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Analogues of nucleotides and oligonucleotides featuring difluorophosphonate, difluorophosphonothioate and difluorophosphinate functional groups

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Abstract

Efforts to stereoselectively install difluorophosphonyl, difluorophosphonothioyl and difluorophosphinyl groups in place of the phosphate linking positions 3′ and 5′ of two furanoses are reviewed. Two equally efficient approaches have been worked out based on either the ionic addition of difluorophosphonothioate reagents **17** or **33** onto a ketone, or on the addition of phosphorus-centered radicals onto *gem*-difluoroalkenes. These methodologies resulted in the successful preparation of 3′-phosphonodifluoromethyl analogues to nucleosides-3′-phosphates, and of key intermediate **83**, featuring two ribofuranosyl nuclei linked by a difluorinated phosphonothioyl unit on positions 3′ and 5′. In addition, the groundwork for the synthesis of *H*-difluorophosphinates and difluorophosphinates has been laid.

Keywords: Difluorophosphonothioate; Difluorophosphonate; *H*-difluorophosphinate; Nucleotide analogues; Antisense strategy

1. Introduction

Twenty-five years ago, Zamecnik and Stephenson reported on the possibility of interfering with the biosynthesis of proteins at the translational stage, and demonstrated the efficacy of oligonucleotides in the process [1]. This pioneering work induced a massive amount of work and efforts aiming at the regulation of gene expression, and resulted in a conceptually new approach in the therapeutic treatment of diseases. Briefly, the so-called antisense strategy is relying on the sequence-specific binding of a modified oligonucleotide (MON) with the messenger RNA (m-RNA) to keep the ribosome from reading and translating the nucleic acid into the protein and thus induce the destruction of the m-RNA strand by nucleases (Fig. 1).

A large number of research groups have initiated work to design the best modified oligonucleotide possible [3]. Replacing the phosphate diesters linking two nucleosides has been one of the early strategies followed by scientists to produce antisense molecules. In this context, the structural and electronic design of a good phosphate isostere relies on several criteria, among which the following: (i) the isostere must help keep the predominant $C_{3'}$-*endo* conformation **1** of the ribofuranoses that favors pairing and stabilizes RNA duplexes (Fig. 2) [4]; (ii) the isostere must be of optimal geometry to maintain the interbase distances, thereby favoring the stabilizing interactions between the complementary strands; (iii) the isostere must be stable in physiological media and, more importantly, towards nucleases. This allows the MON to target another m-RNA molecule, once the first one has been lyzed by enzymes [5].

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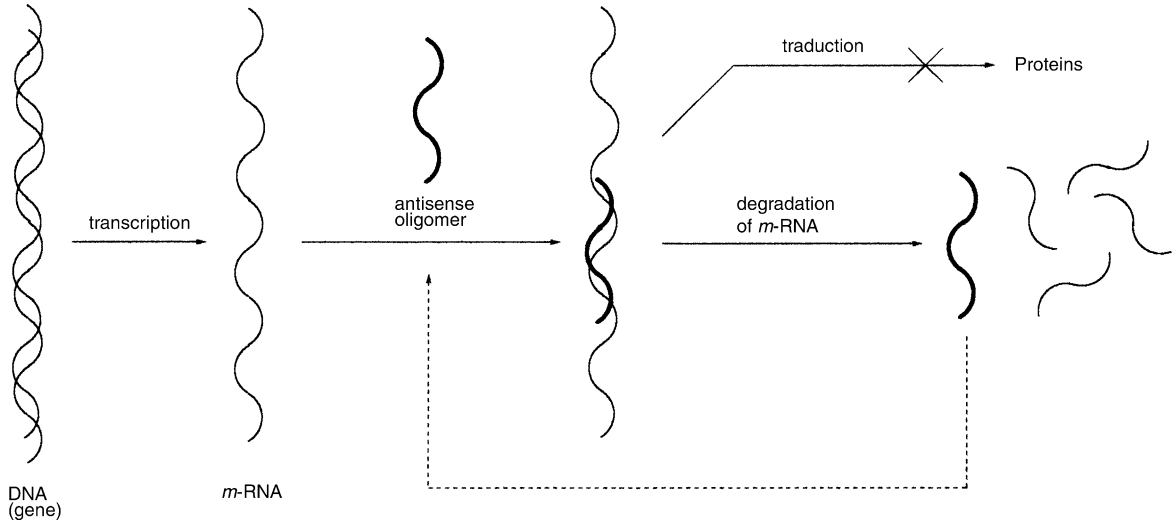


Fig. 1. Blockade of translation by modified oligonucleotides (antisense strategy).

The various functional groups used to mimic the phosphates in oligonucleotides may be categorized in two distinct classes. The first one encompass all the groups devoid of phosphorus atom, such as, for instance, amides, carbamates, acetals or thioethers (e.g. **3**, Fig. 3) [3a,6]. The second class of phosphate mimics will include all the phosphorus-centered isosteres. Among these, those still including the $C_{3'}$ oxygen atom incorporate functional groups such phosphorothioate, phosphoramidate, phosphonate or difluorophosphonate, for instance (**4**) [3a,7]. Vitravene™, a drug approved by the Federal Food and Drug Administration for the treatment of cytomegalovirus infection is a phosphorothioate MON [8]. The MON featuring a phosphorus-centered phosphate mimic in which the $C_{3'}$ oxygen atom has been replaced with another atom include the phosphonates and phosphinates reported by Collingwood, and the phosphorothioamidates recently described by Gryaznov (see **5**, Fig. 3) [9]. These transformations have been shown to result in increased RNA binding affinity, as reflected by the determined positive T_m values [10].

