Nucleic Acids Research, Vol. 19, No. 15 4067–4074

Synthesis and physicochemical properties of oligonucleotides built with either α-L or β-L nucleotides units and covalently linked to an acridine derivative

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Received June 10, 1991; Accepted July 5, 1991

ABSTRACT

Modified deoxynucleosides 2′-deoxy-β-L-uridine, β-L-thymidine, α-L-thymidine, 2′-deoxy-β-L-adenosine and 2′-deoxy-α-L-adenosine were synthesized and assembled as homooligomers, respectively: octa-β-L-deoxyuridylates, octa β-L and α-L-thymidylates and tetra β-L and α-L-deoxyadenylates. These unnatural oligomers were then substituted with an acridine derivative. The binding studies of these modified oligonucleotides with D-ribo- and D-deoxyribopolynucleotides were carried out by absorption spectroscopy. While β-L-d(Up)$_8$m$_5$Acr, β-L-(Tp)$_8$m$_5$Acr, α-L-(Tp)$_8$m$_5$Acr did not interact with poly(rA) and poly(dA), β-L-d(Ap)$_4$m$_5$Acr and α-L-d(Ap)$_4$m$_5$Acr did form double and triple helices with poly(rU) and poly(dT), respectively. Their stability towards nuclease digestion was studied through comparison with that of octa-β-D-thymidylate and tetra β-D-deoxyadenylate covalently linked to an acridine derivative. One endonuclease (nuclease P1 from *Penicillium citrinum*) and two exonucleases (a 3′-exonuclease from *Crotalus durissus* venom and a 5′-exonuclease extracted from calf thymus) were employed. β-L- and α-L-oligomers demonstrate a high resistance toward nuclease digestion.

INTRODUCTION

During the past few years it has been demonstrated that gene expression can be regulated at the translational level by the use of synthetic oligodeoxynucleotides (1) called 'anti-messenger' or 'antisense' oligonucleotides. More recently synthetic oligonucleotides with homopyrimidic sequences were used to inhibit recognition of double helical DNA by sequence-specific DNA binding proteins at a homopurine-homopyrimidine target site (2–4). The efficiency of these synthetic oligonucleotides to regulate gene expression in living cells depends on the thermodynamic stability of the duplex or triplex structures, resistance towards nucleases and cellular uptake. To overcome the limitations due to the nuclease sensitivity of natural oligonucleotides several modifications have been introduced in the oligonucleotide chain. One possibility is to replace the phosphodiester bond by methylphosphonate (5, 6), phosphotriester (7) or phosphorothioate (8) groups. However, these modifications introduce chirality at the phosphorus atom. Thus an oligonucleotide containing n such modified linkages is a mixture of 2^n different molecules which are characterized by different association constants for binding to their complementary sequence. To avoid these difficulties, other kinds of modifications of the oligonucleotide chains have been developed. One of them consists in changing the anomeric configuration at the sugar moiety. α-D-oligodeoxynucleotides exhibit resistance towards nuclease activities and yet retain the ability to base pair with a complementary β-strand to form a parallel-stranded duplex (9, 10). They also form-triple helices at homopurine homopyrimidine sequences of duplex DNA (11).

In the field of modified oligonucleotides built with unnatural nucleotides, some other investigations have been done. The dinucleoside monophosphate ApA containing β-L-ribose possessed similar binding properties with poly(rU) as the parent compound in the β-D-series (12). In the same way a penta-oligoribonucleotide-β-L-5′CAAGG3′ hybridizes with its complementary β-D-ribose sequence (13). In order to develop further investigations in this field, we have synthesized oligonucleotides built with the β- and α-anomers of L-deoxynucleosides (Figure 1).

We describe here the preparation of modified nucleosides and their incorporation into oligonucleotide chains which were then coupled to an acridine derivative (Figure 2). The binding of these modified oligonucleotides to their complementary sequences and the effect of such modifications toward nuclease activity will be discussed.

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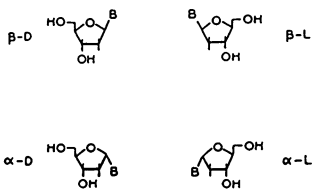
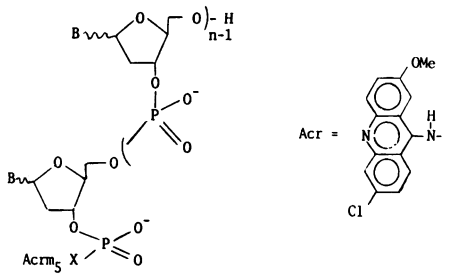


Figure 1. Structures of natural and unnatural deoxynucleosides.



6 : B = Uracile, anomer β, n = 8, X = O
7 : B = Adenine, anomer β, n = 4, X = O
8 : B = Adenine, anomer α, n = 4, X = O
15 : B = Thymine, anomer β, n = 8, X = S
16 : B = Thymine, anomer α, n = 8, X = S
m = $-CH_2-$

Figure 2. Structure of L-oligodeoxynucleotides.

EXPERIMENTAL SECTION

General methods

All chemicals were used as obtained unless otherwise stated. All solvents used were dried, distilled and stored as described in reference 24. Preparation of 2,2'-dithiodiethanol-derivatized support was achieved as described in reference 21. 2-Methoxy-6-chloro-9-(ω-hydroxypentylamino)acridine was obtained as previously described (20). Analytical thin-layer chromatography (TLC) was performed in precoated alumina plates (E. Merck silica gel 60 F 254 ref 5554) and preparative TLC on glass-backed plates of Merck silicagel 60 PF 254. For flash chromatography Merck silica gel 60 (230−400 mesh) (ref 9385), and Merck silica gel 60 (70−230 mesh) (ref 7734) and anhydrous solvents were used. All 4,4-dimethoxytrityl-containing substances were identified as orange colored spot on TLC plates by spraying with 10% perchloric acid solution. HPLC was performed on a Varian 5000 liquid chromatography apparatus equipped with a Varian UV 50 detector operating at 254 nm and at 425 nm. Analysis and purification by ion exchange chromatography were performed with a FPLC apparatus (Pharmacia). Optical rotations were measured on a Perkin-Elmer 141 polarimeter. IR spectra (film, KBr disk) were recorded on a Unicam SP3−300 spectrophotometer. ^1H NMR spectra were recorded in $CDCl_3$ or $(CD_3)_2SO$ using Me_4Si as internal standard, at a frequency 250 MHz with a Bruker AM 250 MHz operating in the FT mode. Absorption spectra were recorded with a Cary 210 spectrophotometer.

