotherapeutic applications resulting from sequence-specific hybridization require oligonucleotides analogues that are resistant to *in vivo* degradation by nucleases and strongly bind to their target nucleic acid. Thus, for the effectiveness of the antisense oligo approach it was necessary to develop chemically modified oligonucleotides. Few years ago, we introduced a series of sugar-modified oligodeoxynucleotides, namely α-oligodeoxynucleotides, consisting exclusively of α-anomeric nucleotide units (6). This structural modification retains the original charge, aqueous solubility and confers the benefit of strong nuclease resistance (7). In addition, α-oligomers form stable parallel duplexes with either complementary natural DNA or RNA single strands (8,9). Although these latter duplexes are not substrates for RNase H (10), α-oligodeoxynucleotides were shown to efficiently inhibit translation initiation in cell-free systems (11) and *Xenopus* oocytes (12). These promising results prompted us to extend our investigations to the synthesis of α-anomeric oligoribonucleotides since it has been found that RNA·RNA duplexes are more stable than DNA·RNA heteroduplexes (13,14). In a previous communication, we reported for the first time, the synthesis of α-hexauridylate and α-dodecauridylate (15). Preliminary results indicated that these non natural α-oligoribonucleotides exhibit a marked resistance to hydrolysis by various purified nucleases including RNase A.

The aim of the present work was to synthesize a dodecaribonucleotide α-[r(UCUUAACCCACA)] presenting a mixed-base sequence in order to evaluate its binding capacity with the complementary sequence β-[d(AGAATTGGGTGT)] as well as its *in vitro* ability to block the *de novo* HIV-1 infection.

MATERIAL AND METHODS

General Methods

Melting points were determined on a Buchi 510 apparatus and are incorrected. Ultraviolet spectra (UV) were recorded on a Uvikon 810 spectrophotometer (Kontron). Optical rotations were measured in a 1 cm cell at 21°C on a Perkin-Elmer model 241 spectropolarimeter. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Bruker AC 250 or WM 360 WB spectrometer in DMSO-d_6 or in $CDCl_3$, at room temperature. Chemical shifts (δ) are expressed in parts per million relative to DMSO-d_5 set at 2.49 ppm or to $CHCl_3$ set at 7.28 ppm as internal reference; the signals are described as: s, singulet; d, doublet; t, triplet; q, quadruplet., m, multiplet. ^{31}P-NMR spectra were recorded in CD_3CN on a Bruker SP 200 spectrometer with ^{1}H broad band decoupling. Chemical shifts are expressed downfield from external 85% H_3PO_4. Fast atom

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bombardment (FAB) mass spectra (m/z) were determined in the positive or negative ion mode on a JEOL DX 300 mass spectrometer. Elemental analyses were determined by the Service Central d'Analyse du CNRS, Division de Vernaison, France. Thin-layer chromatography (TLC) was performed on precoated silica gel sheets 60 F_{254} (Merck, n° 5554) and flash column chromatography was performed with silica gel 60 (Merck n° 7736) at 0.2−0.4 10^5 Pa (N_2).

High performance liquid chromatographic (HPLC) analyses were carried out on a Beckman C_{18} XL-ODS (3 μm) column. The HPLC system (Waters Millipore) included a Model U6K injector, two Model 6000 A pumps, a Model 680 gradient controller, a Model 990 photodiode array detector interfaced with a NEC APC IV computer. A linear gradient of 0−15% acetonitrile in 0.1 M ammonium acetate (pH 5.9) was applied in 20 min at a flow rate of 1 ml min^{-1}.

Long chain alkylamine controlled pore glass (LCA-CPG) beads and bacterial alkaline phosphatase (370 units per ml) were purchased from Sigma.

Capillary gel electrophoresis analyses were carried out on an Applied Biosystems (ABI) Model 270A capillary electrophoresis system. A 50 cm gel-filled Micro-Gel 100 (ABI) capillary was used and the buffer was 75 mM Tris-phosphate, 10% methanol (pH 7.6). Analyses were run at 300 $V.cm^{-1}$ and UV detector was set at 260 nm.

α-Uridine (1a)

This compound was prepared as described in reference 16. Yield 34.3%. UV $@_{max}$in EtOH (nm(ε)) 263 (9,800). $[\alpha]_D$ −71.8° (c 1.17, H_2O). m/z (FAB$^+$, 3-nitrobenzyl alcohol) 245 $[M+H]^+$. ^{1}H-NMR (DMSO-d_6) δ 3.39−3.61 (m, 2H, H5′ and H5″); 3.99−4.07 (m, 2H, H3′ and H4′); 4.17 (m, 1H, H2′); 4.79 (t, 1H, OH5′); 5.06 (d, 1H, OH3′); 5.43 (d, 1H, OH2′); 5.56 (d, 1H, H5); 6.01 (d, 1H, H1′,$J_{1'-2'}$=4.5 Hz); 7.61 (d, 1H, H6, J_{5-6}=8.1 Hz); 11.18 (broad s, 1H, NH).

α-Cytidine

This compound (17) was prepared from α-uridine using a procedure similar to that reported for the preparation of 5-methyl-2′-deoxycytidine from thymidine as described in reference 18. Yield 48.5%, mp 215°C. UV $@_{max}$ in EtOH (nm(ε)) 273 (10,200). $[\alpha]_D$ +60.6° (c 0.94, DMSO). m/z (FAB$^+$, 3-nitrobenzyl alcohol) 244 $[M+H]^+$. ^{1}H-NMR (DMSO-d_6) δ 3.43−3.61 (m, 2H, H5′ and H5″); 3.96 (m, 1H, H4′); 4.05 (m, 2H, H2′ and H3′); 4.75 (m, 1H, OH5′); 4.97 and 5.26 (2d, 1H each, OH3′ and OH5′); 5.68 (d, 1H, H5); 6.02 (d, 1H, H1′,$J_{1'-2'}$=3.9 Hz); 7.14 (broad s, 2H, NH_2); 7.54 (d, 1H, H6, J_{5-6}= 7.5 Hz).