2. Results and discussion

Our interest in the reported close analogy between the phosphate and the α,α-difluorophosphonate [11,12] led us to envision the replacement of the $C_{3'}$ oxygen atom with a CF_2 unit and consider the use of hitherto unreported functional groups as linker between nucleosides, among which the difluorophosphonate, the difluorophosphonothioate and the difluorophosphinate. In this paper, we review our efforts to prepare nucleoside-5′-phosphate analogues in which the phosphate group has been replaced with phosphonodifluoromethyl and phosphonothiodifluoromethyl moieties.

Close inspection of the target monomeric 3′-phosphonodifluoromethyl monomer **6** led us to consider approaches relying on either an ionic introduction of the functional group, or a radical addition-based construction of the desired function. Scheme 1 outlines the first, ionic approach.

The disubstitution of carbon $C_{3'}$ in nucleosides prevented us from using reactant **8a** in a SN_2-type reaction [13]. Instead, a protocol calling for (i) the addition of **8a** onto a keto group and (ii) a radical-based deoxygenation of the resultant adduct **9** was considered [14]. The stereodirecting effect of the base was expected to result in a predominant attack of nucleophilic species **8a** on the α-face of the furanose. However, the same stereodirecting effect acting on the hydrogen quenching of radical intermediate **10** during the deoxygenation step would probably provide the undesired diastereomer **11**. As this last step would probably play a crucial role in installing the requisite configuration on carbon $C_{3'}$, it was decided to start from a furanose derivative featuring a hindered α-face to force the hydrogen quenching to occur on the β-face (thereby positioning the phosphonodifluoromethyl unit on the desired face of the cycle), and to introduce the base in a subsequent step. Glucofuranose derivative **12** was chosen as a readily available substrate, because of the 1,2-dimethyl acetal protection and the known steric hindrance generated by the angular methyl group (Scheme 2) [15].

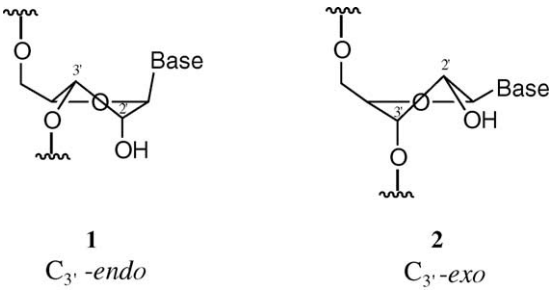


Fig. 2. $C_{3'}$-*endo* confirmation of ribofuranose rings in nucleic acids vs. $C_{3'}$-*exo* confirmation.

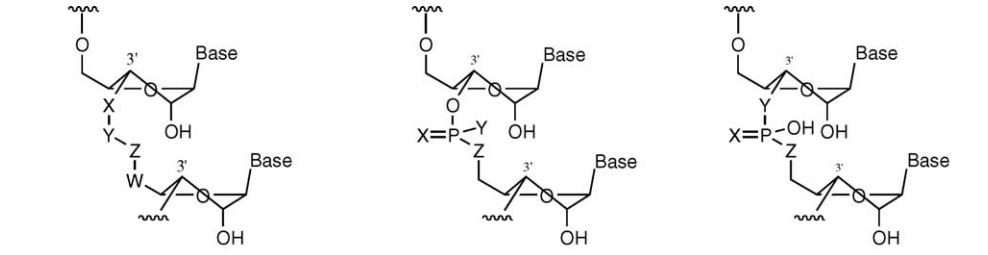
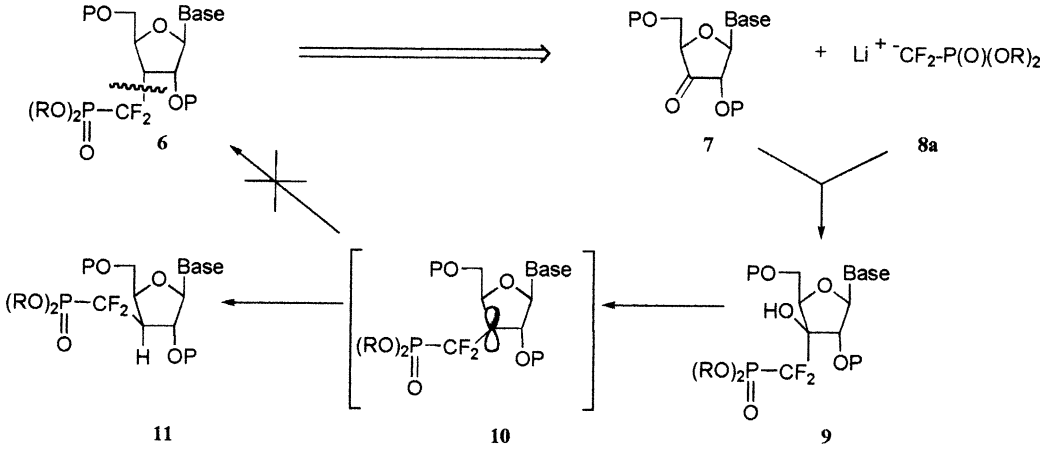
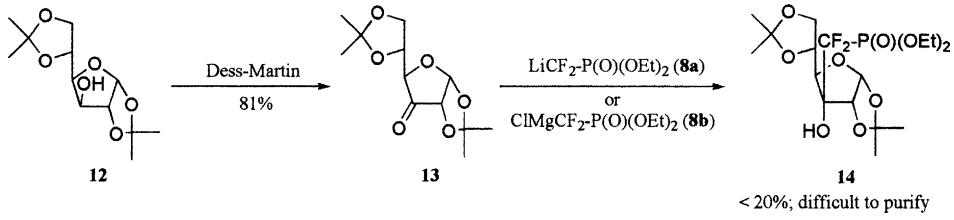


Fig. 3. Modified oligonucleotides encompassing a phosphate mimic.