Extinction coefficients of β-D-d(Ap)$_4$m$_5$Acr were determined after digestion of the oligonucleotide chain by nucleases using a previously described procedure (20). This allowed us to determine $\epsilon_{425} = 8150\ M^{-1}.cm^{-1}$ for β-D-d(Ap)$_4$m$_5$Acr. The L derivatives are resistant to nuclease activity. We chose to use the same ϵ_{425} value for the three oligodeoxyadenylates. Endo and exonucleases were purchased from Boehringer Mannheim. Polynucleotides came from P.L. Biochemicals.

2'-deoxy-β-L-uridine 1

Synthesis of 2'-deoxy-β-L-uridine was achieved as described in refs 14 and 15. Yield 94%, mp 157−9°C (lit. 14 mp 158°C), $[\alpha]_D^{22}$ −28.9° (c 1.5, H_2O). Anal. calcd. for $C_9H_{12}N_2O_5$: C, 47.37; H, 5.29 ; N, 12.27. Found: C, 47.50; H, 5.31; N, 12.39.

β-L-Thymidine 2

2'-Deoxy-β-L-uridine **1** (14.4 mmol, 3.3 g) was added to a solution of 40% aqueous KOH (14.5 mL). The mixture was maintained at 65°C during 4 days with addition of 40% aqueous formaldehyde (3.5 mL) and 1 M aqueous KOH (3.5 mL) twice a day. After cooling, water (72 mL) was added and the pH was adjusted to 3 with IRN 77 resin (H^+ form). The resin was filtered off and the filtrate evaporated to dryness under reduced pressure to yield a residue which was dissolved in EtOH (8 mL), treated with Et_3N (1 mL) and further evaporated to dryness, then coevaporated with toluene (2×10 mL) and dried under reduced pressure at 50°C during 15 hrs. The resulting foamy material was dissolved in MeOH (200 mL), concentrated hydrochloric acid (0.75 mL) was added and the mixture refluxed during 10 hrs, then neutralized and evaporated to dryness under reduced pressure. Flash chromatography (silica gel 50 g, eluted by $CHCl_3$/MeOH, 93:7) afforded a compound which was hydrogenated (10% Pd/C, 750 mg) in MeOH solution (155 mL) during 4 hrs. The catalyst was filtered off on celite, rinsed with MeOH (50 mL) and the filtrate evaporated to dryness under reduced pressure. The resulting white solid was recrystallized from 90% EtOHAcOEt thus yielding **2** (2.15 g, 61.5%). mp 187−9°C, $[\alpha]_D^{23}$ −31.2° (c 1, 1 N NaOH). Anal. calcd. for $C_{10}H_{14}N_2O_5$: C, 49.59; H, 5.82; N, 11.56. Found C, 49.62; H, 5.89; N, 11.60.

3', 5'-di-O-benzoyl-2'-deoxy-α- and-β-L-N^6-benzoyladenosine

BSA (5.2 mL, 21 mmol) was added to a solution of 3', 5'-di-O-benzoyl-2'-deoxy-β-L-uridine (2.2 g, 5 mmol) and N^6-benzoyladenine (3.4 g, 14 mmol) in anhydrous acetonitrile (22 mL). The mixture was heated at 70°C for 15 min under mechanical stirring to afford a clear solution, to which TMS-triflate (1.25 mL, 6.5 mmol) was added. The mixture was stirred at 70°C for 4 hrs. The solvent was removed under reduced pressure and the residue was treated with cold aqueous $HNaCO_3$ (150 mL) and chloroform (150 mL). After vigorous shaking, the mixture was filtered and the organic layer was separated and dried over anhydrous $MgSO_4$. The solvent was evaporated under reduced pressure. The residual yellow sticky gum (6.7 g) was dissolved in a small amount of chloroform and chromatographed on a silica gel column with chloroform-containing methanol as eluent (29.9:0.1; 29.7:0.3; 29.4:0.6; 29.1:0.9). The fractions containing a mixture of α and β nucleosides were combined and the solvent was evaporated. The pure 3',5'-di-O-benzoyl-2'-deoxy-α- and β-L-N^6-benzoyladenosine were obtained by preparative TLC (CH_2Cl_2/THF, 95:5), (α-L-derivative, Rf 0.25, β-L-derivative, Rf 0.35).

2'-deoxy-α-L-N^6-benzoyladenosine 3

1 M aqueous solution hydroxide (11 ml) was added to a solution of 3',5'-di-O-benzoyl-2'-deoxy-α-L-N^6-benzoyladenosine (2.18 g, 3.9 mmol) in a mixture of THF (55 mL), methylene chloride (2 mL), H_2O (12 mL) and methanol (45 mL) at 0°C for 1 hr 15 min. The solution was neutralised with resin amberlite (IRN/pyridinium). The resin was filtered on celite and the solvent was removed under reduced pressure. The residual gum (1.19 g) was purified by preparative TLC (chloroform/methanol 85:15). 0.81 g (59%) of pure α-deoxynucleoside **3** was obtained: mp 110−113°C : $[\alpha]_D^{20}$ −39° (C 1.04, MeOH); R_f = 0.32 eluent chloroform/methanol (8:2); UV λmax (H_2O) 263 nm, ε 13,333; ^{1}H NMR ($CDCl_3$) δ 9.17, 8.8 (2s, 2H, H-2, H-8, 2H ar), 6.36 (dd, 1H, H-1', $J_{1'2'}$: 8.8 Hz), 4.51 (m, 1H, H-3', $J_{3'2'}$ = 8.3 Hz, $J_{3',4'}$ = 3.1 Hz), 3.8 (m, 1H, H-4', $J_{4',3'}$ = 3.1 Hz, $J_{4',5'}$ = 4 Hz), 3.7 (m, 2H, H-5', H-5''), 3.1 (m, 1H, H-2', $J_{2',2''}$ = 13 Hz), 2.6 (m, 1H, H-2'', $J_{2',1'}$ = 8.5 Hz), 2.1 (br s, 2H, 2OH).