4-N-Benzoyl-α-cytidine (1b)

Using a three-step procedure similar to that described for the corresponding β-anomer (19), the title compound was prepared from α-cytidine (7 g, 28.7 mmol.). At the end of the third step, a further treatment with a mixture of water and ethanol overnight at room temperature was necessary for the complete removal of the silyl groups. The solution was evaporated to dryness and the resulting residue was crystallized from methanol. Filtration afforded 9 g (90%) of **1b**, mp 207°C. Anal. Calc. for $C_{16}H_{17}N_3O_6$: C, 55.33; H, 4.93; N, 12.10. Found: C, 55.24; H, 5.12; N, 12.05. UV $@_{max}$ in EtOH (nm (ε)) 306 (4,100), 260 (23,100). m/z (FAB$^-$, thioglycerol) 346 $[M-H]^-$. ^{1}H-(DMSO-d_6) δ 3.44−3.69 (m, 2H, H5′ and H5″); 4.09 (m, 2H, H3′ and H4′); 4.20 (m, 1H, H2′); 4.79 (m, 1H, OH5′); 5.04 (d, 1H, OH3′); 5.40 (d, 1H, OH2′); 6.09 (d, 1H, H1′, $J_{1'-2'}$= 3.9 Hz); 7.31−8.04 (m, 7H, H5, H6 and ArH); 11.05 (s, 1H, NH).

6-N-Benzoyl-α-adenosine (1c)

2′,3′,5′-Tri-O-benzoyl-6-N-benzoyl-α-adenosine was prepared by fusion reaction between 2,3,5-tri-O-benzoyl-1-O-acetyl-D-ribofuranose (2.73 g, 5.41 mmol) and 6-N-benzoyladenine (1.20 g, 5.02 mmol) using the same procedure as described in reference 20. This reaction was repeated twelve times, the combined resulting residues were dissolved in ethanol (250 ml) and the solution was clarified with charcoal, then evaporated to dryness. The reaction products were fractionated by flash column chromatography on silica gel. Fractions containing 2′,3′,5′-tri-O-benzoyl-6-N-benzoyl-α-adenosine (contaminated with corresponding β-anomer) were combined and evaporated to dryness (16 g, foam) and the residue was dissolved in pyridine-ethanol (90 ml, 2/1, v/v). To the resulting cooled (0°C) solution was added 2N sodium hydroxide in water-ethanol (90 ml, 1/1 v/v) and after 15 min. stirring at 0°C the reaction mixture was neutralized with Dowex 50W (pyridinium form) and filtered. The filtrate was evaporated and compound **1c** was isolated from the anomeric mixture by fractional crystallization from ethanol (1.72 g, 4.63 mmol). Yield 7%, mp 200−201°C. UV $@_{max}$ in EtOH (nm(ε)) 280 (19,800). $[\alpha]_D$ +52° (c 1, DMSO). m/z (FAB$^+$, glycerol) 372 $[M+H]^+$. ^{1}H-NMR (DMSO-d_6) δ 3.39−3.63 (m, 2H, H5′ and H5″); 4.13−4.20 (m, 2H, H3′ and H4′); 4.45 (m, 1H, H2′); 4.91 (pseudo t, 1H, OH5′); 5.44 (d, 1H, OH3′); 5.59 (d, 1H, OH2′); 6.46 (d, 1H, H1′, $J_{1'-2'}$=5.5 Hz); 7.53−8.06 (m, 5H, ArH); 8.64 and 8.73 (2s, 2H, H2 and H8); 11.15 (broad s, 1H, NH).

5′-O-Acetyl-2′,3′-O-isopropylidene-2-N-acetyl-6-O-diphenylcarbamoyl-α-guanosine

N,O-Bis (trimethylsilyl) acetamide (8.85 ml, 36.19 mmol) was added to a suspension of 2-N-acetyl-6-O-diphenylcarbamoyl guanine (21) (7.35 g, 18.92 mmol) in dry 1,2-dichloroethane (150 ml) and the resulting mixture was stirred for 15 min at 80°C. The solvent was evaporated under reduced pressure and a solution of 1,5-di-O-acetyl-2,3-O-isopropylidene-D-ribofuranose (22) (6.21 g, 22.66 mmol) in dry toluene (150 ml) and trimethylsilyl trifluoromethanesulfonate (6 ml, 31.04 mmol) were successively added to the viscous residue. The reaction mixture was stirred for 1 h at 80°C, cooled down to room temperature and ethyl acetate (200 ml) was added. Then the mixture was poured into water (200 ml) and the organic layer was washed successively with water (200 ml) and saturated aqueous sodium hydrogencarbonate (200 ml). The organic layer was dried over anhydrous sodium sulfate, filtered and evaporated to dryness. The residue was fractionated by flash column chromatography using increasing proportions of acetone (0 to 50%) in methylene chloride. Fractions containing pure title compound were combined and evaporated to dryness to afford a colorless foam (1.4 g, 12%), ^{1}H-NMR ($CDCl_3$) δ 1.29 and 1.48 (2s, 6H, $(CH_3)_2C$); 2.14 (s, 3H, CH_3-C(O)O-); 2.52 (s, 3H, CH_3-C(O)N-); 4.19−4.31 (m, 2H, H5′ and H5″); 4.55 (m, 1H, H4′); 4.83−4.89 (m, 2H, H2′ and H3′); 6.42 (d, 1H, H1′, $J_{1'-2'}$=3.8 Hz); 7.21−7.42 (m, 10H, ArH); 7.95 (s, 1H, NH); 8.20 (s, 1H, H8).

2',3'-O-Isopropylidene-2-N-acetyl-α-guanosine

A solution of 1M tetrabutylammonium fluoride in tetrahydrofuran (12 ml) was added to a solution of previous compound (1.45 g, 2.4 mmol) in the same solvent (12 ml) and the resulting mixture was stirred at room temperature for 20 hr. The solvent was evaporated, the residue was dissolved in ethyl acetate (50 ml) and the resulting solution was washed with water (3×50 ml). Then the combined aqueous layers containing the title compound were evaporated and the residue was fractionated by silanized silica gel column chromatography (RP-2, Merck n°. 7719, eluent: linear gradient of methanol [0−50%] in water). The appropriate fractions were combined, evaporated to dryness and the residue was lyophilized from water to afford a colorless powder (0.581 g, 66%). m/z (FAB$^+$, thioglycerol) 366 [M+H]$^+$. ^{1}H-NMR (DMSO-d_6) δ 1.23 and 1.31 (2s, 6H, $(CH_3)_2C$); 2.14 (s, 3H, CH_3-C(O)-); 3.61 (m, 2H, H5' and H5"); 4.35 (m, 1H, H4'); 4.80 (m, 1H, H2'); 4.90 (m, 1H, H3'); 5.25 (broad s, 1H, OH5'); 6.32 (d, 1H, H1', $J_{1'-2'}$=4.3 Hz); 7.89 (s, 1H, H8); 11.95 (broad s, 2H, 2NH).

