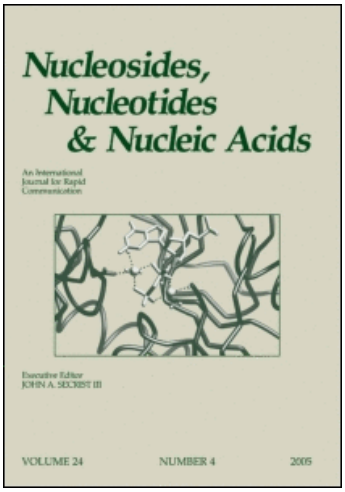

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Solid Phase Synthesis of 2',5'-Oligoadenylates Containing 3'-Fluorinated Ribose§

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Abstract: 2',5'-phosphodiester bond-linked oligoadenylate trimers with 3'-fluoro-3'-deoxyadenosine residues incorporated at specific positions of the nucleotide sequence were synthesized by the solid phase phosphite triester (phosphoramidite) method. The syntheses were in the 2' to 5' direction and were performed manually using commercially available microcolumns. The oligonucleotides were 5'-end phosphorylated on the support before deprotection.

The series of 2',5'-phosphodiester bond linked small oligoadenylates of the general formula ppp5'A2'(p5'A)$_n$ (n≥1) is called 2-5A[1]. With the exception of the dimeric member of this group, these 2',5'-oligonucleotides are potent inhibitors of translation and also can play a role in the degradation of mRNA and rRNA. Their mechanism of action is mediated exclusively through the activation of a latent 2-5A-dependent endonuclease[2,3]. It appears that each individual nucleotide residue of 2-5A may assume a fundamentally different role in binding to and activation of the 2-5A-dependent RNase. Several sites of strong interaction of the endonuclease with its oligonucleotide activator have been uncovered to date[4]. These include the polyphosphate or monophosphate (depending on the source of the enzyme) of the 5'-terminus, the purine N1/N^6-amino moiety of the adenosine residues of the 5'-terminal (first) and the third nucleotide, the purine N7 of the first (5'-terminus) and third residues, and the 3'-hydroxyl group of the second nucleotide of 2-5A[4-8]. Furthermore, there is a vital dependence on the nature of the phosphodiester linkage: a 3',5'-linkage isomer of 2-5A was orders of magnitude less active as a translational inhibitor [9].

Previous studies[7] have shown that only the 3'-hydroxyl group of the second from the 5'-terminus of 2-5A is needed for effective activation of the 2-5A dependent nuclease. To evaluate in

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more detail the contribution of the 3'-OH to the binding and activation of RNase L by the oligonucleotide ligand, we have synthesized analogues in which the 3'-hydroxyl group of one or more specific nucleotide residues of 2-5A trimer have been replaced by fluorine. The rationale for replacing the OH and H groups of biologically significant molecules by fluorine have been extensively reviewed[10]. Fluorine closely mimics hydrogen with respect to steric requirements (the van der Waals' radii of F: 1.35Å; of H: 1.1Å), but it strongly differs from hydrogen in electronegative character. Replacement by fluorine eliminates hydrogen bond donation as an interactive mechanism, but due to the great electronegativity of fluorine, fluorine still can function as a hydrogen bond receptor, albeit not as effectively as oxygen because of it's reduced tendency to share its non-bonded electrons [11]. Furthermore, the conformation of a fluorine compound often resembles that of the parent hydroxyl derivative rather than that of the hydrogen analogue, as was shown by NMR studies [12-14].

Herein we report on the synthesis of 2-5A analogues containing fluorinated ribose by a solid phase phosphite triester (phosphoramidite) methodology as modified for the preparation of oligoribonucleotides with 2',5'-phosphodiester bonds[15]. The biological activity of the fluorinated 2-5A analogues has been described elsewhere[16].