Scheme 1.



Scheme 2.

Oxidation of the $C_{3'}$ hydroxyl group with Dess-Martin periodinane resulted in a clean and smooth production of ketone **13**, which was subjected to the action of the anion of diethyl difluoromethylphosphonate [16]. However, neither the action of the lithium salt **8a**, nor the use of the corresponding organomagnesium reagent **8b** resulted in the clean formation of a 1:1 adduct. By-products, such as bisphosphonate **15**, were isolated, sometimes in good yield, and the expect 1:1 adduct **14** was obtained in low yield (<20%) and found to be contaminated with the hydrate **16** of unreacted ketone **13** (Fig. 4).

The thioanologue **17** of reagent **8a** was then considered. The lithium salt of *O*,*O*-diethyl-phosphonothiodifluoromethyl anion (**17**) may easily be prepared by treating precursor **18** with LDA at low temperature, or by reacting the corresponding bromide **19** with 2 Eq. of *tert*-butyllithium (Scheme 3) [17]. The thermal stability of reagent **17**

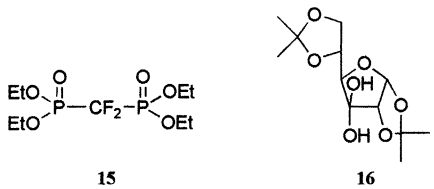
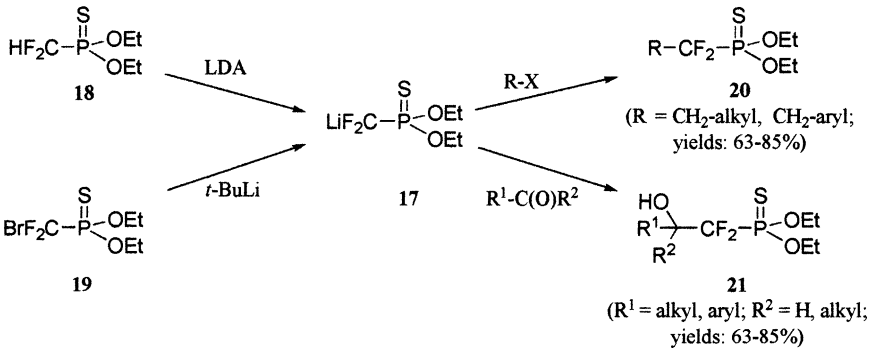
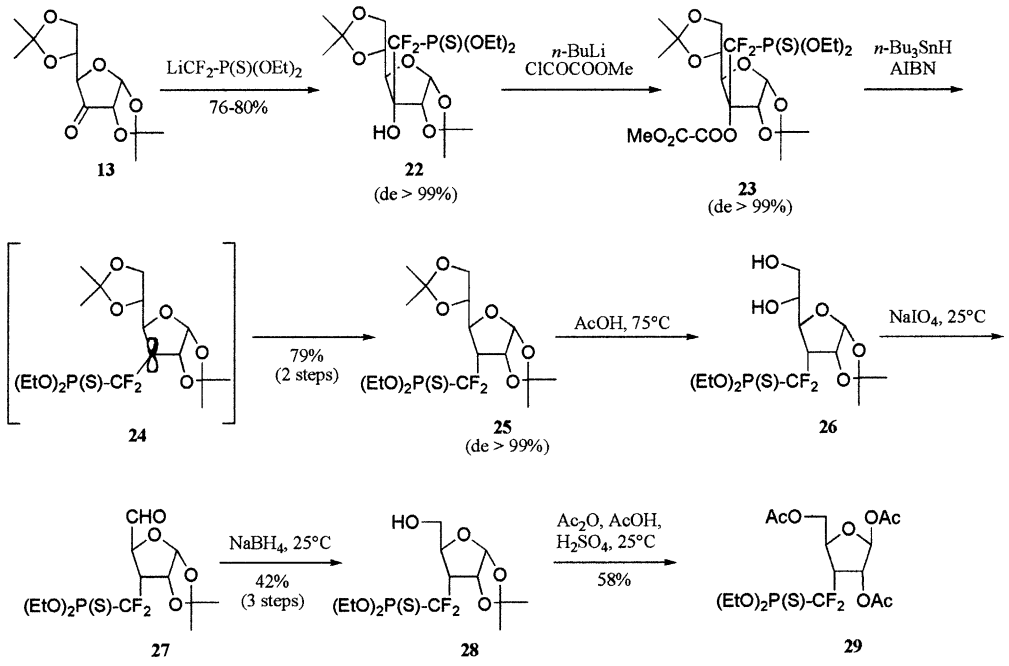


Fig. 4. Byproducts from the reaction between ketone **13** and reagents **8a** or **b**.