2-N-Acetyl-α-guanosine (1d)

2',3'-O-isopropylidene-2-N-acetyl-α-guanosine (0.581 g, 1.6 mmol) was dissolved in 50% aqueous formic acid (8 ml) and the solution was stirred for 4 days at room temperature and then evaporated to dryness. The residue was crystallized from water (0.33 g, 73%), mp 227−229°C. UV $@_{max}$ (EtOH) (nm(ε)) 276 (7,800); 255 (11,000). m/z (FAB$^+$, thioglycerol) 326 [M+H]$^+$.^{1}H-NMR (DMSO-d_6) δ 2.17 (s, 3H, CH_3-C(O)-); 3.41−3.58 (m, 2H, H5' and H5"); 4.09 (m, 1H, H4'); 4.14 (m, 1H, H3'); 4.33 (m, 1H, H2'); 4.89 (t, 1H, OH5'); 5.36 (d, 1H, OH3'); 5.52 (d, 1H, OH2'); 6.09 (d, 1H, H1', $J_{1'-2'}$=5.3 Hz); 8.14 (s, 1H, H8); 11.7 and 12 (2 broad s, 2H, 2NH).

General procedure for the preparation of base-protected 5'-O-dimethoxytrityl-α-ribonucleosides 2a−d

4,4'-Dimethoxytrityl chloride (0.44 g, 1.3 mmol) was added to a solution of N-protected α-ribonucleoside **1a−d** (1 mmol) in dry pyridine (5 ml). The reaction mixture was stirred at room temperature for 2 to 3 hr and methanol (1 ml) was then added. After an additional 10 min stirring period, the reaction mixture was poured into 5% aqueous sodium hydrogencarbonate (25 ml) and the products were extracted with methylene chloride (3×20 ml). The combined organic layers were dried over anhydrous sodium sulfate, filtered and evaporated to dryness under reduced pressure. The residue was fractionated by flash column chromatography using increasing proportions of methanol (0 to 10%) in methylene chloride as eluent. Fractions containing pure 5'-O-dimethoxytrityl derivatives were combined, evaporated to dryness and the residue was crystallized or precipitated from pet.ether (<40°C) to afford an amorphous powder.

5'-O-Dimethoxytrityl-α-uridine (2a)

Yield 94% as an amorphous powder. m/z (FAB$^-$, thioglycerol) 545 [M−H]$^-$. ^{1}H-NMR ($CDCl_3$) δ 3.05−3.50 (m, 3H, H5', H5" and OH3'); 3.78 (s, 6H, 2 O-CH_3); 4.25 (m, 1H, H3'); 4.34 (m, 1H, H4'); 4.78 (m, 1H, H2'); 5.36 (broad s, 1H, OH 2'); 5.67 (d, 1H, H5); 6.25 (d, 1H, H1', $J_{1'-2'}$=4.7 Hz); 6.81−7.43 (m, 13H, ArH); 7.65 (d, 1H, H6, J_{5-6}=8.1 Hz); 10.4 (broad s, 1H, NH).

5'-O-Dimethoxytrityl-4-N-benzoyl-α-cytidine (2b)

Yield 93% as an amorphous powder. m/z (FAB$^-$, thioglycerol) 648 [M−H]$^-$. ^{1}H-NMR ($CDCl_3$) δ 3.11 (broad s, 1H, OH3'); 3.21−3.47 (m, 2H, H5' and H5"); 3.78 (s, 6H, 2 O-CH_3); 4.29 (m, 1H, H3'); 4.38 (m, 1H, H4'); 4.86 (m, 1H, H2'); 5.54 (broad s, 1H, OH2'); 6.27 (d, 1H, H1', $J_{1'-2'}$=4.6 Hz); 6.75−8.07 (m, 20H, H5, H6 and ArH); 8.99 (broad s, 1H, NH).

5'-O-Dimethoxytrityl-6-N-benzoyl-α-adenosine (2c)

Yield 82% as an amorphous powder. m/z (FAB$^-$, thioglycerol) 672 [M−H]$^-$. ^{1}H-NMR ($CDCl_3$) δ 3.16−3.45 (m, 2H, H5' and H5"); 3.76 (s, 6H, 2 O-CH_3); 4.37 (m, 1H, H3'); 4.53 (m, 1H, H4'); 4.91 (m, 1H, H2'); 5.27 (broad s, 1H, OH3'); 5.52 (broad s, 1H, OH2'); 6.43 (d, 1H, H1', $J_{1'-2'}$=6 Hz); 6.80−7.99 (m, 18H, ArH); 8.21 and 8.53 (2s, 2H, H2 and H8); 9.25 (broad s, 1H, NH).

5'-O-Dimethoxytrityl-2-N-acetyl-α-guanosine (2d)

Yield 84% as colorless crystals from methylene chloride, mp 242−243°C. m/z (FAB$^-$, thioglycerol) 626 [M−H]$^-$. ^{1}H-NMR ($CDCl_3$) δ 2.18 (s, 3H, CH_3-C(O)-); 3.00−3.32 (m, 2H, H5' and H5"); 3.74 (s, 6H, 2 O-CH_3); 4.14 (m, 1H, H3'); 4.27 (m, 1H, H4'); 4.40 (m, 1H, H2'); 5.42 (broad s, 1H, OH3'); 5.63 (broad s, 1H, OH2'); 6.18 (d, 1H, H1',$J_{1'-2'}$=5.2 Hz); 6.83−7.41 (m, 13H, ArH); 8.18 (s, 1H, H8); 11.70 and 12.04 (2 broad s, 2H, 2NH).

General procedure for the preparation of base-protected 5'-O-dimethoxytrityl-2'-O-*tert*-butyldimethylsilyl-α-ribonucleosides (3a−d) and their 3'-O-*tert*-butyldimethylsilyl isomers (4a−d)

5'-O-dimethoxytrityl-N-acyl-α-ribonucleoside **2a−d** (1 mmole) and imidazole (0.177 g, 2.6 mmol) were dissolved in anhydrous pyridine (10 ml). *tert*-Butyldimethylsilyl chloride (0,196 g, 1.3 mmol) was added and the solution was stirred at room temperature for 24 hr to 72 hr. Then, the reaction mixture was poured in 5% aqueous sodium hydrogencarbonate (30 ml) and the products were extracted with methylene chloride (3×20 ml). The combined organic layers were washed with water, dried over sodium sulfate and evaporated to dryness under reduced pressure. The residue was fractionated by flash column chromatography using a 0−10% acetone in CH_2Cl_2 stepwise gradient to yield pure 2'-O-silylated isomer. The last fractions containing mainly the 3'-O-*tert*-butyldimethylsilylated derivative were dissolved in ethanol and stirred at room temperature overnight. The solvent was evaporated to dryness. The residue was fractionated as previously described. Repetition of this procedure three times allowed the recovery of additional pure 2'-*tert*-butyldimethylsilylated derivative that was lyophilized from dioxane to afford a colorless powder.