2'-deoxy-β-L-N^6-benzoyladenosine 4

Treatment of 3',5'-di-O-benzoyl- 2'-deoxy-β-L-N^6-benzoyl-adenosine (1.5 g, 2.7 mmol) as described for the preparation of compound **3** afforded 0.534 g (50%) of pure β-deoxynucleoside **4**: mp 105−107°C; $[\alpha]_D^{20}$ 8.4° (c 1.02, MeOH); R_f = 0.43 eluent chloroform/methanol (8:2) UV λmax (H_2O) 283 nm, 16,700 : ^{1}H NMR ($CDCl_3$) δ 9.05, 8.02 (2s, 2H, H-2, H-8, 2H ar), 8.05 (s, 1H, N-H), 7.9−7.95 (m, 2H, 2H ar), 7.4−7.6 (m, 3H, 3H ar), 6.4 (dd, 1H, H-1', $J_{1',2'}$ = 8.5 Hz, $J_{1',2''}$ = 5.8 Hz), 4.81 (m, 1H, H- 3', $J_{3',4'}$ = 4.4 Hz), 4.27 (m, 1H, H-4', $J_{4',5'}$ = 6.64 Hz), 3.97−3.85 (m, H, H-5', H-5'',$J_{5',5''}$ = 12.7 Hz), 3.06 (m, 1H. H-2', $J_{2',1'}$ = 8.5 Hz), 2.34 (m, H, H-2'', $J_{2',2''}$ = 13.5 Hz), 2.01 (br s, 2H, 2OH).

1-O-acetyl-2,3,5-tri-O-benzoyl-α- and -β-L-arabinofuranoside

A solution of methyl-2,3,5-tri-O-benzoyl- α-L-arabinofuranoside (obtained as described in refs 25−27) (8.0 g, 16.4 mmol) in glacial acetic acid (84.5 mL) and acetic anhydride (16.9 mL) was stirred at 5−10°C while concentrated sulfuric acid (4.9 mL) was added dropwise (28). After completion of the reaction (4 hrs at room temperature) the reaction mixture was poured into ice-water (300 mL) and extracted with chloroform (2×100 mL). The extracts were combined, washed successively with saturated aqueous $HNaCO_3$ (2×50 mL) and water (50 mL), dried over anhydrous $MgSO_4$, filtered and evaporated under reduced pressure. A syrup, containing the two anomers (8.0 g, 95%) was obtained and used in the next step without purification.

1-[2',3',5'-tri-O-benzoyl-α-L-arabinofuranosyl] thymine

To a mixture of thymine (2.06 g, 16.4 mmol) and 1-O-acetyl-2,3,5-tri-O-benzoyl-α- and β-L-arabinofuranoside (8 g, 16.4 mmol) in 150 ml of anhydrous CH_3CN, were successively added hexamethyldisilazane (2.74 mL, 13.1 mmol), trimethylchlorosilane (1.6 mL, 13.1 mmol) and $SnCl_4$ (2.3 mL, 19.7 mmol) (29, 30). The resulting clear solution was refluxed for 1 hr and TLC indicated completion of the reaction. The reaction mixture was concentrated to a small volume, diluted with CH_2Cl_2 (200 mL), washed with saturated aqueous $NaHCO_3$ (2×75 mL) and with water (3×50 mL). The organic phase was dried over $MgSO_4$, filtered and evaporated to give a white foam (7.53 g, 80%) which was chromatographed on a silica gel column ($CHCl_3$/acetone 9:1). Evaporation of the appropriate fractions afforded 4.52 g (60%) of pure product: mp 85°C; $[\alpha]_D^{20}$ −6.6° (c 0.6, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 6.2 (d, 1H, H-1', $J_{1',2'}$ = 3.1 Hz), 5.95 (t, 1H, H-2', $J_{2',3'}$ = 2.9 Hz), 5.75 (t, 1H, H-3', $J_{3',4'}$ = 3 Hz), 4.95 (m, 1H, H-4', $J_{4',5'}$ = 5.9 Hz, $J_{4',5''}$ = 4.8 Hz), 4.6−4.8 (m, 2H, H-5', H-5'', $J_{5',5''}$ = 12 Hz), 8.8 (br s, 1H. N-H), 7.3 (s, 1H, H-6), 1.95 (s, 3H, CH_3), 7.3−8.2 (m, 15H, -CO- C_6H_5). Anal. calcd. for $C_{31}H_{26}O_9N_2$: C, 65.26; H, 4.56; N, 4.91. Found: C, 65.47; H, 4.61; N, 4.67.

α-L-thymidine 5

The compound obtained above was debenzoylated according to ref 14 and 1[3', 5'-O-tetraisopropyldisiloxane-1, 3-diyl)-α-L-arabinofuranosyl] thymine was prepared as described in refs 17 and 31a, b. Treatment with phenyl chlorothionoformate followed by reduction with $n(Bu)_3SnH$ (18) and deprotection of the obtained nucleoside (17, 31) led to α-L-thymidine; m.p. 187−188°C ; litt (16) m.p. 188−190°C for the α-D isomer. $[\alpha]_D^{20}$ −11.55° (c 1.0, NaOH 1N); ^{1}H NMR (D_2O), δ 7.6 (d, 1H, H-6, $J_{H\text{-}6,CH}$ = 1 Hz), 6.0 (dd, 1H, H-1', $J_{1',2'}$ = 7.4 Hz, $J_{1',2''}$ = 3.1 Hz), 4.3.−4.15 (m, 2H, H-3', H-4'), 3.6−3.4 (m, 2H, H-5', H-5'', $J_{5',5''}$ = 12.3 Hz, $J_{5',4'}$ = 5.2 Hz, $J_{5',4'}$ = 3.8 Hz), 2.65−2.5 (m, 1H, H-2', $J_{2',2''}$ = 13.9 Hz), 2.1−1.95 (m, 1H, H-2''), 1.75 (s, 3H, CH_3); MS, mz 242.23, 243 (100, M + 1), 260 (29.4, M + NH_3). Anal. calcd. for $C_{10}H_{14}O_5N_2$: C, 49.5; H, 5.8; N. 11.6. Found: C 49.38; H, 5.82; N, 11.43.