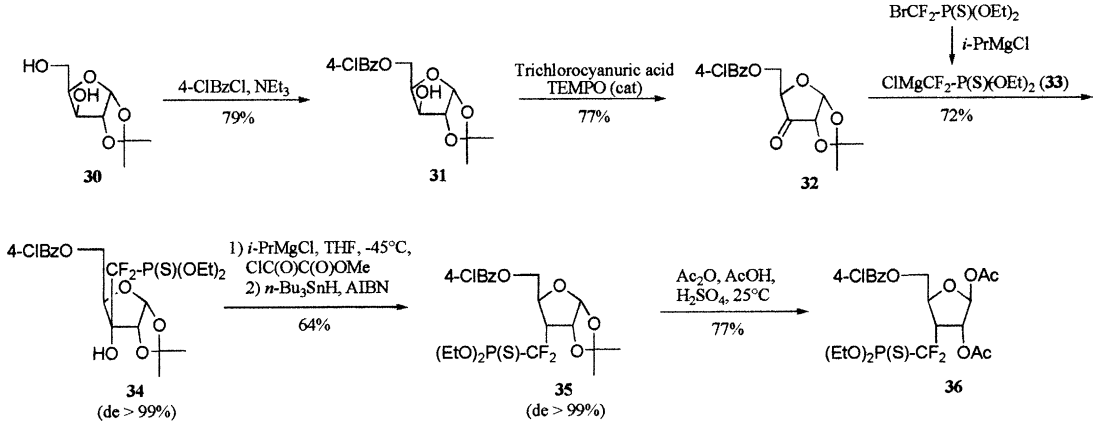
Scheme 3.



Scheme 4.

has been reported to be around −20 °C and thus contrasts favorably with the documented thermal lability of the fully oxygenated analogue **8a** (−78 °C) [13]. Its reactivity was found to parallel that of **8a** and, in particular, successfully allowed the isolation of compounds of the type **21**, by interaction with aldehydes and ketones [17].

Treatment of ketone **13** with reagent **17** at −78 °C, followed by a classical work-up, allowed the isolation of a



Scheme 5.

1:1 adduct in perfectly reproducible fashion (76–80% isolated yield). Diastereoselection proved to be complete based on ^{1}H, ^{19}F and ^{31}P NMR data, and we assumed the structure to correspond to compound **22** (Scheme 4). Deoxygenation of carbon $C_{3'}$ was carried out according to the procedure of Berkowitz [14b]. Thus, reaction of **22** with *n*-butyl lithium and *O*-methyloxalyl chloride, isolation of the resultant oxalate **23** and interaction of the latter with tri-*n*-butyltin hydride in the presence of a catalytic amount of azobis*iso*butyronitrile (AIBN) furnished **25** as a single diastereomer, isolated in 79% yield. Analysis of the NMR data confirmed the positioning of the phosphonothiodifluoromethyl unit on the α-face, the result of the stereodirecting influence of the 1,2-acetonide protecting group, and thus of the exlusive hydrogenation quenching of radical intermediate **24** on the convex face. Functional group manipulation allowed the transformation of the glucofuranose into a ribofuranose derivative **28**, and included deprotection of the 5,6-acetal unit, oxidative cleavage to **27** and reduction. The final, one-pot operation achieved the preparation of precursor **29** by deprotection of the 1,2-acetonide unit and the protection of the three hydroxyl groups in the form of acetates. This first preparation thus resulted in the isolation of precursor **29** of the desired modified nucleotides in 10% overall yield.

Critical analysis of this synthesis led to the identification of several drawbacks impeding its use in larger scale preparation of **29**. Thus, LDA or *tert*-butyllithium were used as base, and chromatography had to be used to obtain several intermediates in the pure form. The involvement of the somewhat hazardous Dess-Martin periodinane was also considered as a limitation to the development of a larger scale preparation. Moreover, the six-carbon starting substrate implied additional steps to obtain the five-carbon skeleton of ribofuranose **29**. An improved synthesis was thus worked out, starting from α-D-xylose derivative **30** (Scheme 5). The required 1,2-dimethylacetal protection was completed by the installation of a *para*-chlorobenzoyl unit on the 5'-hydroxyl group. Indeed, *para*-chlorobenzoate have often been noticed for their propensity to crystallize [18].

Alcohol **31** was oxidized by a mixture of trichlorocyanuric acid and tetramethylpiperidine oxide (TEMPO) in catalytic amount to deliver ketone **32** in 77% isolated yield [19]. Lithium reagent **17** was replaced with the organomagnesium reagent **33**, readily prepared by treating bromide **19** with 1.1 Eq. of freshly prepared *iso*propyl magnesium chloride in diethyl ether [20]. For the reason cited above, adduct **34** was obtained as a single diastereomer. Once again, radical deoxygenation of adduct **34** resulted in a clean stereochemical inversion of the phosphonothiodifluoromethyl moiety, and phosphonothioate **35** was obtained in 64% isolated yield (two steps) as a single diastereomer. Treatment of this product with acetic anhydride in a mixture of acetic acid and sulfuric acid removed the 1,2-acetonide and furnished triester **36** as a single stereoisomer. This scheme resulted in the formation of a slightly different

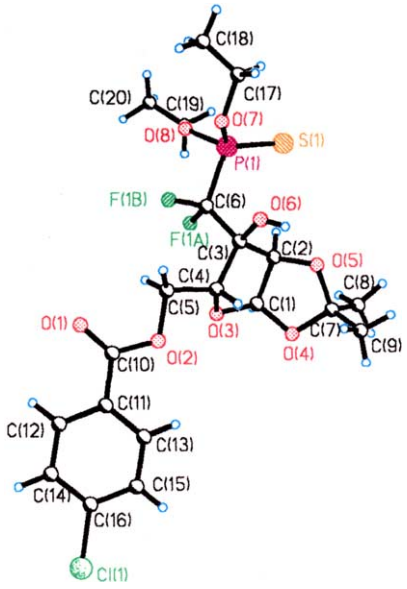
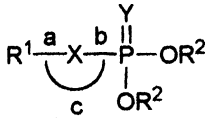


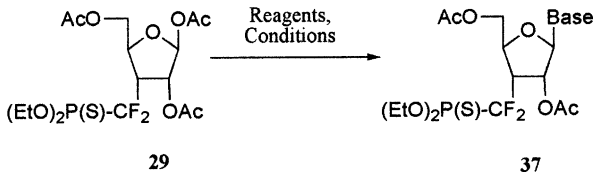
Fig. 5. ORTEP graphic of adduct **24**.