5'-O-Dimethoxytrityl-2'-O-*tert*-butyldimethylsilyl-α-uridine (3a)

Yield 42%, m/z (FAB$^-$, thioglycerol) 659 [M−H]$^-$. ^{1}H-NMR ($CDCl_3$): see reference 15.

5'-O-Dimethoxytrityl-3'-O-*tert*-butyldimethylsilyl α-uridine (4a)

^{1}H-NMR ($CDCl_3$): see reference 15.

5'-O-Dimethoxytrityl-2'-O-*tert*-butyldimethylsilyl-4-N-benzoyl-α-cytidine (3b)

Yield 85%, m/z (FAB$^-$, thioglycerol) 762 [M−H]$^-$. ^{1}H-NMR ($CDCl_3$) δ 0 and 0.15 (2s, 6H, $(CH_3)_2Si$); 0.82 (s, 9H, $(CH_3)_3C$); 2.48 (d, 1H, OH3'); 3.13−3.51 (m, 2H, H5' and H5"); 3.79 (s, 6H, 2 O-CH_3); 4.19 (m, 1H, H3'); 4.38 (m, 1H, H4'); 4.87 (pseudo t, 1H, H2'); 6.58 (d, 1H, H1', $J_{1'-2'}$=5.2 Hz); 6.83−8.01 (m, 20H, H5, H6 and ArH); 8.67 (broad s,1H,NH).

5'-O-Dimethoxytrityl-3'-O-*tert*-butyldimethylsilyl-4-N-benzoyl-α-cytidine (4b)

^{1}H-NMR ($CDCl_3$) δ −0.08 and −0.01 (2s, 6H, $(CH_3)_2Si$); 0.80 (s, 9H, $(CH_3)_3C$); 2.97 (broad s, 1H, OH2'); 3.05-3.48 (m, 2H, H5' and H5"); 3.79 (s, 6H, 2 O-CH_3); 4.30 (m, 1H, H4'); 4.44 (pseudo t, 1H, H3'); 4.56 (m, 1H, H2'); 6.42 (d, 1H, H1',$J_{1'-2'}$=3.9 Hz); 6.82−8.03 (m, 20H, H5, H6 and ArH); 8.73 (broad s,1H,NH).

5'-O-Dimethoxytrityl-2'-O-*tert*-butyldimethylsilyl-6-N-benzoyl-α-adenosine (3c)

Yield 66%; m/z (FAB$^+$, glycerol) 788 [M+H]$^+$. ^{1}H-NMR ($CDCl_3$) δ −0.23 and 0.07 (2s, 6H, $(CH_3)_2Si$); 0.60 (s, 9H, $(CH_3)_3C$); 3.11−3.58 (m, 2H, H5' and H5"); 3.67 (d, 1H, OH3'); 3.80 (s, 6H, 2 O-CH_3); 4.17 (m, 1H, H3'); 4.53 (m, 1H, H4'); 5.07 (t, 1H, H2'); 6.67 (d, 1H, H1', $J_{1'-2'}$=6.5 Hz); 6.84−8.38 (m, 18H, ArH); 8.83 and 8.89 (2s, 2H, H2 and H8); 9.34 (s, 1H, NH).

5'-O-Dimethoxytrityl-3'-O-*tert*-butyldimethylsilyl-6-N-benzoyl-α-adenosine (4c)

^{1}H-NMR ($CDCl_3$) δ −0.021 and 0.029 (2s, 6H, $(CH_3)_2Si$); 0.83 (s, 9H, $(CH_3)_3C$); 3.43 (d, 1H, OH2'); 3.10−3.55 (m, 2H, H5' and H5"); 3.79 (s, 6H, 2 O-CH_3); 4.39 (m, 1H, H4'); 4.45 (m, 1H, H3'); 4.64 (m, 1H, H2'); 6.70 (d, 1H, H1', $J_{1'-2'}$=4.9 Hz); 6.85−8.04 (m, 18H, ArH); 8.37 and 8.79 (2s, 2H, H2 and H8); 9.39 (broad s, 1H, NH).

5'-O-Dimethoxytrityl-2'-O-*tert*-butyldimethylsilyl-2-N-acetyl-α-guanosine (3d)

Yield 43%. m/z (FAB$^-$, thioglycerol) 740 [M−H]$^-$. ^{1}H-NMR ($CDCl_3$) δ −0.19 and 0.04 (2s, 6H, $(CH_3)_2Si$); 0.69 (s, 9H, $(CH_3)_3C$); 2.28 (s, 3H, CH_3-C(O)-); 3.32 (d, 1H, OH3'); 3.12−3.52 (m, 2H, H5' and H5"); 3.79 (s, 6H, 2 O-CH_3); 4.16 (m, 1H, H3'); 4.42 (m, 1H, H4'); 4.93 (t, 1H, H2'); 6.25 (d, 1H, H1', $J_{1'-2'}$=6.6 Hz); 6.83−7.42 (M, 13H, ArH); 8.00 (s, 1H, H8); 8.36 and 11.87 (2broad s, 2H, 2NH).

5'-O-Dimethoxytrityl-3'-O-*tert*-butyldimethylsilyl-2-N-acetyl-α-guanosine (4d)

^{1}H-NMR ($CDCl_3$) δ −0.005 and 0.045 (2s, 6H, $(CH_3)_2Si$); 0.83 (s, 9H, $(CH_3)_3C$); 2.24 (s, 3H, CH_3-C(O)-); 3.05−3.53 (m, 2H, H5' and H-5"); 3.71 (d, 1H, OH2'); 3.78 (s, 6H, 2 O-CH_3); 4.34−4.38 (m, 1H, H4'); 4.44 (pseudo t, 1H, H3'); 4.66−4.72 (m, 1H, H2'); 6.28 (d, 1H, H1', $J_{1'-2'}$=4.7 Hz); 6.81−7.45 (m, 13H, ArH); 7.99 (s, 1H, H8); 8.16 and 11.75 (2s, 2H, 2NH).