5'-O-Dimethoxytrityl-β-L-thymidine (Scheme)

Compound **2** (0.242 g, 1 mmol) was dissolved in anhydrous pyridine and evaporated (three times) and then dissolved in anhydrous pyridine (8 mL) and 4,4'-dimethoxytrityl chloride (0.355 g, 1.05 mmol) was added at room temperature with stirring. After 2 h. methanol (1 mL) was added and after additional 10 min the mixture was poured into 5%, $NaHCO_3$ solution (10 mL), extracted with CH_2Cl_2 (2×20 mL), and the combined organic layer dried over Na_2SO_4 and evaporated to dryness. The residue was separated by flash chromatography over silica gel. Eluent CH_2Cl_2/MeOH 99:1 v/v to CH_2Cl_2/MeOH 95:5 v/v, yield 95%.

5'-O-Dimethoxytrityl-α-L-thymidine

Treatment of **5** as described for **2** afforded 5'-O-dimethoxytrityl-α-L-thymidine (yield 94%).

5'-O-Dimethoxytrityl-β-L-thymidine-3'-[(2-cyanoethyl)-N,N-diisopropyl] phosphoramidite 9

5'-O-Dimethoxytrityl-β-L-thymidine (0.272 g, 0.5 mmol) was dried overnight under high vacuum, then dissolved in 20 ml of anhydrous CH_2Cl_2 ; tetrazole (0.035 g, 0.5 mmol) as well as (2-cyanoethyl) bis-(diisopropylamido) phosphite (0.250 g, 0.75 mmol) were added with stirring. Stirring was continued for 30 min, then the mixture poured into 18 mL of saturated $NaHCO_3$ solution and extracted with 3×30 mL of CH_2Cl_2, and the combined organic layer extracted once with 12 mL of saturated NaCl solution and evaporated. The residue was taken up in 5 mL of CH_2Cl_2 and the product precipitated with 180 mL of pentane. The precipitate was dried and isolated as white powder (yield 95%). TLC (CH_2Cl_2/acetone/NEt_3, 75:20:5 v/v) Rf = 0.77 and 0.75 (mixture of diastereoisomers).

5'-O-Dimethoxytrityl-α-L-thymidine-3'-[(2-cyanoethyl)-N,N-diisopropyl] phosphoramidite 10

Treatment of 5'-O-dimethoxytrityl-α-L-thymidine as described for 5'-O-dimethoxytrityl-β-L-thymidine afforded compound **10** (yield 94%). TLC (CH_2Cl_2/acetone/NEt_3, 75:20:5 v/v) Rf = 0.75 and 0.73 (mixture of diastereoisomers).

Preparation of the derivatized support 11

A mixture of **9** (0.038 g, 0.027 mmol) and tetrazole (0.007 g, 0.1 mmol) in anhydrous acetonitrile was shaked by hand with the derivatized support.

HO-CH_2-CH_2-S-S-CH_2-CH_2O-C(O)-CH_2-CH_2-C(O)-NH-CH_2-CH_2-CH_2-Fractosil 500

(0.1 g, 0.055 mmol) (obtained as described in reference 21) for 5 min under nitrogen atmosphere. After filtration to remove the excess of phosphoramidite **9** and wash with anhydrous acetonitrile, sulfurization step was achieved by shaking the derivatized support with a solution (2 mL) of S_8 (0.004 mg, 0.12 mmol) in CS_2/pyridine (2:1 v/v) for 30 min. After wash to remove the excess of reagent the unreacted hydroxyl groups were capped as described in reference 24. The loading of support **11** is approximately 0.060 mmol of nucleoside phosphorothioate derivative per gram.

Preparation of the derivatized support 12

By using compound **10** and operating as described for the preparation of support **11**, derivatized support **12** was obtained with a loading of approximately 0.054 mmol of nucleoside phosphorothioate derivative per gram.

Oligonucleotides synthesis

The octadeoxyuridylate **6** and the two tetradeoxyadenylates **7** and **8** were obtained by using the phosphotriester method in solution as previously described for β-D-oligonucleotide synthesis (19, 20).

The octathymidylate-3'-phosphorothioates **13** and **14** containing either β-L-T or α-L-T nucleoside units were synthesized on a Pharmacia synthesizer by using a modified phosphoramidite procedure. The sequential growth of each oligonucleotide chain was performed in 5 μmole scale on support **11** and **12** using 28 μmole of phosphoramidites respectively **9** and **10** with a cycle time of 21 min as shown in Table II.

Unblocking of the oligonucleotides

The unblocking was achieved according to the previously described procedure (20) in the case of oligodeoxyuridylate and oligodeoxyadenylates obtained via the phosphotriester method in solution. The protocol reported in reference 21 was used in the case of the oligothymidylate synthesized using the phosphoramidite method on solid-phase.

Oligonucleotide purification and analysis

After unblocking and extraction of the organic impurities, the crude reaction product **6–8, 13, 14**, were purified by ion exchange chromatography using a Pharmacia FPLC system equipped with a Mono P HR 5/5 column (50 mm×5 mm diameter) from Pharmacia.

A 10^{-2} mM NaH_2PO_4 buffer, pH 6.8, in acetonitrile/water 20:80 v/v with a linear gradient of NaCl, ranging from 0 to 1 M over 25 min at 1 ml/min was used as eluent. The fractions were monitored by absorption at 254 nm. After desalting, the purity of all the oligonucleotides described here has been checked by using two chromatographic systems (retention time are given in Table II).

A second run was performed using the same ion exchange system with the same elution conditions[a]. After this, reverse phase analysis was performed on a Varian 5000 liquid chromatography apparatus equipped with a Varian UV 50 detector operating at 254 nm for all the compounds and also at 425 nm for those containing the acridine ring. Two systems were used, a reverse phase column (Lichrosorb RP-18, 5 μm, 150 mm×3 mm) from Merck with a linear gradient of acetonitrile from 13.5% to 18.1% over 20 min, in 120 mM aqueous ammonium acetate pH 5.9, with a flow rate of 0.8 mL/min[b] or a reverse phase column (Lichrosorb RP-18, 5 μm, 125 mm×4 mm) from Merck with a linear gradient of acetonitrile from 23.75% to 38.75% over 20 min in 100 mM triethylammonium acetate buffer pH 7 at 1 mL/min[c].

