Bioorganic & Medicinal Chemistry Letters, Vol.2, No.2, pp. 145-148, 1992
Printed in Great Britain

0960-894X/92 $3.00 + .00

SYNTHESIS OF ACID STABLE 5'-O-FLUOROMETHYL PHOSPHONATES OF NUCLEOSIDES. EVALUATION AS INHIBITORS OF REVERSE TRANSCRIPTASE.

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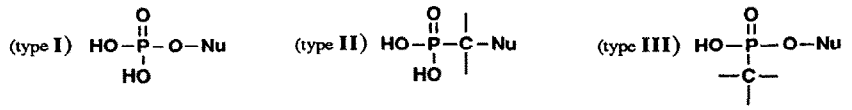
(*Received* 15 *November* 1991)

Key words : nucleoside 5'-fluoromethylphosphonate, acid stable, reverse transcriptase inhibition

Abstract:
The synthesis and the reverse transcriptase inhibitory activity of new 5'-O-mono-,di- and trifluoromethylphosphonate derivatives of nucleosides and 2'-deoxynucleosides are described.

In the biosynthetic pathway leading to DNA, several enzymes using mono-, di-, and trinucleotides as substrates play a critical role. Most of the known inhibitors of these enzymatic reactions are nucleotides. However, these nucleotides are labile in vivo and are not therapeutically useful.

Phosphonate esters have been used extensively to replace naturally occuring phosphates (type I)[2], either the bridging oxygen (type II) or one of the phosphate hydroxyl functions (type III) being replaced by a carbon atom. The resulting analogs were presumed to be less susceptible to either acidic or enzymatic cleavage.[3]



The 5'-methylene[4,5] and 4'- [6,7,8] or 5'-methylenoxy[9] (type II), 5'-methyl-,[10,11,12] 5'-hydroxymethylphosphonate[13] (type III), of nucleosides have been described to mimic mononucleotides, but no significant biological activity of such derivatives has been reported so far.

The potential of the fluorine atom in the synthesis of nucleotide analogs has been illustrated by Blackburn *et al* [14] and very recently by Hebel *et al* [15] in their work on di- and triphosphates of nucleoside analogs. In both cases a fluoromethylene moiety replaces the β,γ bridging oxygen in the terminal pyrophosphate function in order to obtain an isosteric and isopolar pyrophosphate function.

Fluorine atom incorporation into organic molecules has often been associated with profound changes in their biological profile compared to their non fluorinated counterparts. Such differences are the consequences of the extreme electronegativity of the fluorine atom and its versatility in replacing either an hydrogen atom without notable steric consequences, or a hydroxyl group, the fluorine atom being able to form hydrogen bonds.[16,17]

In order to combine these isosteric and isopolar effects of the fluorine atom, we propose mono-, di -, and trifluoromethylphosphonate functions of type III as "bioisomers" of the phosphate group in mononucleotides. In this manner, the fluorinated moiety could mimic the missing hydroxyl function.

Recently, an oligonucleotide containing a difluoromethylenephosphonate diester moiety was synthesized for ^{19}F NMR studies, but no biological data has been reported yet.[18]

We describe here a general and straightforward route to this class of compounds, starting from the appropriately protected nucleosides or 2'-deoxynucleosides and from mono-,[19] di-,[20] or trifluoromethylphosphonic acids[21] (scheme 1, table 1 or scheme 2, table 2, respectively).

Scheme 1: Synthesis of 5'-mono-, di-, and trifluoromethyl phosphonates of nucleosides :

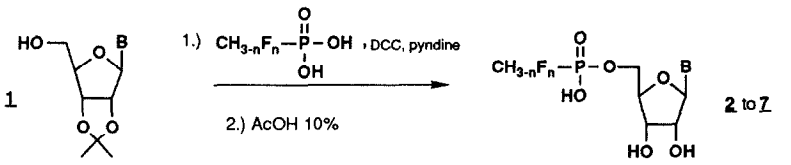


Table 1 :

Product	Starting material	n	Nucleic base B	yield	$CH_{3-n}F_n$-P- Nu NMR (δ ppm) ^1H(360 MHz)	^{19}F(338.8 MHz)	J_{HF}	J_{HP}	J_{FP} (Hz)
2	1a	1	Guanine	51%	4.55 (dd)	-170.1 (dt)	46.5	4.4	59.3
3	1a	2	Guanine	38%	6.0 (dt)	-58.9 (dd)	49	24.5	81
4	1a	3	Guanine	21%	-	2.6 (d)	-	-	109
5	1b	2	Adenine	27%	5.87 (dd)	-59.93 (dd)	49	4.5	81
6	1c	2	Cytosine	64%	6.05 (dt)	-59.9 (dd)	49	25	81
7	1d	2	Uracil	88%	5.82 (dt)	30.4 (dd)	49.7	20.8	71

The **general procedure** for their synthesis is based on that described by Myers *et al.* :[10]

The protected nucleoside 1[22] or 8[23] (1mmol) and the fluoromethylphosphonic acid (1.2 mmol) are dissolved in 2 ml anhydrous pyridine and the solvent is removed at 35°C *in vacuo* (20 mm Hg). This procedure is repeated three times to provide an anhydrous reaction mixture. The residue is dissolved in anhydrous pyridine (40 ml) and dicyclohexylcarbodiimide (0.495 g, 2.4 mmol) is added in one batch. The reaction mixture is stirred at 35-40°C for 40 h, then hydrolysed with water (10 ml) for 0.5 h at room temperature. After usual work-up, the dicyclohexylurea is filtered off, and the protecting group is removed by acidic treatment for the 2',3'-isopropylidene, or by reaction with tetra-N-butylammoniumfluoride for the 3'-O-tertiobutyldiphenylsilyl protected 2'-deoxynucleoside. The residue obtained after either of these treatments is taken up in water, purified by ion exchange chromatography (Dowex AG1X4, formate form), eluted sequentially with 4N formic acid (for the removal of the excess fluorophosphonic acid), and 1N hydrochloric acid. The fluoromethylphosphonate of the nucleoside is obtained as a white hygroscopic solid after lyophilisation.[24]

For long term storage of such compounds it is advisable to transform the fluorophosphonic acid into its sodium salt (cation exchange column CHELEX 100 sodium form).