Table 1
Selected bond lengths and angle values for the phosphate, the difluorophosphonate and the difluorophosphonothioate groups

X	Y	*a* (Å)	*b* (Å)	*c* (°)	
O	O	1.59	1.43	118.5	
CF_2	O	1.85	1.50	116.5	
CF_2	S	1.88	1.51	120.3	

precursor *with an improved overall yield of 21%* from xylose derivative **30**. Noteworthy is the fact that no chromatography separation is needed, and that the preparation of **36** has been carried out on a 10-g scale [21].

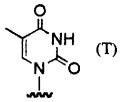
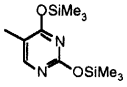
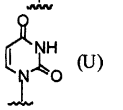
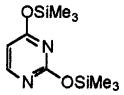
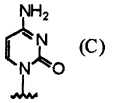
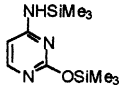
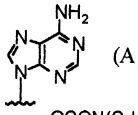
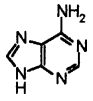
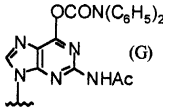
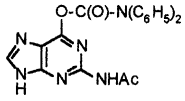
Adduct **34** was isolated as a crystalline solid which from provided monocrystals of size and quality good enough for X-ray analysis. The computer-generated ORTEP graphic clearly demonstrates that the nucleophilic attack occurred exclusively on the β–face, as expected (Fig. 5). In addition, this provided the first structural comparison between a difluorophosphonate and a difluorophosphonothioate. Values in Table 1 clearly indicate that the C_3–CF_2 and CF_2–P bond lengths in difluorophosphonotioate **34** compare favorably with those reported by Chambers et al. on difluorophosphonates [22]. Perhaps the most interesting feature is the wider angle formed by these two bonds



29 **37**

Scheme 6.

Table 2
Structures and yields of nucleotides analogues **37**

Entry	Base	Reagent	Conditions	Yields **37** (%)
1	 (T)		TMSOTf, $ClCH_2CH_2Cl$, 25 °C	92
2	 (U)		TMSOTf, $ClCH_2CH_2Cl$, 25 °C	81
3	 (C)		TMSOTf, $ClCH_2CH_2Cl$, 80 °C	95
4	 (A)		$SnCl_4$, CH_3CN, 25 °C	87
5	 (G)		TMSOTf, $ClCH_2CH_2Cl$, 80 °C	66

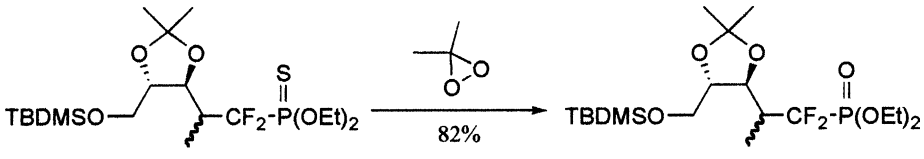
(120.3°), when compared to values reported for the phosphate (118.5°) and the difluorophosphonate (116.5°).

The stereoselective introduction of the bases on the phosphonothiodifluoromethyl-bearing furanose ring was achieved by reacting triester **29** and the requisite protected or unprotected base under Lewis acid catalysis (Scheme 6, Table 2). The use of Vörbruggen's modification of the Hilbert-Johnson protocol proved especially efficient to introduce the monocyclic bases [23], while adenine or protected guanine were installed by using the procedure of Saneyoshi and Robins [24]. Isolated yields were good to excellent and a complete diastereoselectivity was observed, as a result of the steric hindrance generated by both the 2-acetoxy group and the 3-phosphonothiodifluoromethyl unit. It might be suggested that the longer P=S bond (1.886 Å versus 1.580 Å for the P=O bond) allows the latter group to participate in the stabilisation of the intermediate cation. Such an analogous participation has recently been described with 2,3-dideoxy-3-phosphonothiofuranose derivatives [25]. Of particular note is the excellent regiochemical control observed in the induction of the purine bases: a single regioisomer was found to have formed.

Interconversion between difluorophosphonates and difluorophosphonothioates was studied a few years ago. Thus, transformation of the former to the thioanalogues may be efficiently achieved by simple refluxing a toluene solution of the substrate and Lawesson's reagent, while the reverse process (P=S → P=O) is carried out with an oxidating agent such as a perfluorinated oxaziridine, dioxirane or, more simply, *meta*-chloroperoxybenzoic acid (*m*-CPBA) [26], These methods are mild enough and tolerant of various functional groups, as demonstrated by the efficient transformation of acyclic difluorophosphonothioate **38** into the corresponding phosphonate **39**, when reacted with dioxirane (Scheme 7).