Coupling of 2-methoxy-6-chloro-9-(ω-bromopentylamino) acridine with the unblocked oligomer 13

Lyophilized oligonucleotide **13** (potassium salt) (20 OD units) was dissolved in MeOH (1 ml) in the presence of Crown-6 ether (0.001 g); an excess of acridine derivative (≈0.002 g) dissolved in MeOH (0.5 mL) was added. The mixture was incubated with stirring at room temperature for 48 h. The mixture was then concentrated to dryness. The residue was dissolved in H_2O/MeOH mixture and the excess of acridine derivative was separated by gel filtration on G10 column (from Pharmacia).

The unreacted oligonucleotide **13** and the reaction-product (yellow-colored solution) were first collected. FPLC analysis by anion exchange chromatography on a Mono P column, as described above, gave a yield of about 80%. After being purified as described above the acridine-derivatized β-L-oligothymidylate **15** was analyzed on reverse phase system. Retention times are given in Table II.

Coupling of 2-methoxy-6-chloro-9-(ω-bromopentylamino) acridine with the unblocked oligomer 14

By using the oligomer **14** and operating as described for compound **13**, the acridine derivatized α-L-oligothymidylate **16** was obtained in about 75% yield (for retention time, see Table II).

RESULTS AND DISCUSSION

Oligonucleotide synthesis

The sole L-pentose abundant in nature, L-arabinose, was used as starting material for the synthesis of the nucleosides employed in this study but different strategies were followed depending on the particular nucleoside.

2'-Deoxy-β-L-uridine **1** was prepared by construction of the heterocyclic ring at the anomeric carbon of the sugar (14, 15), the β configuration of the obtained nucleoside is controlled by the C-2 hydroxyl. Hydroxymethylation of **1** with formaldehyde under basic conditions followed by acid catalyzed etherification in methanol and reduction afforded β-L-thymidine **2**.

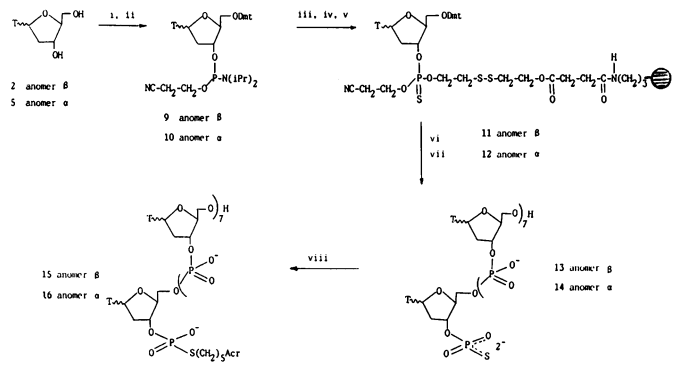
2'-Deoxy-α- and β-L-N^6-benzoyladenosine **3** and **4** were prepared in a different way because construction of the purine ring is more difficult than that of the pyrimidine ring. Since coupling of activated N^6-benzoyladenine with a L-arabinose derivative would afford only the α anomer, we decided to prepare an α/β mixture by acid-catalyzed exchange (16). Treatment of

3',5'-di-O-benzoyl-2'-deoxy-β-L-uridine with an excess of N^6-benzoyladenine in the presence of bis(trimethylsilyl)-acetamide (BSA) and trimethylsilyl trifluoromethanesulfonate (TMS-triflate) in anhydrous acetonitrile (70°C, 4 h) afforded a mixture of 3',5'-di-O-benzoyl-2'-deoxy-α- and β-L-N^6-benzoyladenosine. The two anomers were separated by preparative TLC and the ester groups removed under basic conditions yielding pure 2'-deoxy-α-L-N^6-benzoyladenosine **3** and 2'-deoxy-β-L-N^6-benzoyladenosine **4**.

The preparation of 1-[2', 3', 5'-tri-O-benzoyl-α-L-arabinofuranosyl] thymine was carried out by stereospecific glycosylation of thymine with 1-O-acetyl-2,3,5-tri-O-benzoyl-α- and β-L-arabinofuranoside. After debenzoylation and selective protection of 3' and 5' hydroxy groups (17), followed by a Barton deoxygenation reaction (18), and final deprotection of the protected deoxynucleoside, α-L-thymidine **5** was then obtained.

The octamer of β-L-deoxyuridylate and tetramers of β-L-deoxyadenylate and α-L-deoxyadenylate, covalently linked through their 3' terminal hydroxyl to the 9-amino group of 9-amino-6-chloro-2-methoxyacridine via a pentamethylene phosphate linker [β-L-d(Up)$_8$m$_5$Acr **6**, β-L-d(Ap)$_4$m$_5$Acr **7**, α-L-d(Ap)$_4$m$_5$Acr **8**] were synthesized by the phosphotriester method in solution described for β-D-oligodeoxynucleotides (19, 20).

The synthesis of the two octa-β-L-thymidylate and octa-α-L-thymidylate derivatized at their 3' end with 9-amino-6-chloro-2-methoxyacridine via a pentamethylene thiolophosphate linker [β-L-(Tp)$_7$Tpsm$_5$Acr **15**, α-L-(Tp)$_7$Tpsm$_5$Acr **16**] was carried out using a different procedure. The method consists first in the automated preparation of oligonucleotides carrying a thiophosphate group at their 3' end [β-L-(Tp)$_7$Tps **13**, α-L-(Tp)$_7$Tps **14**]. This was achieved by adaptation of the method previously described for β-D-nucleotides (21). This procedure needs the preparation of both nucleoside-phosphoramidite **9** and **10** and modified solid support **11** and **12** for each modified oligonucleotide (Scheme).



Scheme: Reagents and conditions: i) dimethoxytritylchloride, pyridine; ii) NC-CH_2-CH_2-O-P($NiPr_2$)$_2$, tetrazole, CH_3CN; iii) HO-CH_2-CH_2-S-S-CH_2-CH_2-O-C(O)-CH_2-CH_2-C(O)-NH-$(CH_2)_3$-Fractosil 500, tetrazole, CH_3CN; iv) S_8, CS_2/Py; v) Ac_2O, dimethylaminopyridine; vi) elongation of the oligonucleotide chain (see Table I); vii) NH_4OH, dithiothreitol, H^+; viii) Acr$(CH_2)_5$-Br, 6-crown-18, MeOH.