EXPERIMENTAL

Ultraviolet spectra were recorded on a Varian DMS 200 UV-Visible spectrophotometer. The ^{1}H NMR spectra were determined with a Varian VXR 300 MHz spectrophotometer with TMS as internal standard; for ^{31}P NMR spectra 5% H_3PO_4 was used as external standard. Precoated Uniplate™ silica gel GHLF plates (Analtech, Inc.) were used for TLC. Column chromatography was performed on silicagel 60 (Fluka Chemika). Reverse phase high performance liquid chromatography on a Beckman Ultraspere ODS column (4.6mm x 25cm, flow rate: 1mL/min.) was executed with a Beckman System Gold HPLC with two model 112 pumps and a Knauer variable wavelength monitor operating at 260 nm. Ion-exchange column chromatography on DEAE-Sephadex A-25 (Pharmacia LKB) was carried out at 4°C using various concentrations of triethylammonium bicarbonate (TEAB), pH 7.5, as an elution buffer. A stock solution of TEAB (1M) was prepared by introducing carbon dioxide in a 1 M solution of triethylamine in water at 4°C. Buffer was removed by repeated coevaporation with water and finally with anhydrous ethanol. Long chain alkylamine controlled pore glass (LCAA-CPG, pore size 500Å) support was obtained from Pierce Chemical Company (Rockland, IL). Adenosine, anhydrous solvents (Sure/Seal™) and reagents used in the solid phase synthesis were purchased from Aldrich Chemical Company. The syntheses were performed manually in commercially available microcolums (Applied Biosystems, Inc.) as described by Uznanski *et al.* [17]. Snake venom phosphodiesterase was a product of Cooper Biomedical, alkaline phosphatase was obtained from Sigma (St. Louis, MO).

Preparation of Fully Protected Monomers

5'-O-Dimethoxytrityl-N^6-benzoyladenosine was prepared by 5'-tritylation and transient protection of OH groups by trimethylsilyl chloride for N-benzoylation[18]. Sugar hydroxyl groups of adenosine were protected with the *tert* - butyldimethylsilyl protecting group according to the method of Ogilvie *et al.* [19]. The 2'-silyl isomer was utilized for derivatization of the solid support

via a succinate linkage, while the 3'-silyl isomer was converted into the 2'-O-(ß-cyanoethyl)-N,N-diisopropyl phosphoramidite as described by Usman *et al.* [20]. Derivatization of 3'-fluoro-3'-deoxyadenosine for solid phase oligoribonucleotide synthesis has not been previously reported, so the preparation of the fully protected monomers **4,5** is detailed here.

N^6-Benzoyl-3'-fluoro-3'-deoxyadenosine (2)

A portion of 538.5 mg (2 mmol) of 3'-fluoro-3'-deoxyadenosine[21, 22] (dried by coevaporation with anhydrous pyridine) in 20 mL of anhydrous pyridine was cooled to 0°C in an ice-bath and 1.25 mL (20 mmol) of trimethylchlorosilane (Fluka) was added. After stirring at 0° for 30 min, benzoyl chloride (Fluka, 0.58 mL, 5 mmol) was added. The colored reaction mixture was stirred at room temperature for 2 h. After cooling (ice-bath) 4 mL of cold water was added with stirring at 0° for 15 min, then 4 mL of concentrated ammonia was added with additional stirring at 0° for 30 min. After evaporation the mixture was purified on silica gel using ethyl acetate first, then ethyl acetate-methanol (95:5) as an eluent. Crystallization from MeOH afforded 531 mg (1.42 mmol, 71%) of the title compound as a colorless amorphous powder. TLC in ethyl acetate-methanol (95:5): R_f = 0.5. CI MS (NH_3) 374 $(M+H)^+$. ^1H NMR ($CDCl_3$ + DMSO-d_6 (20% v/v)) δ: 3.72 (m, H-5', H-5"); 4.35 (broadened d, $J_{H-4',F}$ = 27.8 Hz); 4.90 (m, H-2'); 5.03 (dd, J = 4.2 Hz, $J_{3',F}$ = 54.6 Hz, H-3'); 5.72 (m, 2'-OH, 5'-OH); 5.99 (d, J = 7.9 Hz, H-1'); 7.45 (m, 3H, Ar-H); 7.98 (d, J = 7.4Hz, 2H, Ar-H); 8.28 (s, H-2); 8.61 (s, H-8); 10.41 (s, 6-NH).