General procedure for the preparation of α-ribonucleoside 3'-O-(methyl N,N-diisopropylphosphoramidites) 5a−d

This procedure is similar to that already described in reference 22. After flash column chromatography, the appropriate fractions were combined and evaporated to dryness. The residue was lyophilized from benzene affording a colorless powder.

5'-O-Dimethoxytrityl-2'-O-*tert*-butyldimethylsilyl-α-uridine 3'-O-(methyl N,N-diisopropylphosphoramidite) (5a)

Yield 75%, as a diastereoisomeric mixture. m/z (FAB$^-$, polyethylene glycol 400) 820 [M−H]$^-$. ^{31}P-NMR (CD_3CN) δ 151.25 and 152.37. ^{1}H-NMR ($CDCl_3$) δ −0.17, −0.13, 0.06 and 0.09 (4s, 6H, $(CH_3)_2Si$); 0.82 (2s, 9H, $(CH_3)_3C$); 0.97−1.13 (m, 12H, 2 $(CH_3)_2C$); 3.01−3.06 (m, 1H, H5' or H5"); 3.19 and 3.34 (2d, 3H, CH_3O-P, J_{P-H}=13.3 Hz); 3.37−3.43 (m, 1H, H5' or H5"); 3.44−3.54 (m, 2H, $2CH(Me)_2$); 3.769 and 3.772 (2s, 6H, 2 O-CH_3); 4.13−4.17 (m, 1H, H3'); 4.34−4.41 (m, 1H, H4'); 4.73−4.79 (m, 1H, H2'); 5.59 and 5.63 (2d, 1H, H5); 6.44 (pseudo t, 1H, H1'); 6.80−7.42 (m, 13H, ArH); 7.85 and 7.88 (2d, 1H, H6); 8.04 (broad s, 1H, NH).

5'-O-Dimethoxytrityl-2'-O-*tert*-butyldimethylsilyl-4-N-benzoyl-α-cytidine 3'-O-(methyl N,N-diisopropylphosphoramidite) (5b)

Yield 81%, as a diastereoisomeric mixture. m/z (FAB$^-$, polyethyleneglycol 400) 923 [M−H]$^-$. ^{31}P-NMR (CD_3CN) δ 151.26 and 152.40. ^{1}H-NMR ($CDCl_3$) δ −0.15, −0.13, 0.08 and 0.10 (4s, 6H, $(CH_3)_2Si$); 0.79 and 0.80 (2s, 9H, $(CH_3)_3C$); 0.93−1.18 (m, 12H, $2(CH_3)_2C$); 3.11 (m, 1H, H5' or H5"); 3.19 and 3.32 (2d, 3H, CH_3O-P, J_{P-H}=13.2 and 13.4 Hz); 3.43−3.62 (m, 3H, 2 $CH(Me)_2$ and H5" or H5'); 3.77 (s, 6H, 2 O-CH_3); 4.24−4.33 (m, 1H, H3');4.38 and 4.44 (2m, 1H, H4'); 4.71 (m, 1H, H2'); 6.46 and 6.49 (2d, 1H, H1', $J_{1'-2'}$=4.6 Hz); 6.79−7.92 (m, 19H, H5 and ArH); 8.14 (pseudo t, 1H, H6); 8.60 (broad s, 1H, NH).

5'-O-Dimethoxytrityl-2'-O-*tert*-butyldimethylsilyl-6-N-benzoyl-α-adenosine 3'-O-(methyl N,N-diisopropylphosphoramidite) (5c)

Yield 88%, as a diastereoisomeric mixture. m/z (FAB$^-$, polyethyleneglycol 400) 949 [M−H]$^-$. ^{31}P-NMR (CD_3CN) δ 151.66 and 152.43. ^{1}H-NMR ($CDCl_3$) δ −0.22 and 0.04 (2 pseudo d, 6H, $(CH_3)_2Si$); 0.66 (s, 9H, $(CH_3)_3C$); 0.97−1.28 (m, 12H, $2(CH_3)_2C$); 3.17 and 3.38 (2d, 3H, CH_3O-P, J_{P-H}=13.5 Hz); 3.04−3.63 (m, 4H, H5', H5" and 2 $CH(Me)_2$); 3.78 (s, 6H, 2 O-CH_3); 4.26−4.43 (m, 2H, H3' and H4'); 4.99−5.08 (m, 1H, H2'); 6.68 (pseudo t, 1H, H1', $J_{1'-2'}$=7 Hz); 6.81−8.87 (m, 20 H, H2, H8 and ArH); 9.11 and 9.16 (2s, 1H, NH).

5'-O-Dimethoxytrityl-2'-O-*tert*-butyldimethylsilyl-2-N-acetyl-α-guanosine 3'-O-(methyl N,N-diisopropylphosphoramidite) (5d)

4,4-Dimethylaminopyridine was omitted in this case (23). Elution from silica gel column was performed with triethylamine-methylene chloride-acetonitrile (1/99/0 to 1/49/50 v/v/v). Yield 79%, as a diastereoisomeric mixture. m/z (FAB$^-$, polyethyleneglycol 400) 901 [M−H]$^-$. ^{31}P-NMR (CD_3CN) δ 151.37 and 152.47. ^{1}H-NMR ($CDCl_3$) δ −0.16, −0.15 and 0.05 (3s, 6H, $(CH_3)_2Si$); 0.72 (2s, 9H, $(CH_3)_3C$); 0.99−1.25 (m, 12H, $2(CH_3)_2C$); 2.27 and 2.28 (2s, 3H, CH_3-C(O)-); 3.18 and 3.40 (2d, 3H, CH_3O-P, J_{P-H}=13.3 Hz); 3.10−3.49 (m, 2H, H5' and H5"); 3.51−3.61 (m, 2H, 2 $CH(Me)_2$); 3.80 (s, 6H, 2 O-CH_3); 4.34 (m, 2H, H3' and H4'); 4.93 (m, 1H, H2'); 6.12 (pseudo t, 1H, H1', $J_{1'-2'}$=6.8 Hz); 6.83−7.45 (m, 13H,

ArH); 8.32 (broad s, 1H, NH); 8.32 and 8.39 (2s, 1H, H8); 11.82 (broad s, 1H, NH).

General procedure for the preparation of 5'-O-dimethoxytrityl-3'(2')-O-*tert*-butyldimethylsilyl-N-protected-α-ribonucleoside 2'(3')-O-pentachlorophenylsuccinates (7a–c)

The compounds were prepared from **4a–c** according to the procedure described in reference 24. Overall yields ranged from 54 to 72%.