Automated synthesis of the oligonucleotides **13** and **14** was performed using the cycle described in Table I.

After the unblocking step and the purification, the oligonucleotides with a terminal 3'-thiophosphate **13**, **14** were coupled with the bromoalkyl derivative of the acridine-linker compound to give the expected thiolester derivatives **15** and **16** (Scheme).

The oligomers described above were purified by ion exchange chromatography. Purity of every oligonucleotide was checked by analysis on ion exchange column and reverse-phase chromatography. Retention times are given in Table II.

Step N°	Reagent	Flow (ml.min^{-1})	Time (min)
1	$C_2H_4Cl_2$	2.5	1.5
2	$Cl_2CHCO_2H/C_2H_4Cl_2$ 3:97 v/v	2.5	2.4
3	CH_3CN	2	1
4	Recycling 0.35 M Te 0.01 M 9 (or **10**) in CH_3CN (1.4 mL)	2.5	6
5	0.01 M I_2 in CH_3CN/H_2O/collidine 65:30:6 v/v	2.5	1
6	CH_3CN	2.5	0.3
7,9,11,13	0.5 M DMAP in CH_3CN	1	0.1
8,10,12,14	Ac_2O/collidine/CH_3CN 20:30:50 v/v	1	0.1
15	CH_3CN	2.5	2

Table I. Modified cycle synthesis used for automated preparation of compounds **13** and **14**.

Compound	Anion exchange (a)	Reverse phase
β-L-d(Up)$_8$m$_5$Acr **6**	14 min 15 sec	10 min 56 sec (c)
β-L-d(Ap)$_4$m$_5$Acr **7**	8 min	11 min 17 sec (b)
α-L-d(Ap)$_4$m$_5$Acr **8**	8 min	9 min 4 sec (b)
β-L-(Tp)$_7$Tps **13**	18 min 15 sec	
α-L-(Tp)$_7$Tps **14**	16 min 52 sec	
β-L-(Tp)$_7$Tpsm$_5$Acr **15**	14 min 45 sec	14 min 18 sec (c)
α-L-(Tp)$_7$Tpsm$_5$Acr **16**	14 min	15 min 7 sec

Table II. Retention time of modified oligomers obtained using different chromatographic systems. Eluents a, b, c are described in the experimental section (see oligonucleotide analysis part).

Stability toward nucleases

Stability of the modified oligonucleotides **6**, **7**, **8**, **15** and **16** towards endonucleases and exonucleases was studied through comparison with the parent β-D-oligomers, [β-D-(Tp)$_8$m$_5$Acr and β-D-d(Ap)$_4$m$_5$Acr] previously synthesized (20). One endonuclease (nuclease P1 from *Penicillium citrinum*) and two exonucleases (a 3'-exonuclease extracted from *Crotalus durissus* venom and a 5'-exonuclease extracted from calf thymus) were used. As expected, attachment of the acridine at the 3' end of the oligonucleotides protected them against degradation by the 3'-exonuclease extracted from *Crotalus durissus*.

With calf thymus 5'-exonuclease at a concentration which allowed nearly complete digestion of β-D-d(Ap)$_4$m$_5$Acr and β-D(Tp)$_8$m$_5$Acr, in 5 and 7 min respectively, no degradation of the corresponding L compounds (either β or α) was observed after 72 hrs of incubation. Previous results obtained with α-D-(Tp)$_8$m$_5$Acr showed that the same level of degradation in these

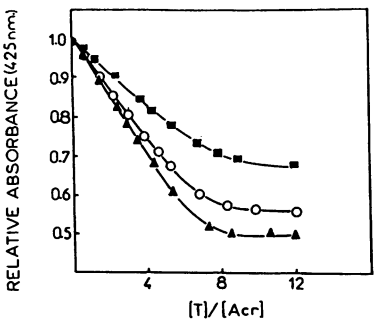


Figure 3. Change in absorbance at 425 nm of β-D-d(Ap)$_4$m$_5$Acr (▲), α-L-d(Ap)$_4$m$_5$Acr (O) and β-L-d(Ap)$_4$m$_5$Acr (■), upon addition of D-poly(dT). Measurements were carried out at 2°C in a pH 7 buffer containing 0.01 M sodium cacodylate and 0.1 M NaCl. The oligonucleotide concentration was 10 μM.

	Stoichiometry [T]/[Acr]	Hypochromicity at 425 nm, 2°C	Tm °C	Isosbestic λnm
β-D-d(Ap)$_4$m$_5$Acr	7.5	50 %	52.6	350,367,457
α-L-d(Ap)$_4$m$_5$Acr	8.0	44 %	14.4	348,367,457.5
β-L-d(Ap)$_4$m$_5$Acr	8.0	33 %	9.8	351,369,459.5

Table III. Stoichiometries, hypochromicities, melting temperatures and isosbestic wavelengths of complexes of α-L- and β-L-oligodeoxyadenylates with D-poly(dT). Total oligonucleotide concentration was 10 μM. Measurements were carried out in a pH 7 buffer containing 0.01 mM sodium cacodylate and 0.1 M NaCl.