5'-(4,4'-O-Dimethoxytrityl)-N^6-benzoyl-3'-fluoro-3'-deoxyadenosine (3)

A mixture of 500 mg (1.34 mmol) of N^6-benzoyl-3'-fluoro-3'-deoxyadenosine (dried by coevaporation with anhydrous pyridine) and 550 mg (1.62 mmol) of 4,4'-dimethoxytrityl chloride (Aldrich) in 10 mL of anhydrous pyridine was stirred at room temperature until TLC-analysis showed the disappearance of the starting material (2 h). Methanol (0.5 mL) was added. After evaporation, the residue was taken up in 10 mL of water and extracted twice with 10 mL of ether. The organic layer was dried over Na_2SO_4, solvent was evaporated and the residue purified by column chromatography in the presence of 0.2% of pyridine using first dichloromethane, then CH_2Cl_2-MeOH (98:2) to yield 616 mg (0.91 mmol, 68%) of the title compound as a pale yellow foam. TLC in chloroform-methanol (95:5): R_f = 0.66. ^1H NMR [$CDCl_3$ + pyridine-d_5 (1% v/v)] δ: 3.22 and 3.40 (2 x dd, J = 3.2 Hz, J = 10.7 Hz, H-5', H-5"); 3.65 (s, 6H, CH_3O-); 4.45 (broadened d, $J_{4',F}$ = 26.9 Hz, H-4'); 5.06 (m, H-2'); 5.06 (dd, J = 4.4 Hz, $J_{3',F}$ = 55.1 Hz, H-3'); 6.02 (d, J = 7.2 Hz, H-1'); 6.64-7.21 (m, 13H, dimethoxytrityl); 7.44 (m, 3H, Ar-H); 7.70 (broadened d, J = 7.1 Hz, 2'-OH); 7.91 (d, J = 7.2, 2H, Ar-H); 8.12 (s, H-2); 8.61 (s, H-8); 9.02 (bs, 6-NH).

5'-(4,4'-O-Dimethoxytrityl)-N^6-benzoyl-3'-fluoro-3'-deoxyadenosine-2'-O-(ß-cyanoethyl)-N,N-diisopropyl phosphoramidite (4)

To a stirred solution of 405 mg (0.6 mmol) of 5'-(4,4'-O-dimethoxytrityl)-N^6-benzoyl-3'-fluoro-3'-deoxyadenosine and 42 mg (0.6 mmol) of tetrazole (Aldrich) in 10 mL of dry dichloromethane (distilled from CaH_2), 180.8 mg (0.6 mmol) of ß-cyanoethyl)-N,N,N',N'-tetraisopropyl phosphorodiamidite (Aldrich) was added with a syringe. After stirring for 1 h at room temperature under a nitrogen atmosphere, the reaction mixture was evaporated and purified by flash

column chromatography using benzene-hexane-triethylamine (7:2:1) to yield 352 mg (0.4 mmol, 67%) of the title compound as a colorless foam. TLC in benzene-hexane-TEA (7:2:1): R_f = 0.46 and 0.34 for the diastereomers, respectively. ^{31}P NMR ($CDCl_3$ + 1% pyridine-d_5) δ: 152.2 ppm (1P) and 151.4, 151.3 ppm (1P) J_{P-F}: 9.1 Hz.

5'-(4,4'-O-Dimethoxytrityl)-N^6-benzoyl-3'-fluoro-3'-deoxyadenosine-2'-O-succinate (5)

To a solution of 270.3 mg (0.4 mmol) of 5'-(4,4'-O-dimethoxytrityl)-N^6-benzoyl-3'-fluoro-3'-deoxyadenosine (dried by coevaporation with anhydrous pyridine) in 2.4 mL of anhydrous pyridine containing 4-dimethylaminopyridine (24.4 mg, 0.2 mmol), succinic anhydride (120.8 mg, 1.2 mmol, Fluka) was added. The yellow solution was stirred for 36 h at room temperature and monitored by TLC using dichloromethane-methanol (95:5). No starting material was left. After evaporation the residual pyridine was removed by coevaporation with dry toluene (3 x 5 mL). The residue was dissolved in 15 mL of chloroform and washed with saturated NaCl solution (3 x 15 mL) and then with water (1x15 mL). The organic layer was dried over $CaCl_2$, evaporated and purified by column chromatography in the presence of 0.2% of pyridine using a stepwise gradient of dichloromethane-methanol from 95:5 to 9:1 yielding 240 mg (0.31 mmol, 77%) of the title compound as a pale yellow amorphous powder. TLC in ethyl acetate-acetone-water (5:10:1) containing 0.5% pyridine R_f = 0.61. ^1H NMR ($CDCl_3$ + 1% pyridine-d_5) δ: 2.46 (broad s, CH_2CH_2); 3.19 and 3.63 (2 x m, H-5', H-5''); 3.63 (s, 6H, CH_3O-); 4.38 (broadened d, $J_{4',F}$ = 26.9 Hz, H-4'); 5.29 (broad d, $J_{3',F}$ = 57.9 Hz, H-3'); 5.98 (m, H-2'); 6.23 (broad s, H-1'); 6.64-7.25 (m, 13H, dimethoxytrityl); 7.30 (m, 3H, Ar-H); 7.84 (m, 2H, Ar-H); 8.10 (s, H-2); 8.47 (s, H-8); 9.20 (bs, 6-NH).