General method for the functionalization of the controlled pore glass solid supports 8a–c

LCA-CPG beads were reacted with **7a–c** according to the procedure described in reference 25. Spectrophotometric measurement at 500 nm of the amount of dimethoxytrityl cation released by treating portions of the derivatized glass beads with 0.1 M toluene-sulfonic acid in acetonitrile indicated loadings of 18 to 26 μmol g^{-1}.

Solid-phase synthesis of α-anomeric dodecaribonucleotides

α-[r(UCUUAACCCACA)] was synthesized on a Applied Biosystems 381 A synthesizer employing the same cycle as described for the β-oligoribonucleotides (23). The synthesis was carried out using a column containing 1 μmmole (0.035 g) of immobilized α-adenosine **8c**. After completion of the assembling, the solid support was then successively treated with thiophenol-triethylamine-dioxane (1/1/2, v/v/v, 0.5 ml) for 30 min at room temperature, washed with methanol (10 ml), and incubated in concentrated (32%) aqueous ammonia-ethanol (3/1,v/v, 2 ml) for 5 hr at 55°C. Solvents were removed and 1.1 M tetrabutylammonium fluoride in THF (0.5 ml) was added to the dry residue. The resulting solution was kept for 24 hr at room temperature and then the reaction was quenched with an equal volume of 0.05 M NH_4OAc. Organic solvent was removed and the residue was fractionated on a DEAE A25 Sephadex (HCO_3^- form) column using triethylammonium hydrogencarbonate buffer (pH 7.5, linear gradient from 0.001 M to 0.8 M over 200 ml). The main UV absorbing fraction was eluted with 0.6–0.75 M buffer and contained the pure (97% as determined by reverse-phase HPLC analysis) dodecamer (55 A_{260} units, 52% yield). α-[r(ACUAAAUUCCCC)] was synthesized following the same procedure.

Hydrolysis of α-[r(UCUUAACCCACA)] with sodium hydroxide and alkaline phosphatase

To a solution of α-dodecaribonucleotide (1 A_{260} unit) in water (0.020 ml) was added 0.1 N sodium hydroxyde (0.1 ml). After 20 h incubation at 37°C, the mixture was neutralized by addition of glacial acetic acid (0.005 ml) and water was removed under reduced pressure. The resulting residue was dissolved in 0.1 M tris-HCl buffer (pH 9, 0.01 M with respect to $MgCl_2$) and alkaline phosphatase (stock solution, 0.002 ml) was added. After 2 h incubation at 37°C, HPLC analysis of the hydrolysate indicated complete degradation into α-cytidine, α-uridine and α-adenosine in the molar ratio of 5/2.8/4.1.

Annealing Experiments

Optical measurements were performed on a UVIKON 810 spectrophotometer (KONTRON) interfaced with an IBM PC compatible microcomputer. The temperature control was through a HUBER PD415 temperature programmer connected to a refrigerated ethylene glycol-water bath (HUBER Ministat). Cuvettes were 1 cm pathlength quartz cells and nitrogen was continuously circulated through the cuvette compartment. Prior to the experiments, the sequences to be studied were mixed together in 1 M NaCl, 0.01 M sodium cacodylate (pH 7.0) and allowed to incubate at 90°C for a length of time sufficient to allow the optical density of the mixture to be perfectly stable (about 1 hour). Digitized absorbance and temperature values were stored on the computer for subsequent plotting and analysis. The temperature variation was 0.5 °C/min. Prior the recording of the data from the melting experiments (from 0°C up to 90°C) the mixture was stored at 5°C overnight. The temperature variation was the same as above.

Mixing curve titrations

Mixing curves for α-RNA:β-DNA hybrids were obtained by varying the molar ratio of each strand, keeping the total strand concentration (5 μM) and temperature (4.7°C) constant. Buffer was 1 M NaCl, 0.01 M sodium cacodylate (pH 7.0).

Cell culture and virus isolate

MT4 cells (human T cell leukaemia virus type 1 (HTLV-1)-transformed human leukaemic $CD4^+$ cell line provided by N. Yamamoto, NIH, Tokio, Japan (26) were maintained at 37°C

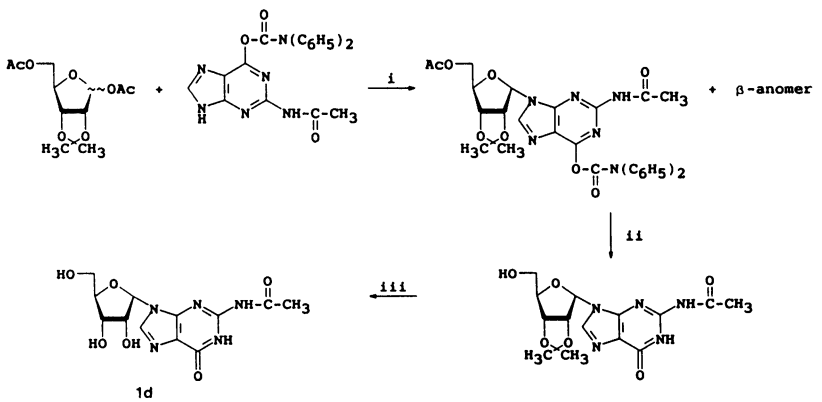

Figure 1. Synthesis of 2-N-acetyl-α-guanosine. Reactions conditions (i) N,O-bis(trimethylsilyl)acetamide/1,2-dichloroethane, trimethylsilyl trifluoromethanesulfonate/toluene; (ii) 1 M tetrabutylammonium fluoride/tetrahydrofuran; (iii) 50% aqueous formic acid.

and 5% CO_2 in RPMI medium supplemented with 10% (v/v) decomplemented fetal calf serum, 2 mM glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin.

HIV-1 BRU (27) isolate, provided by L. Montagnier (Institut Pasteur, Paris, France) was maintained and amplified on CEM cells.

Assays for HIV inhibition

MT4 cells, harvested during the exponential growth phase, were concentrated to 3×10^6 cells ml^{-1}, infected with an equal volume of virus containing medium (1000 $TCID_{50}$) and incubated for 30 min at 4°C. Cells were washed, diluted to 3×10^5 cells ml^{-1} and incubated at 37°C in the presence of oligonucleotide for 5 days.

Culture samples were removed to determine syncytia formation (28) and reverse transcriptase (RT) activity. RT assays were performed upon 1 ml samples of infected cell supernatant as already described (29).