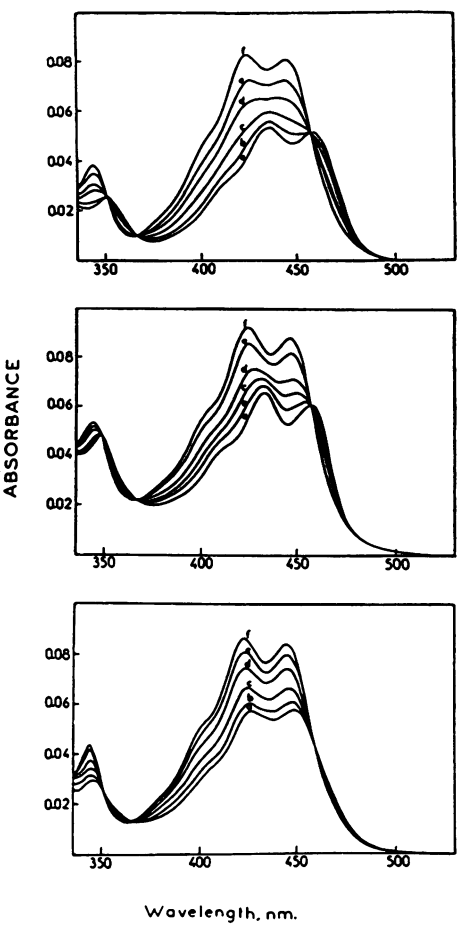


Figure 4. Change in absorbance spectrum with temperature of 2:1 (T to A) mixtures of D-poly(dT) with β-D-d(Ap)$_4$m$_5$Acr (upper), α-L-d(Ap)$_4$m$_5$Acr (middle) and β-L-d(Ap)$_4$m$_5$Acr (lower). Temperatures are: (upper) a, 12.1°C; b, 47.9°C; c, 50.7°C; d, 52.7°C; e, 53.9°C; f, 59.1°C. (middle) a, 3.4°C; b, 6.3°C; c, 8.4°C; d, 10.2°C; e, 11.3°C; f, 22.1°C. (lower) a, 3.0°C; b, 11.4°C; c, 13.1°C; d, 14.5°C; e, 16.6°C; f, 28.3°C. Measurements were carried out in a pH 7 buffer containing 0.01 mM sodium cacodylate and 0.1 M NaCl. The oligonucleotide concentration was 10 μM.

conditions required 40 hrs of incubation (9). With endonuclease P1 the same results were obtained. Nuclease P1 concentration, which hydrolyzed α-D-oligothymidylates in 42 hrs, did not induce any degradation of β-L- and α-L-oligonucleotides after 72 hrs of incubation.

Interaction with complementary sequences

Interaction of the oligomers β-L-d(Up)$_8$m$_5$Acr **6**, β-L-d(Ap)$_4$m$_5$Acr **7**, α-L-d(Ap)$_4$m$_5$Acr **8**, β-L-(Tp)$_7$Tpsm$_5$Acr **15** and α-L-(Tp)$_7$Tpsm$_5$Acr **16** with the ribo and the deoxyribopolynucleotides poly(rU), poly(rA), poly(dA) and poly(dT) was followed by absorption spectroscopy in the visible absorbance band of the acridine derivative and in the UV range. Two behaviours were observed depending on the investigated compound.

Upon addition of poly(rA) or poly(dA) to a solution of β-L-d(Up)$_8$m$_5$Acr, α-L-(Tp)$_7$Tpsm$_5$Acr or β-L-(Tp)$_7$Tpsm$_5$Acr at 2°C, no change of the absorption spectra could be observed neither in the presence of 0.1 M NaCl nor at 1 M NaCl concentration. When the temperature of the 1:1 mixtures (A to U or A to T) was increased from 2°C to 80°C no change was observed in the absorption spectra between λ = 220 nm and λ = 530 nm. These results showed that neither β- and α-L-oligothymidylates **15** and **16** nor β-L-oligodeoxyuridylates **6** hybridized with the complementary polypurine sequences poly(rA) and poly(dA). These results are in agreement with the literature reports which showed that β-L-d(Up)$_{17}$dU did not interact with poly(dA) (15) neither did α- and β-L-(Tp)$_8$(CH$_2$)$_3$OH with β-D-d(Ap)$_7$A or with poly(rA) (22).

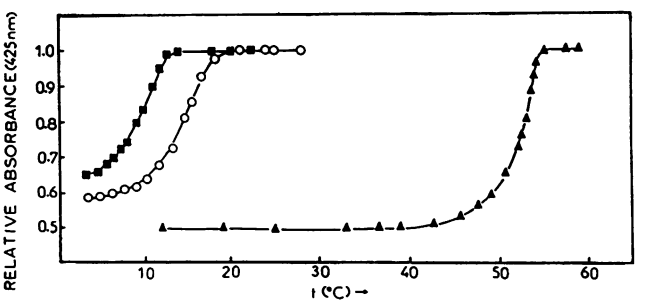


Figure 5. Temperature dependence of absorbance at 425 nm for 2:1 (T to A) mixtures of D-poly(dT) with β-L-d(Ap)$_4$m$_5$Acr (■), α-L-d(Ap)$_4$m$_5$Acr (O) or β-D-d(Ap)$_4$m$_5$Acr (▲). Same conditions as for Figure 4.

In contrast studies carried out with β-L-d(Ap)$_4$m$_5$Acr and α-L-d(Ap)$_4$m$_5$Acr showed that these compounds did interact with D-poly(dT) and D-poly(rU). Addition of D-poly(dT) to a solution of β-L-d(Ap)$_4$m$_5$Acr, α-L-d(Ap)$_4$m$_5$Acr or β-D-d(Ap)$_4$m$_5$Acr

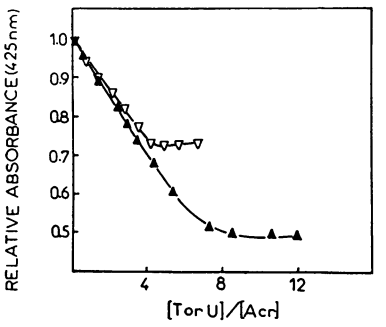


Figure 6. Change in absorbance at 425 nm of β-D-d(AP)$_4$m$_5$Acr upon addition of D-poly(dT) (▲) or D-poly(rU) (▽). Same conditions as for Figure 3.