Derivatization of the solid support

A mixture of 100 μmol of fully protected 3'-fluoro-3'-deoxyadenosine 2'-succinate (5) (for adenosine the 3'-succinate was used), 500 mg of pre-activated LCAA-CPG[23], 6 mg (50 μmol) of DMAP, 40 μL of TEA and 191 mg (1 μmol) of N-(dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (Fluka) in 5 mL of anhydrous pyridine was first sonicated for 2 min, and then shaken at room temperature for 2 d. After shaking, the solid support was filtered off and washed successively with pyridine, methanol and chloroform and then dried under vacuum over P_2O_5. The unreacted sites were then capped using 100 μl of acetic anhydride in 1.5 ml of anhydrous pyridine. After shaking for 30 min, the CPG was filtered off and washed successively with pyridine, methanol and ether and then dried under vacuum over P_2O_5. Colorimetric dimethoxytrityl assay[24] indicated a loading of 35 μmol/g for 3'-fluoro-3'-deoxyadenosine and 59 μmol/g for adenosine, respectively.

Solid phase synthesis of oligonucleotides

Oligonucleotides were synthesized by the phosphoramidite method[25] using ß-cyanoethyl as the phosphate protecting group[26]. The syntheses were performed manually in commercially available microcolumns using adaptors and gas-tight syringes[17] on a 1-2 μmol scale depending on the solid support loading. The chain elongation was carried out in the 2' to 5' direction. The synthetic cycle used was as follows: detritylation, 2% TCA in dichloromethane; washing, 2%

pyridine in CH_3CN, followed by CH_3CN, then dry N_2; coupling, 0.5 M tetrazole & 0.2 M phosphoramidite in CH_3CN; washing with CH_3CN, then dry N_2; oxidation with 0.1 M I_2 in THF-lutidine-H_2O (7:2:1); washing with CH_3CN, then dry N_2; capping with 6.5% DMAP in THF (w/v) and Ac_2O-2,6-lutidine (2:3); then washing with CH_3CN followed by dry N_2. Syntheses were monitored by colorimetric dimethoxytrityl assay[24]. After the desired sequence had been obtained, the 5'-terminal nucleoside was converted to the 5'-phosphate by reaction with bis-(ß-cyanoethyl)-N,N-diisopropylphosphoramidite using tetrazole as catalyst and subsequent oxidation with iodine/water[27,28].

Deprotection and purification

The oligonucleotides synthesized were cleaved from the solid support by treatment with a mixture of concentrated ammonia and ethanol (3:1, v/v) at room temperature for 2 h. The resulting solutions were further incubated for 6 h at 55°C to remove the N^6-benzoyl protecting groups as well as the ß-cyanoethyl phosphate protecting groups. Finally, the 3'-O-(*tert*-butyldimethylsilyl) protection of the adenosine moieties was removed by treating the oligomers with 1M tetrabutylammonium fluoride in THF at room temperature for 4-6 h[20, 29]. The synthesized oligonucleotides were purified by ion-exchange chromatography using DEAE-Sephadex column eluted with a linear gradient of 0.0-0.5M TEAB buffer (pH 7.5). Final desalting was achieved by reverse phase C18 SEP-PAK cartridges (Waters Millipore) using methanol/water = 1:1 (v/v) as eluent.

Determination of hypochromicities of oligonucleotides

The appropriate oligonucleotide (0.5 A_{260} unit) in 200 mM Tris-HCl, pH 8.5 containing 50 mM $MgCl_2$ was digested by adding snake venom phosphodiesterase (0.5 U, 0.5 µL) until a constant A_{260} value was obtained. The total volume of the reaction mixture was 1 mL. The hypochromicity values were determined by the ratio $A_{initial}/A_{final}$ and the oligonucleotide extinction coefficients calculated by multiplying the respective hypochromicity values by the sum of the constituent nucleoside micromolar extinction coefficients at 260 nm.