RESULTS AND DISCUSSION

One of the most powerful strategies to synthesize oligoribonucleotides is the use of solid-phase technique in combination with application of the phosphoramidite methodology (23,30). Application of an analogous strategy for α-oligoribonucleotide synthesis requires the preparation of the suitably protected α-ribonucleoside phosphoramidites **5a–d**.

α-Uridine **1a** was prepared according to an already published procedure (16). Treatment of 2',3',5'-tri-O-acetyl-α-uridine with *o*-chlorophenyl phosphorodichloridate and 1,2,4-triazole (18) afforded the putative 4-triazolyl-pyrimidinone intermediate which upon further treatment with aqueous ammonia in dioxane yielded α-cytidine in 48% yield. Using the transient protection method (19), α-cytidine was then converted into the corresponding 4-N-benzoylated derivative **1b**. On the other hand, 6-N-benzoyl-α-adenosine **1c** was obtained in poor yield as previously described (20) by acid-catalyzed fusion reaction of 6-N-benzoyladenine with 2,3,5-tri-O-benzoyl-1-O-acetyl-ribofuranose followed by deprotection of the sugar hydroxyls. Several attempts to repeat the procedure reported in 1968 by Y. Furukowa and coworkers (31) for the synthesis of α-guanosine by the use of boron trichloride complex of methyl D-ribofuranoside were unsuccessful in our hands. Although the presence of α-guanosine was detected by HPLC analysis of the crude reaction mixture, its high salt content considerably hindered the scale up of the procedure. Alternatively, we considered the possibility of using a procedure described by Robins and coworker (21) for the regioselective glycosylation at N-9 of guanine in combination with a D-ribofuranose derivative exhibiting no participating protecting group at OH-2 (32), thus favoring the formation of α-guanosine derivative. Trimethylsilyl derivative of 2-N-acetyl-6-O-diphenylcarbamoylguanine was reacted with 1,5-di-O-acetyl-2,3-O-isopropylidene-D-ribofuranose (22) in the presence of trimethylsilyl trifluoromethanesulfonate in toluene (Fig. 1). TLC analysis of the crude reaction mixture revealed the presence of several products, only two of them giving a distinct dark blue spot upon spraying with sulfuric acid and heating (33). These two products were isolated by silica gel chromatography and identified as anomeric 5'-O-acetyl-2',3'-O-isopropylidene-2-N-acetyl-6-O-diphenylcarbamoyl-α—(low eluting) and β—(fast eluting) guanosines. The α-anomer (12% yield) was successively treated with 1M tetrabutylammonium fluoride in tetrahydrofuran and 50% aqueous formic acid to afford crystalline 2-N-acetyl-α-guanosine in 6% overall yield. Although the yield was low, the reaction and purification conditions have not yet been optimized. Acetyl group was shown to be a good substitute to the classical isobutyryl group for the protection of NH_2-2 of guanine base since half-life time of the former in 32% aqueous ammonia/ethanol (3/1 v/v) at room temperature is 2.5 h as compared to 6 h for the latter.

We have previously reported the conversion of α-uridine **1a** into the corresponding protected nucleoside 3'-O-phosphoramidite **5a** (15). The same three step protocol was applied with the three other α-ribonucleosides **1b–d** (Fig. 2). Individual treatment of the three 5'-O-dimethoxytrityl-α-ribonucleosides **2b–d** with *tert*-butyldimethylsilyl chloride (TBDMS-Cl) in pyridine and in the

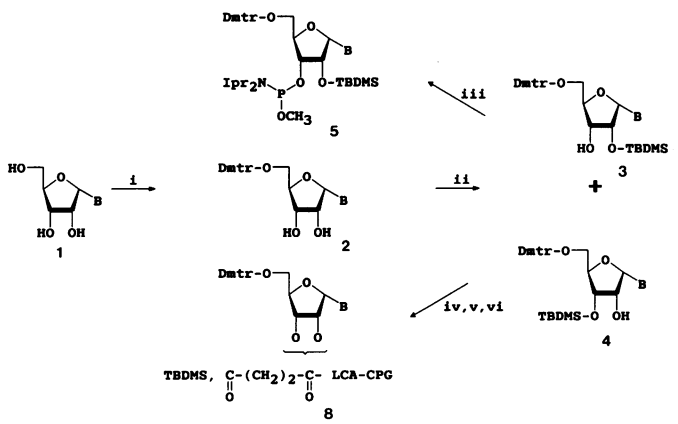

Figure 2. Synthesis of α-ribonucleoside phosphoramidites and α-ribonucleoside derivatized solid supports. a: B = uridin-1-yl; b: B = 4-N-benzoylcytosin-1-yl; c: B = 6-N-benzoyladenin-9-yl; d: B = 2-N-acetylguanin-9-yl. Reactions conditions (i) 4,4'-dimethoxytrityl chloride/pyridine; (ii) *tert*-butyldimethylsilyl chloride/imidazole/pyridine; (iii) chlorodiisopropylaminomethoxyphosphine/N,N,N-diisopropylethylamine/4-dimethylaminopyridine (this catalyst was ommited during the synthesis of **5d**)/methylene chloride; (iv) succinic anhydride/4-dimethylaminopyridine/triethylamine/methylene chloride; (v) pentachlorophenol/1,3-dicyclohexylcarbodiimide/1,2-dimethoxyethane; (vi) long chain alkylamine controlled pore glass/pyridine and then acetic anhydride/2,6-lutidine/4-dimethylaminopyridine/tetrahydrofuran.

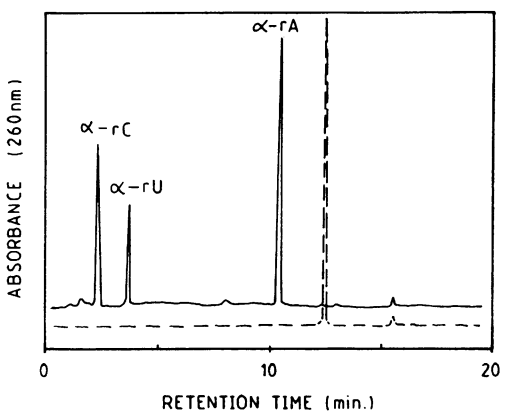

Figure 3. Reverse-phase HPLC profiles of purified α-[r(UCUUAACCCACA)] before (---) and after (——) hydrolysis with 0.1 M sodium hydroxide and alcaline phosphatase.

presence of imidazole (34) invariably produced, apart from traces of a highly lipophylic compound which is most likely the 2',3'-di-O-TBDMS derivative, a mixture of 2'-(fast eluting) and 3'-(slow eluting) TBDMS derivatives **3b–d** and **4b–d** respectively which were separated by silica gel chromatography and characterized by ^{1}H-NMR spectroscopy decoupling experiments. In the last step, the phosphoramidites **5b–d** were obtained according to a procedure similar to that already reported by Usman and coworkers for the synthesis of β-ribonucleoside phosphoramidites (23). Yields ranged from 79 to 88% and, as determined by ^{31}P-NMR analyses, the purity of these phosphoramidites was better than 90%, the main contaminant beeing the corresponding ribonucleoside 3'-phosphonate (doublet at about 10 ppm). Besides, the ^{31}P-NMR spectrum exhibited, as expected, only one pair of diastereoisomeric signals (Rp and Sp) for each α-ribonucleoside phosphoramidite, thereby establishing the isomeric purity of these compounds. No migration of the TBDMS group was detected during the phosphitylation reactions.

