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A High-Yield Synthesis of Deoxy-2-Fluoroinosine and its Incorporation into Oligonucleotides.

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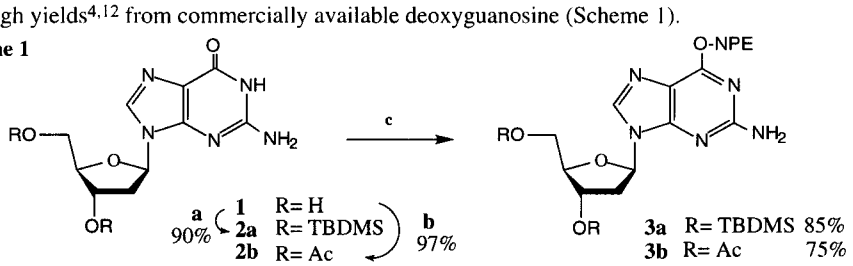
Abstract: An improved synthesis of the deoxy-2-fluoroinosine nucleoside is described, that makes use of mild fluorination (polyvinylpyridinium polyhydrogenfluoride) and O-silyl deprotection (triethylamine trihydrofluoride) reactions. The derived 5'-dimethoxytrityl-2-fluoroinosine-3'-phosphoramidite was incorporated into 10-, 15- and 20- mer oligonucleotides containing up to 7 non-natural bases.

Covalent modification of nucleic bases has been widely used as a way to precisely position a residue in a groove of DNA. Reaction of a primary amine with the appropriate activated nucleic base can lead to substitutions at N^4 of cytidine[1] and N^6 of adenine[2,3] which protude in the major groove, and to substitution at N^2 of guanine in the minor groove. The latter type of compounds has attracted much interest to medicinal chemists since they are thought to be the covalent adducts formed after binding of polycyclic aromatic carcinogens[3] and of antitumor drugs[4] to DNA. Recently deoxyguanosine (dG) has been N^2-substituted also with minor groove-binding cations[5,6,7]. Most of these derivatives were obtained from a common intermediate, namely 2-fluoroinosine, which was synthesized from dG via diazotation in aqueous[8,9] or anhydrous conditions[3,4,9] in rather low yields. Oligonucleotides containing one or two such modifications have been synthesized with 2-fluoroinosine *in lieu* of N^2-substituted dG, the desired substitution being introduced post-oligomerization by heating the precursor resin-bound oligonucleotide with an excess of primary amine[7,9,10]. Here we attempted to extend this strategy to a larger number of substitutions, which required a fair amount of 2-fluoroinosine.

As guidelines to improving the synthesis of 2-fluoroinosine, milder fluorination conditions and less polar intermediate compounds were considered to be important. Moreover, prior to fluorination, O^6 derivatization in Mitsunobu conditions[11] required protection of the hydroxyl groups, but not necessarily that of the amine function. Both silylation and careful acetylation reactions fulfilled these conditions and provided the precursors **3a** and **3b** in high yields[4,12] from commercially available deoxyguanosine (Scheme 1).

Scheme 1



(a) TBDMS-Cl / Imidazole / DMF, 16h - RT (b) Ac_2O / DMAP (cat) / CH_3CN / Et_3N, 2h - RT (c) NPE / P(Ph)3 / DEAD / Dioxane, 12h - RT

At the time, the most satisfactory way to introduce fluorine at the C^2 position seemed to be the method of Robins *et al.*[13]. Accordingly, we looked for similar anhydrous diazotation conditions with milder fluorination reagents. The diazonium salt was generated *in situ* with t-butyl nitrite[14], and SbF_3 or polyvinylpyridinium polyhydrogenfluoride (PVPHF) were tested as sources of fluorine.

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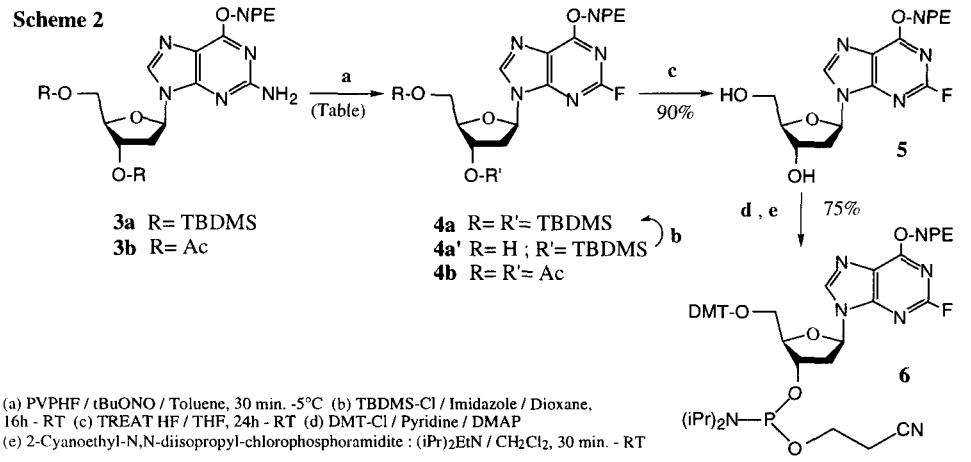
Scheme 2