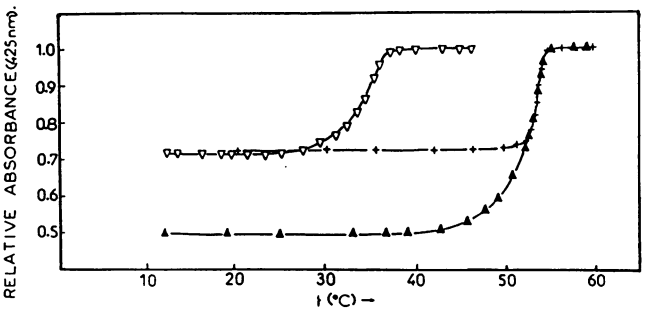


Figure 7. Melting curves for complexes of β-D-d(Ap)$_4$m$_5$Acr with D-poly(rU) (▽) [1:1 (U to A) mixture]; D-poly(dT) (+) [1:1 (T to A) mixture] and D-poly(dT) (▲) [2:1 (T to A) mixture]. Same conditions as for Figure 4.

induced hypochromicity in the visible absorbtion band of the acridine derivative. Plotting relative absorbance versus [T]/[Acr] led to a stoichiometry of about 8, indicating that a triple helix (2 T to 1 A) was formed in all cases. As expected no changes in the visible absorbance band of the acridine derivative were observed upon addition of D-poly(rA) to the three tetraadenylate solutions at 2°C.

Titration curves are shown in Figure 3 and the hypochromicities measured at 425 nm for a T to A ratio of 2:1 are given in Table III. The changes observed in the absorption spectra are not the same for the three oligodeoxyadenylates. Hypochromicities are 44%, 33% and 50% for α-L, β-L- and β-D compounds respectively (Table III). The most striking difference is observed in the red-shift of the visible absorbance band of the acridine derivative. A large red-shift was induced upon binding of either β-D-d(Ap)$_4$m$_5$Acr (425 → 433 nm) or α-L-d(Ap)$_4$m$_5$Acr (425 → 435 nm) to D-poly(dT) but not upon binding of β-L-d(Ap)$_4$m$_5$Acr to poly(dT) (425 → 427 nm). In all cases, isosbestic points could be observed (see Table III and Figure 4).

Increasing the temperature of a 2 T:1 A complex induced a reversal of the spectroscopic effects observed upon adding D-poly(dT) to the acridine-substituted oligodeoxyadenylates (Figure 4). Temperatures of half-dissociation (Tm) are given in Table III at a total oligonucleotide concentration of 10 μM. It can be seen that complexes of α-L- and β-L-tetradeoxyadenylates with D-poly(dT) are less stable than that of the corresponding β-D-tetradeoxyadenylate and D-poly(dT). The complex formed with α-L-d(Ap)$_4$m$_5$Acr is a little more stable than that obtained with the corresponding β-L-anomer (Figure 5).

The same studies were also performed with polyribonucleotide D-poly(rU). Addition of D-poly(rU) to a solution of any of the tetradeoxyadenylates at 2°C also induced changes of the visible absorbance band of the acridine. But when absorbance at 425 nm was plotted versus ([U]/[A]) concentration ratio, the break occurred at a 1:1 ratio indicating the formation of a double helix for the binding of β-D-d(Ap)$_4$m$_5$Acr with D-poly(rU) (Figure 6). The melting temperature was lower (Tm ≈ 34.4°C) than that observed for the complexes of β-D-d(Ap)$_4$m$_5$Acr with D-poly(dT) (Tm ≈ 52.6°C for [T]/[A] = 2/1 and Tm ≈ 53.7 for [T]/[A] = 1/1) (Figure 7). Both at a 1:1 and 2:1 ratio β-D-d(Ap)$_4$m$_5$Acr formed a triple helix with D-poly(dT). The hypochromism observed at a 1:1 ratio is about half of that obtained at a 2:1 ratio, as expected at constant concentration of the acridine substituted oligomer.

In the case of complexes formed with either α-L-d(Ap)$_4$m$_5$Acr or β-L-d(Ap)$_4$m$_5$Acr and D-poly(U) the binding curves suggested that at 2°C the double helix was partially melted. In contrast with the results observed with β-L-d(Ap)$_4$m$_5$Acr, a recent report (23) showed interaction of the non substituted β-L-d(Ap)$_5$A with D-poly(U) but not with D-poly(dT).

CONCLUSIONS

Starting from L-arabinose, a natural sugar, five different L-nucleosides were synthesized following different strategies. 2'-deoxy-β-L-uridine was obtained by complete construction of the uracil base at the anomeric carbon atom of the sugar. β-L-thymidine was obtained by introduction of a methyl group at the C5 atom of β-L-deoxyuridine, α-L-thymidine was prepared via stereospecific glycosylation of thymine with a L-arabinose derivative. 2'-deoxy-α-L- and β-L-N^6-benzoyladenosine were synthesized as an α/β mixture by acid catalyzed exchange from 5', 3'-protected β-L-deoxyuridine and then separated as pure α and β anomer. The structure of each of these five L-nucleosides was ascertained by ^{1}H NMR spectroscopy and by optical rotation measurements and comparison with the values of the D-isomers.

By adaptation of the previously described procedures every unnatural L-nucleoside was used to prepare the homooligonucleotides covalently linked through their 3'-terminus to an acridine derivative. The purity of these oligomers was checked by HPLC analysis using two systems: anion exchange and reverse-phase chromatography. As expected these acridine-derivatized L-deoxyhomooligonucleotides were resistant towards nuclease hydrolysis. The degradation of these compounds by a 5'-exonuclease, a 3'-exonuclease and an endonuclease was much slower than that of the previously described α-D-deoxyoligonucleotides.

The interactions of L-oligomers with the complementary deoxy- and ribo-β-D-homopolynucleotides were studied by absorption spectroscopy. Whereas β-L-d(Up)$_8$m$_5$Acr or β-L and α-L-d(Tp)$_7$Tpsm$_5$Acr did not form complexes with complementary β-D-oligo(A)$_n$ sequences, β-L and α-L-d(Ap)$_4$m$_5$Acr formed complexes with β-D-oligo(dT) sequences. A triple helix was formed with the stoichiometry 2 T:1 A as observed with β-D-d(Ap)$_4$m$_5$Acr. However the stability was less for the α-L- and β-L-derivatives than for the β-D-oligomer. It should also be noted that the spectral changes observed with the α-L-oligomer were quite similar to those seen with the β-D-oligomer (Figure 3) which indicates that the environment of the acridine ring is similar in

both cases. A different situation was found with the β-L-oligomer which also formed the less stable complex.

ACKNOWLEDGEMENTS

This work was supported in part by the Agence Nationale de Recherches sur le SIDA, the Fédération Nationale des Centres de Lutte contre le Cancer, the Fondation pour la Recherche Médicale, and Rhône-Poulenc-Santé.

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