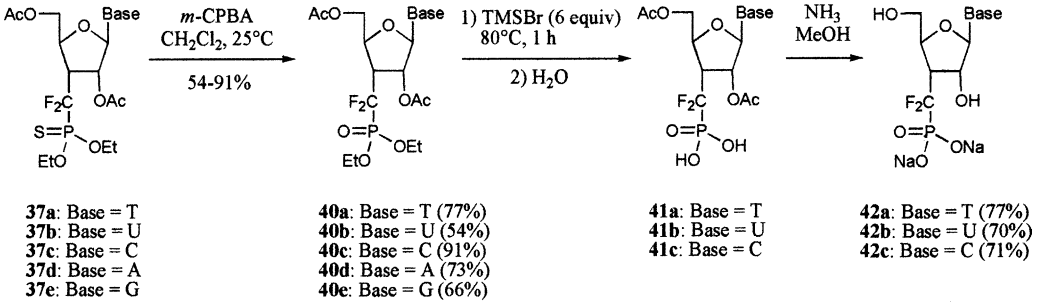
In the case of nucleotide analogues **37**, the P=S/P=O conversion was carried out in presence *m*-CPBA and yielded the desired phosphonates **40** in yields ranging from 54 to 91% (Scheme 8). The synthesis was then completed by fully deprotecting the difluorophosphonates **40**. Thus, sequentially treating **40a–c** with 6 Eq. of trimethylsilylbromide at 80 °C for 1 h, and water delivered phosphonic acid **41a–c**, which were converted to the final nucleotide analogues **42** by the action of ammonia in methanol; the target molecules were conveniently isolated as sodium salts by standard purification with Dowex 50X-2 resin [27–29].

In addition to this successful synthesis of the target nucleotide analogues, another approach was being simulta-

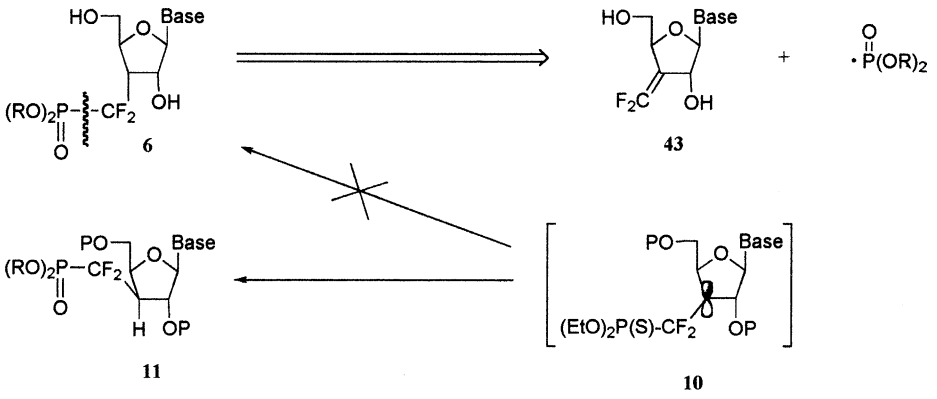


38 **39**

Scheme 7.



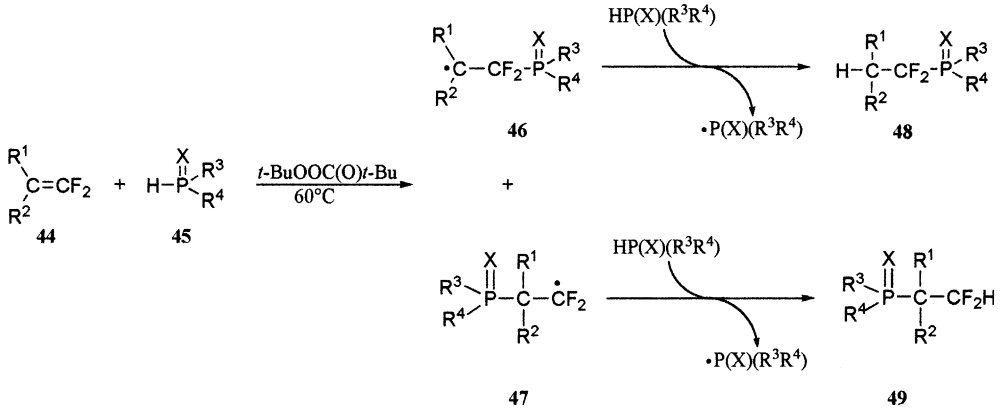
Scheme 8.



Scheme 9.

neously developed. This time, the retrosynthetic analysis called for the construction of the desired functional group by way of a phosphorus-centered radical addition onto a *gem*-difluoroalkene of the type **43** (Scheme 9). Here again, as the stereodirecting effect of the nucleobase was expected to induce hydrogen quenching of the intermediate radical adduct **10** on the α-face, and deliver diastereomer **11**, it was decided to start from a carefully protected furanose. The positive experience we had gathered with furanose derivative **13** led us to use it as starting material.

Phosphonyl and phosphonothioyl radicals generated from phosphate or thiophosphite have been shown to add onto *gem*-difluoroalkenes to deliver a 9:1 to 95:5 mixture of regioadducts **48** and **49**, respectively. (Scheme 10) [30]. The yields are good, but it has to be noted that side-reactions may occur with some phosphite, e.g. diphenylphosphite and dibenzylphosphite. Thiophosphites bear a clear synthetic advantage over their fully oxygenated counterparts, in view of both their greater ease to undergo homolytic substitution, and the higher yields in which the expected adducts are



Scheme 10.