3a R= TBDMS
3b R= Ac

4a R= R'= TBDMS
4a' R= H ; R'= TBDMS
4b R= R'= Ac

(a) PVPHF / tBuONO / Toluene, 30 min. -5°C (b) TBDMS-Cl / Imidazole / Dioxane, 16h - RT (c) TREAT HF / THF, 24h - RT (d) DMT-Cl / Pyridine / DMAP
(e) 2-Cyanoethyl-N,N-diisopropyl-chlorophosphoramidite : $(iPr)_2EtN$ / CH_2Cl_2, 30 min. - RT

Fluorination with SbF_3 was an extension of halogenation using $SbCl_3$ or $SbBr_3$[13]; it gave slightly better results than HF/pyridine (Table). The mild PVPHF reagent developped by Olah *et al.*[15] appeared to be a good candidate too, being a safe and an easily removable polymer. Reaction proceeded smoothly in several solvents with reasonable yields. Methylene chloride gave an interesting 70% yield, however it appeared to be a 6:4 mixture of 2-fluoro- and 2-chloro-inosine[16]. Chlorination was presumably a consequence of radical abstraction from the halogenated solvent as previously observed in similar conditions[17]. The best results were obtained in toluene. Surprisingly, the fluoride- and acid-sensitive TBDMS protecting group was essentially stable (scheme 2). The reaction workup was straigthforward[18] thanks to the lipophily of compound **4a**. Some 5'-desilylation (< 20%) nevertheless occured, yet **4a'** could either be quantitatively protected back to **4a** or further deprotected to **5**. The combined diazotation, fluorination and reprotection steps led to an overall yield of 75%.

Table

O-Protection	O^6-Protection	Fluorination agent	Solvent	Yield
none	benzyl	HBF_4	water	24%[8]
none	NPE	HBF_4	water	35%[9]
none	NPE	HF-pyridine	pyridine	20%[9]
TBDMS	NPE	HF-pyridine	pyridine	32%[4]
TBDMS	NPE	SbF_3	toluene	49%
acetyl	NPE	SbF_3	toluene	44%
acetyl	NPE	PVPHF	toluene	71%
TBDMS	NPE	PVPHF	CH_2Cl_2	70% (60:40 F/Cl)
TBDMS	NPE	PVPHF	toluene	75%

Removal of TBDMS with $Bu_4N.HF$ was shown to cleave the O-NPE moiety[19]. On the contrary, triethylamine trihydrofluoride (TREAT HF) was described[20] as a very mild source of solvated fluoride devoid of basicity. This reagent was used here to remove the 5'- and 3'-O-silyl protections in high yield (90%), i.e. without base-promoted β-elimination. The 6-O-p-nitrophenylethyl-2-fluoroinosine **5** was then tritylated (87%) and converted to the phosphoramidite derivative **6** (86%) using standard methods (scheme 2)[21].

The activated nucleoside synthon was incorporated into oligonucleotides[22]: a 10-mer of sequence 5'-$CI_FAACI_FTI_FTA$-3' (**7**), a 15-mer $TI_FTI_FACI_FAACI_FTI_FTA$ (**8**) and a 20-mer $TI_FAI_FATI_FTI_FACI_FAACI_FTI_FTA$ (**9**) encompassing the Afl III restriction site in the luciferase gene of the pGL2-control plasmid (Promega). Standard stepwise nucleotide coupling efficiencies (97%), as measured online by the amount of released trityl cation, decreased to 70-80% for **6**. Resin-bound **7** was released trityl-on and deprotected (30%NH_3, 25°C, 48h). Reverse phase HPLC led to a pure DMT-oligonucleotide (figure 3) which was subsequently detritylated (80%AcOH, 30 min). HPLC of the mixture of nucleosides obtained following snake venom phosphodiesterase and alkaline phosphatase digestions[23] was in agreement with the ratio of nucleosides in the sequence of **7** (dC/dT/dA/NPE-dG = 2/2/3/3, figure 3). As a model reaction for C^2(dG) modification, resin-bound **8** was reacted with a primary amine (1.1 M diaminobutane in methanol, 60°C, 24 h) and purified as above. Enzymatic digestion led to the expected ratio of dC, dT, dA and (NPE) dG-nucleosides, N^2-aminobutyl-(NPE)deoxyguanosine being more polar than NPE-dG (retention time = 13 min, conditions as in figure 3). ES-MS confirmed that C and A deprotection, as well as fluorine substitution had occured since three major peaks corresponding to the fully C^2-substituted N^2-aminobutyl-dG oligonucleotide **8** with up to two NPE groups were detected. However HPLC profiles of the crude oligonucleotides and of their enzymatic digestion products, as well as ES/MS spectra clearly showed that neither modified nucleoside coupling nor post-oligomerization substitution were clean reactions. Yields decreased exponentially as the number of substitutions increased, and amine-substituted **9** could not be isolated.