Scheme 2 : Synthesis of 5'-mono-, di-, and trifluoromethyl phosphonates of 2'-deoxynucleosides :

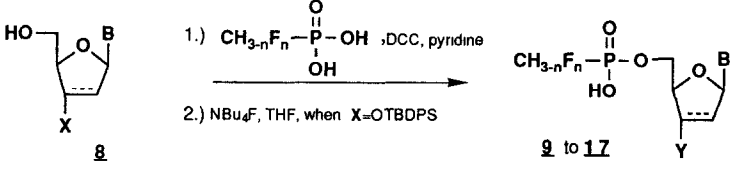


Table 2 :

product		starting material		nucleic	n	Yield	CH_3-xFx-P- Nu NMR (δ ppm)				
Nr	Y	Nr	X	Base			1H(360 MHz)	19F(338.8 MHz)	J_{HF}	J_{HP}	J_{FP} (Hz)
9	N_3	8a	N_3	Thymine	1	57%	4.75 (dd)	-169.8 (dt)	47	4	59
10	N_3	8a	N_3	Thymine	2	85%	6.0 (dt)	-58.72 (dt)	49	22.5	81
11	N_3	8a	N_3	Thymine	3	73%	-	2.77	-	-	109
12	OH	8b	OTBDPS	5-fluorouracil	1	61%	6.42 (m)	-170 (dt)	34	4	59
13	OH	8b	OTBDPS	5-fluorouracil	2	48%	6.0 (dt)	-58.95 (dd)	49	24.5	81
14	OH	8b	OTBDPS	5-fluorouracil	3	70%	-	2.68 (d)	-	-	102
15	H Δ2'-3'	8c	H Δ2'-3'	Thymine	1	58%	4.58 (ddd)	-169.97 (dt)	47	4	59
16	H Δ2'-3'	8c	H Δ2'-3'	Thymine	2	75%	5.91 (dt)	-58.7 (dd)	49	24	81
17	H Δ2'-3'	8c	H Δ2'-3'	Thymine	3	46%	-	2.78 (d)	-	-	103

The chemical stability of these pseudonucleotides has been evaluated in various mediums. For example, as shown in table 3, no significant hydrolysis was found for the 5'-fluoromethylphosphonate of AZT at pH=7.35 for 12 days at 50°C, and only a slight hydrolysis at pH=1.15 was observed during the same period .[25]

Table 3 :

50°C for 12 days in :	% of unchanged compound			
	AZT	9	10	11
Buffer PO_4^{3-} pH=7.35	100	> 99	> 99	> 99
HCl 0.1N pH=1.15	> 99	> 96	> 97	> 97

These compounds were evaluated as substrate and as inhibitors of the cellular kinases susceptible to recognize them. At a concentration eight times higher than the Km values of GMP and AMP for GMP kinase, compounds 3 and 5 were neither substrates nor inhibitors of these enzymes. Similarly, at a concentration 5 times higher than the Km value of TMP for TMP kinase, compounds 9, 10, 11, 15, 16, 17 did not either display substrate or inhibitory activity.

Despite of the fact that they are not triphosphate analogs these compounds were evaluated as inhibitors of both avian myeloblastosis virus (AMV) or recombinant HIV-1 reverse transcriptases[26] and where compared to AZTTP one of the best HIV-1 RT inhibitor known.

Experimental procedure : The reaction mixture (60 µl) contained 42 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 17 µg bovine serum albumin, 33 mM KCl, 0.02 units poly(rA)-oligo dT_{12-18}, 2.5 µCi [^{2}H]TTP (specific activity 25 Ci/mmol; 5µM final concentration), 4 µl of various concentrations of test compound and 1-2 units reverse transcriptase (RT). Due to the high acidity of some of the test compounds, RT enzyme was buffered to pH 8.0 with phosphate buffer and the final pH of the reaction mixture containing drug was never lower than 7. Reactions were incubated at 37°C for 30 minutes, applied to Whatman 3MM discs, washed extensively in TCA, ethanol rinsed dried and counted for radioactivity.

Table 4 : Inhibitory activity against reverse transcriptase

NT = Not Tested

compound	AMV RT IC_{50} (µg/ml)	HIV RT IC_{50} (µg/ml)	compound	AMV RT IC_{50} (µg/ml)	HIV RT IC_{50} (µg/ml)
AZT	600	800	**9**	46	40
AZTMP	900	> 1000	**10**	45	21
AZTTP	0.06	0.12	**11**	15	12
3	150	NT	**13**	135	NT
4	60	NT	**14**	31	18
5	> 400	NT	**15**	90	NT
6	61	NT	**16**	300	NT
7	> 400	NT	**17**	300	NT

As shown in table 4, AZTTP was as expected a very good inhibitor of both RT enzymes whereas AZT and AZTMP were inactive consistent with the known mechanism of action of AZT. Surprisingly, several of the fluorophosphonates had some activity against RT. The AZT phosphonates possessed the best overall activity profile and the trifluorophosphonate derivative **11** was the most potent in this series. The mechanism of this unique activity against RT is the subject of further investigation and will be published in details later. These compounds do not require phosphorylation to be active and offer new perspectives in the design of reverse transcriptase inhibitors.

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