Characterization of oligonucleotides

The appropriate oligonucleotide (1 A_{260} unit) in 10 mM Tris-HCl, pH 8.5 containing 10 mM $Mg(OAc)_2$ was digested by adding snake venom phosphodiesterase (0.3 U, 0.3 µL) for 2 h at 37°C. The total volume of the reaction mixture was 100 µL. Alkaline phosphatase (0.5 U, 1.7 µL) then was added. After a further 2 h incubation, the sample was analyzed by reverse phase HPLC using an elution program of 0-50% buffer B into buffer A over 30 min followed by a linear gradient of 50-100% buffer B over 5 min followed by100% buffer B for 10 min. Buffer A was 20 mM ammonium phosphate pH 7.0 (Condition I) or 60 mM potassium dihydrogen phosphate + 5 mM PIC A reagent (Waters), final pH 5.0 (Condition II). For both conditions, buffer B was 50% methanol-water (v/v). The nucleoside compositions were calculated from the peak area integrals by comparison with that of a standard nucleoside mixture.

RESULTS AND DISCUSSION

The structures of fluorinated 2',5'-oligoadenylates prepared for this study are presented in Figure 1. Oligonucleotides were synthesized by the phosphoramidite method[25] as modified

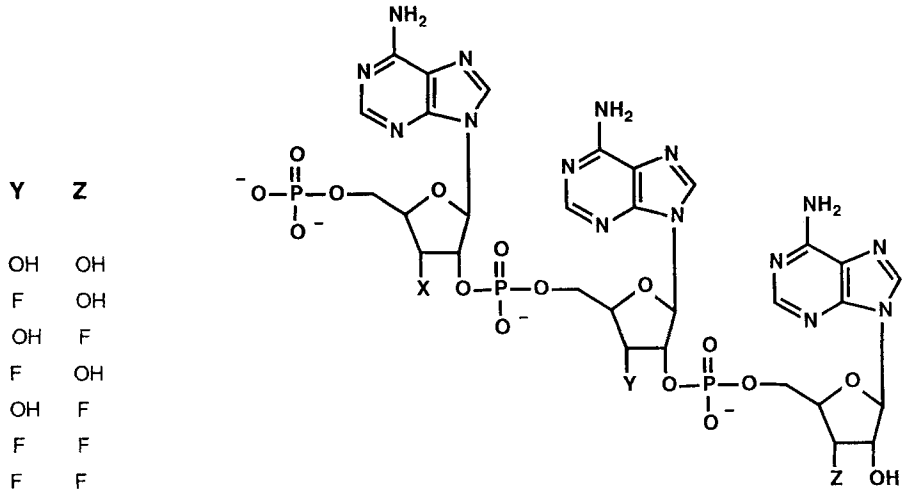

Figure 1. Structure of the 2',5'-linked 3'-fluoro-oligoadenylates.

for the preparation of oligoribonucleotides with 2',5'-phosphodiester bonds[15]. We have used benzoyl and ß-cyanoethyl[26] as protection for the N^6-amino and phosphate groups, respectively. For protection of the sugar hydroxyl groups of adenosine, we have utilized the *tert*-butyldimethylsilyl protecting group[29-31]. The reaction sequence for the 3'-fluoro-3'-deoxyadenosine building blocks, 5'-(4,4'-O-dimethoxytrityl)-N^6-benzoyl-3'-fluoro-3'-deoxyadenosine-2'-O-(ß-cyanoethyl)-N,N-diisopropyl phosphoramidite (**4**) and 5'-(4,4'-O-dimethoxytrityl)-N^6-benzoyl-3'-fluoro-3'-deoxyadenosine-2'-O-succinate (**5**),was straightforward and can be described as follows. Using transient protection compound **1**, 3'-fluoro-3'-deoxyadenosine, was first N^6-benzoylated giving N^6-benzoyl-3'-fluoro-3'-deoxyadenosine (**2**) in 71% yield. Subsequent reaction of the primary hydroxyl group with 4,4'-dimethoxytrityl chloride afforded **3** , 5'-(4,4'-O-dimethoxytrityl)-N^6-benzoyl-3'-fluoro-3'-deoxyadenosine (**3**) in 68% yield. Compound **3** having the necessary 2'-OH free was either phosphitylated yielding **4**, 5'-(4,4'-O-dimethoxytrityl)-N^6-benzoyl-3'-fluoro-3'-deoxyadenosine-2'-O-(ß-cyanoethyl)-N,N-diisopropyl phosphoramidite in 67% yield as the fully protected monomer for chain elongation or **3** was converted into compound **5**, 5'-(4,4'-O-dimethoxytrityl)-N^6-benzoyl-3'-fluoro-3'-deoxyadenosine-2'-O-succinate (**5**), in a good yield (77%). The building block **5** was then utilized for the derivatization of the solid support *via* succinate linkage with a loading of 35 µmol/g for 3'-fluoro-3'-deoxyadenosine.