In summary, besides improving the synthesis of 2-fluoroinosine, we have extended stepwise the number of N^2-substituted dG residues contained in oligonucleotides from one[3,10] or two[6b,7] to five. Obviously, fair yields or larger numbers of substitutions will require a different strategy, such as the substitution of fluoroinosine prior to the oligomerization step[24].

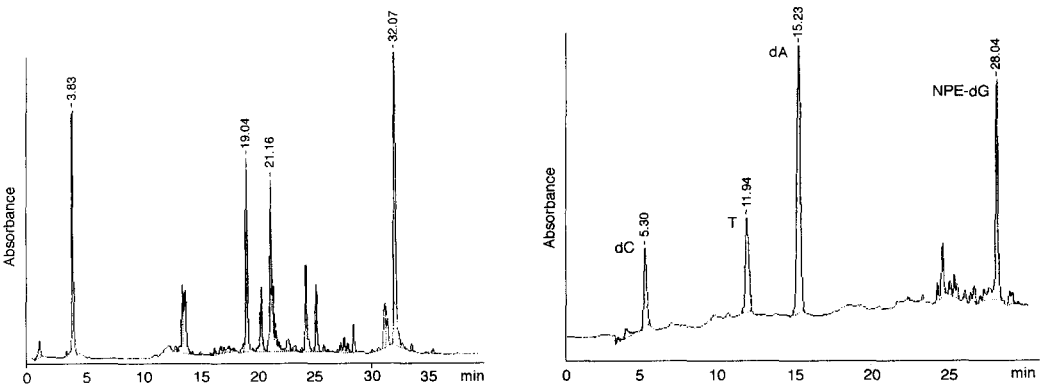


Figure 3. C_{18} reverse-phase HPLC chromatograms of (left) the crude reaction mixture of oligonucleotide DMT-**7** (32.07 min; 0.1 M AcOTEA pH 7.5, 4 - 60% acetonitrile); right: enzymatic digestion mixture of nucleosides from **7** (1 - 5% acetonitrile within 10 min and 5 - 50% within 20 min, 0.1 M Tris-acetate pH 7.5).

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16. The mixture of chloro and fluoroinosine was analysed by FAB mass spectrometry m/e: 648 for fluoroinosine and 664 for chloroinosine.
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18. To a polyethylene bottle containing 0,7 g of 3',5'O-Bis(tert-butyldimethylsilyl)-O^6(p-nitrophenylethyl)-2'-deoxyguanosine (1,1 mmoles) in 20 ml of toluene was added 2g of PVPHF (59% HF) and 0,47 ml of tBuONO (2,2 mmoles) at -10°C. The reaction was stirred for 30 min at -5°C - 0°C and poured into sodium bicarbonate (0,1 M). Extraction with AcOEt afforded an oil. Chromatography using silicagel and 2% MeOH in CH_2Cl_2 as eluent yielded 420 mg of 3',5'O-Bi(ter-butyldimethylsilyl)-O^6(p-nitrophenylethyl)-2'-fluoroinosine (R_f= 0,9) and 100 mg of 3'-O-mono(ter-butyldimethylsilyl)-O^6(p-nitrophenylethyl) fluoroinosine that was resilyled (TBDMSCl/Imidazole). The total yield was 75%. ^{1}H NMR (200 MHz, $CDCl_3$): 0,05 (6H, s, Si-CH_3); 0,07 (6H, s, Si-CH_3); 0,88 (9H, s, Si-C-CH_3), 0,92 (9H, s, Si-C-CH_3); 2,4 (1H, m, $H^{2'}$); 2,6 (1H, m, $H^{2'}$); 3,3 (2H, t, $OCH_2\underline{CH_2}$); 3,8 (2H, t, $H^{5'}$); 4,0 (1H, m, $H^{4'}$); 4,6 (1H, m, $H^{3'}$); 4,85 (2H, t, $O\underline{CH_2}CH_2$); 6,4 (1H, t, $H^{1'}$); 7,5 (2H, d, ArH); 8,15 (2H, d, ArH); 8,25 (1H, s, H^8); ^{19}F NMR (288,2 MHz, $CDCl_3$): -50,0 (s); FAB m/e: 648 (M^+ +1).
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22. Oligonucleotide synthesis was carried out on a Biosearch 8600 DNA Synthesizer on a 1 µmol scale. The standard protocol was modified by increasing the coupling time to 45 sec.
23. 3 nmoles of modified oligonucleotide were digested at 37 °C for 10 h with 8 µg of snake venom phosphodiesterase (Boehringer-Mannheim) and 8 U calf intestine alkaline phosphatase (Sigma) in 0.05 M Tris-HCl, 0.1 M $MgCl_2$ (50 µL), pH 8.1.
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