Table 3
Structure of phosphites, thiophosphites and difluoroalkenes, and yields of adducts **48**

Entry	**44**	R^1	R^2	**45**	X	R^3	R^4	Yields **48** (%)
1	a	$-(CH_2)_5-$		a	O	OMe	OMe	66
2	b	C_9H_{19}	CH_3	b	O	OEt	OEt	72
3	b	C_9H_{19}	CH_3	c	S	OEt	OEt	95
4	c	C_6H_5	CH_3	b	O	OEt	OEt	0
5	c	C_6H_5	CH_3	c	S	OEt	OEt	30
6	a	$-(CH_2)_5-$		e	O	OBn	OBn	8
7	b	C_9H_{19}	CH_3	f	S	OBn	OBn	65

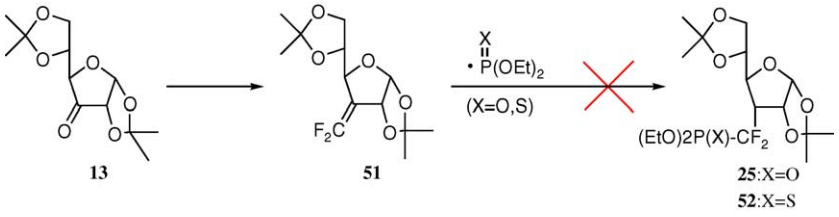
isolated (compare entries 2 with 3, 4 with 5, and 6 with 7, Table 3).

Hence, for instance, the two-step process involving the addition of a thiophosphite on difluoroalkene **50**, followed by a conversion of the P=S bond in adduct **38** into P=O bond was more efficient (62% overall yield) than the direct, one step addition of the phosphonyl radical, as illustrated in Scheme 11 [26].

Furanose **13** was transformed into difluoroalkene **51** by using literature procedures [31]. However, all attempts to react this substrate with phosphonyl or phosphonothioyl radicals failed to deliver the expected adducts **25** or **52**, respectively, the starting material being recovered in each case (Scheme 12) [27].

Other functionalized cyclic molecules, similar or different, and bearing an exocyclic difluoromethylene unit failed also to yield any adduct under a variety of conditions (e.g. compounds **53a–53c**, Fig. 6). Indeed, the few successful cases of addition of these radicals on analogous molecules reported in the literature show that the products are obtained (albeit in yields ranging from low to fair) with *gem*-difluorinated enol ethers (see compounds **54a** and **54b**, Fig. 6) [30b–d]. The electronic influence of the ether group on this reaction might thus be indicative of the electrophilic nature of phosphonyl and phosphonothioyl radicals, and has a strong effect on the course of the reaction by overcoming the electronic and steric action of the fluorine atoms [32]. It is noteworthy that the unfluorinated analogue of **51** undergoes efficient addition of both phosphonyl and

Scheme 11.



Scheme 12.

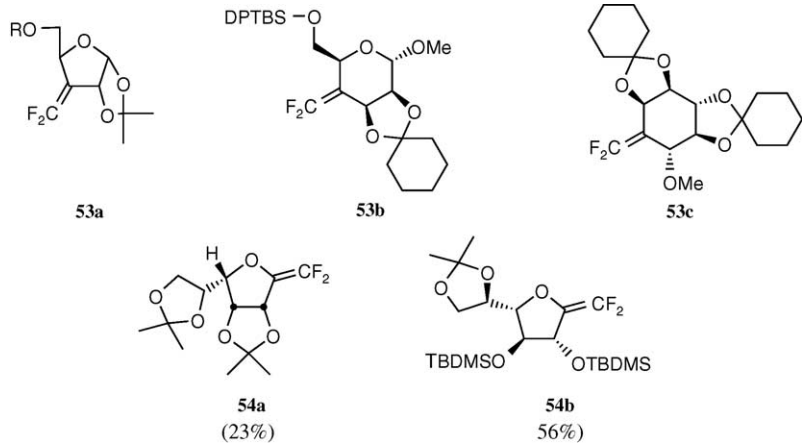
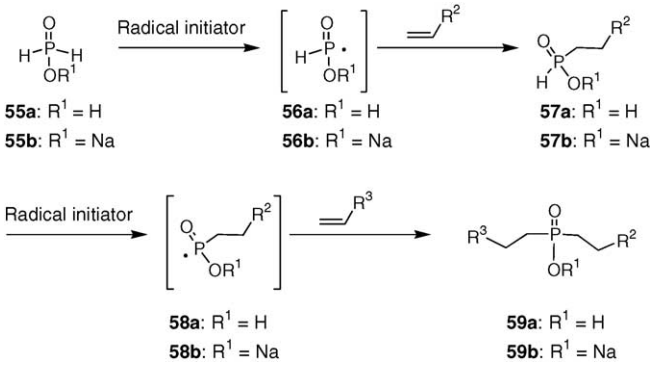


Fig. 6. Structure of cyclic derivatives **53** and **54**.



Scheme 13.

phosphonothioyl radicals to deliver the expected adducts in good to excellent yields [33].