The synthetic cycle used for the solid phase oligonucleotides synthesis is described in the experimental section. With an extended coupling time (up to 8 min) average coupling efficiencies for 3'-fluoro-3'-deoxyadenosine were 95-98 % and for adenosine 93-95 % as determined by colorimetric quantitation of the dimethoxytrityl fractions [24].

These stepwise yields were somewhat lower than those of generally accepted in automatic DNA syntheses. One explanation is the steric hindrance generated by the bulky 3'-O-*tert* -

Table 1.
HPLC Behavior and Molar Extinction Coefficients of 2',5'-Linked Oligonucleotides[a].

Oligomer	Retention time (min)	Condition	e (M^{-1} cm^{-1}x10^3)
pdAflpApA	15.88	I	37.4
pApdAflpA	15.96	I	34.8
pApApdAfl	31.14	II	36.4
pdAflpdAflpA	20.49	I	36.2
pdAflpApdAfl	33.95	II	36.1
pdAflpdAflpdAfl	33.47	II	38.2

Abbreviation: dAfl, 3'-fluoro-3'-deoxyadenosine.
a For Conditions (I or II) see Experimental.

butyldimethylsilyl group of the adenosine residues on the coupling rates of the corresponding phosphoramidite monomers during chain elongation. Using competitive phosphitylation experiments Kierzek *et al.* have studied the steric aspects of the 2'-protective groups on the synthesis of oligoribonucleotides by the phosphoramidite approach and have shown a strong dependence on the steric hindrance of the 2'-substituents both for the 3'-phosphitylation and condensation steps[32]. On the other hand, in the case of 3'-fluoro-3'-deoxyadenosine moieties, direct steric influence seems unlikely based on the similarity of the van der Waals radii of hydrogen (1.1Å) and fluorine (1.35Å). A more plausible explanation is the decreased reactivity of the modified 2'-phosphoramidites by the electron-withdrawing inductive effect of fluorine[33]. A similar observation was made during the solid phase synthesis of oligodeoxyribonucleotides containing the anti-cancer drug 2',2'-difluoro-2'-deoxycytidine[34].

In order to study the binding of the fluorinated 2'5'-oligoadenylates to RNase L the 5'-terminal monophosphate functions were required[35]. The 5'-end phosphorylation could be easily achieved by the reaction with bis-(ß-cyanoethyl)-N,N-diisopropylphosphoramidite in the coupling step of the synthetic cycle with a shorter reaction time (4 min) followed by oxidation with iodine/water [27,28]. The oligomer 5'-phosphates were then removed from the solid support and were deprotected as described in the Experimental part. Final yields, after purification and desalting, for the fluorinated 2',5'-oligoadenylates were between 65-81%. All oligonucleotides were pure, as judged by analytical HPLC.

The structure of each modified oligoadenylate was confirmed by NMR spectra of the intermediates, HPLC behavior, and analysis of enzymatic hydrolysis products. For the latter, the oligomers were subjected to complete digestion with snake venom phosphodiesterase, followed by alkaline phosphatase digestion. The products obtained were analyzed by HPLC, and the determined 3'-fluoro-3'-deoxyadenosine/ adenosine ratios were found to be consistent with the proposed sequences. The molar extinction coefficients were calculated for all oligonucleotides from the increase in UV absorbance obtained after the enzymatic digestion to the constituent nucleosides (Table 1).

To summarize, solid-phase synthesis using phophoramidite chemistry provides a good yield of 3'-fluoro-3'-deoxyadenosine 2',5'-oligonucleotides as homo-oligonucleotides or as mixed

sequence-specific oligonucleotides with adenosine itself. The oligonucleotides containing 3'-fluoro-3'-deoxyadenosine residues presented here have been used successfully as probes of hydrogen bonding in enzymes of the 2-5A system[16]. The facile preparation of 2',5'-oligoadenylates and their analogues is of particular interest in light of the recent description of 2-5A-antisense composite oligonucleotides which provide a technology for the targeted destruction of RNA and a new potential for oligonucleotide therapeutics[36-39].

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