The preparation of the α-ribonucleoside derivatized supports **8a–c** was achieved via the formation of a succinyl linkage between the 2'-hydroxyl group of 3'-TBDMS-α-ribonucleosides **4a–c** and the amino function of the long chain alkylamine on controlled pore glass beads (LCA-CPG). The pentachlorophenyl succinate of 3'-5' protected α-ribonucleosides **7a–c** was reacted with LCA-CPG (25) and yielded loaded supports ranging from 18 to 26 μmol g^{-1}.

For the demonstration of the efficient synthesis of α-oligoribonucleotides, we describe the synthesis of the dodecamer α-[r(UCUUAACCCACA)]. This sequence was expected to be complementary in parallel orientation to the splice acceptor site of the HIV-1 tat mRNA, a target sensitive to unmodified and modified oligodeoxynucleotides (35–38).

This oligomer was assembled automatically on a DNA synthesizer using 1 μmole of protected α-adenosine loaded support. The condensation time required to obtain high coupling yields (≥97%) was 15 min using 0.5 M tetrazole as activator and 0.15 M phosphoramidite **5** in acetonitrile. The oligomer was deprotected under previously described conditions (14) and obtained in 52% yield. Figure 3 shows the C_{18} reverse phase HPLC elution profile of the 12 mer. Its homogeneity was ascertained by capillary gel electrophoresis analysis. Its nucleoside composition was confirmed after hydrolysis with 0.1 M sodium hydroxide and alkaline phosphatase followed by HPLC analysis of the hydrolysate which indicated complete degradation of the starting oligomer into α-rC, α-rU and α-rA in a molar ratio of 5/2.8/4.1 (Fig. 3).

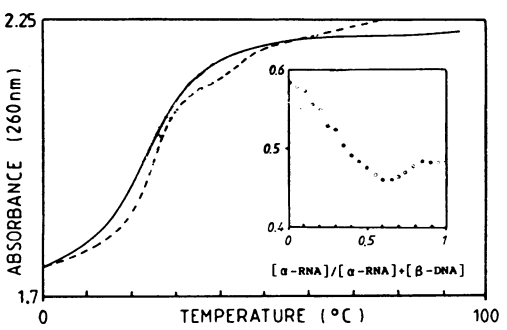

Figure 4. UV hybridization (——) and melting (---) profiles of an equimolar mixture of α-[r(UCUUAACCCACA)] and β-[d(AGAATTGGGTGT)]. Each strand concentration was 10 μM in 1M NaCl, 0.01 M sodium cacodylate (pH 7). Inset shows the mixing curve of the two α- and β-strands incubated at 4.7°C in the same buffer as above for various molar fractions of α-RNA strand.

A special feature of α-anomeric oligonucleotides, in both deoxy- and ribo series, is their high nuclease-resistance (7,15). For instance, in conditions where β-hexauridylate was fully degraded by RNase A, the corresponding α-hexamer was found intact (15). In fact the sterile conditions required during the handling and storage of β-oligoribonucleotides were not applied during the purification process of α-[r(UCUUAACCCACA)] and this α-oligomer was found intact after beeing stored frozen for several months at −20°C.

Base-pairing between α-[r(UCUUAACCCACA)] and its complementary DNA strand either in antiparallel or parallel orientation was studied by UV absorption spectroscopy. Whereas no significant absorbance variation versus temperature of an equimolar mixture of α-[r(UCUUAACCCACA)] and β-[d(TGTGGGTTAAGA)] was detected between 80 and 0°C, a clear transition (Tm 25.5°C) was observed in the case of an equimolar mixture of α-[r(UCUUAACCCACA)] and β-[d(AGAATTGGGTGT)] (Fig. 4). In contrast, the melting curve of the same mixture after beeing stored at 5°C overnight exhibited an additional transition (Tm 44.4°C). Moreover the mixing curve titration of these two latter oligomers suggests the formation at low temperature of a triplex involving two α-RNA and one β-DNA strands (Fig. 4, inset).

These results may be tentatively assigned to the slow formation at low temperature of the following triple stranded species:

```
5' α-r(UCUUAACCCACA) 3'
      | | | |  | | | |
5' ß-d(AGAATTGGGTGT) 3'
      | | | |  | | | |
5' α-r(UCUUAACCCACA) 3'
```

in which the three strands run parallely and exhibit 5 C·G·C and 3 U·A·U base triplets. The dodecamer α-[r(UCUUAACCCACA)] complementary in parallel orientation to the splice acceptor site of HIV-1 tat mRNA and the HIV-1 unrelated α-[r(ACUAAAUUCCCC)] which exhibits the same length and base composition were evaluated for their ability to block the *de novo* HIV-1 infection in MT4 cells. The antisense α-RNA was found to inhibit both syncytia formation and reverse transcriptase synthesis (10 μM < ED_{50} < 20 μM), whereas the antisense β-[d(ACACCCAATTCT)] was unefficient at concentration up to 100 μM. However the HIV-1 unrelated α-RNA dodecamer exhibited the same efficacy as its antisense α-RNA homolog. Further investigations on base-pairing properties of α-RNA will probably provide information on this lack of specificity.

In conclusion, we have synthesized for the first time an α-anomeric oligoribonucleotide presenting a mixed-base sequence and the corresponding phosphoramidites synthons have been fully described. As expected, we have shown that this α-oligoribonucleotide anneals in parallel orientation with its complementary DNA strand. However a 2 α-RNA/1 β-DNA stoichiometry was surprisingly shown and complementary work is currently in progress to determine the exact structure of such a triplex.

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