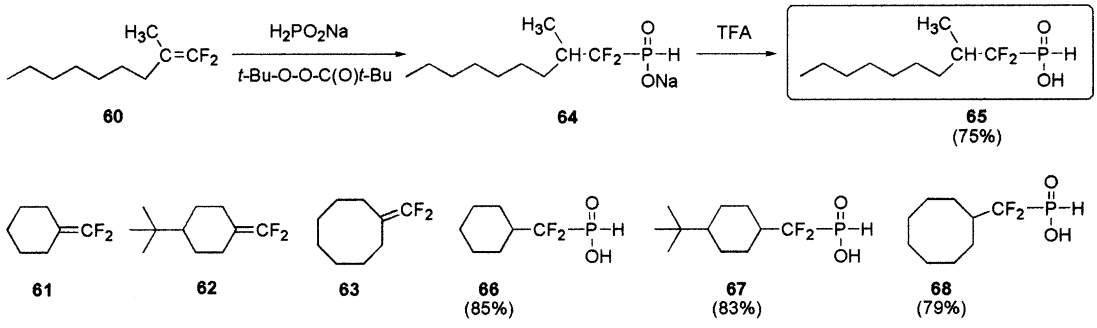
In this context, hypophosphorous acid **55a** constitutes a potentially useful precursor of phosphorus-centered radical **56a**, albeit at a lower oxidation state. A literature search indicates that, in the presence of both a radical initiator and alkenes, the expected 1:1 adducts **57a** are obtained in good yields (Scheme 13) [34]. The remaining P–H bond in the thereby-formed *H*-phosphinates can be cleaved to generate a new phosphorus radical **58a** which adds onto alkenes, thus delivering phosphinates **59a**. Of special interest is the fact that the sodium salt species **55b** and **58b** of hypophosphorous acid react similarly, thus allowing to envision their use in the presence of acid-labile protecting groups.

The reactivity of **55b** towards *gem*-difluoroalkenes under radical-initiating conditions was first verified by testing model difluoroalkenes. Thus, subjecting either of the four substrates **60–63** to refluxing methanol conditions in the presence of sodium salt **55b** and a catalytic amount of *tert*-butyl perpivalate as radical initiator and, second, a classical work-up resulted in the isolation of expected adducts **65–68** in 75–83% isolated yields after purification (Scheme 14).

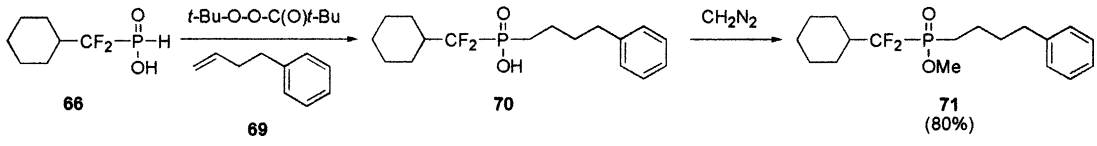
The α,α-difluoro-*H*-phosphinates thus obtained are stable at room temperature under standard conditions and constitute a class of useful intermediates *en route* to a number of difluorinated functional groups. Reacting **66** with 4-phenyl-1-butene (**69**) in the presence of the same radical initiator induced a second radical reaction to take place (Scheme 15). Interaction of the crude product **70** with excess diazomethane furnished methyl α,α-difluorophosphinate **71** in 80% isolated yield.

The tautomeric equilibrium between *H*-phosphonates and phosphites led us to quantitatively transform the α,α-difluoro-*H*-phosphinates to *O*,*O*-bisilylated phosphites by treatment with 3 Eq. of trimethylsilyl chloride in the presence of pyridine (Scheme 16) [35]. The air-sensitive phosphites **72** and **73** now encompass a nucleophilic phosphorus which smoothly reacted with electrophilic centers. Thus, reaction between **72** and methyl vinyl ketone followed by treatment with diazomethane delivered the 1,4-adduct, isolated in the form of its methyl ester **74** (57% yield) [36]. Phosphite **73** interacted with pivalaldehyde to furnish, after work-up and treatment with diazomethane, methyl α,α-difluoro-α′-hydroxyphosphinate **75** in 64% yield [37]. It is noteworthy that the presence of the CF_2 unit did not keep the α,α-difluoro-*H*-phosphinates from reacting smoothly with electrophiles.

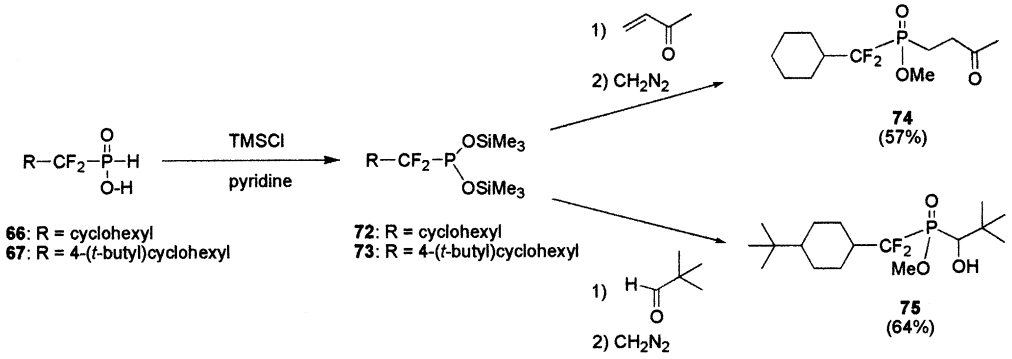
These early results constituted a solid ground on which the reactivity of protected ribofuranose derivatives were tested. Thus, when difluoroalkenes **76a** or **b** were reacted with hypophosphorous acid sodium salt **55b** in the presence of triethylborane and air, a complete consumption of the substrates occurred and adducts **77** were isolated as a single diastereomer in each case (Scheme 17) [38]. Despite the smaller size of the reacting radical (when compared to



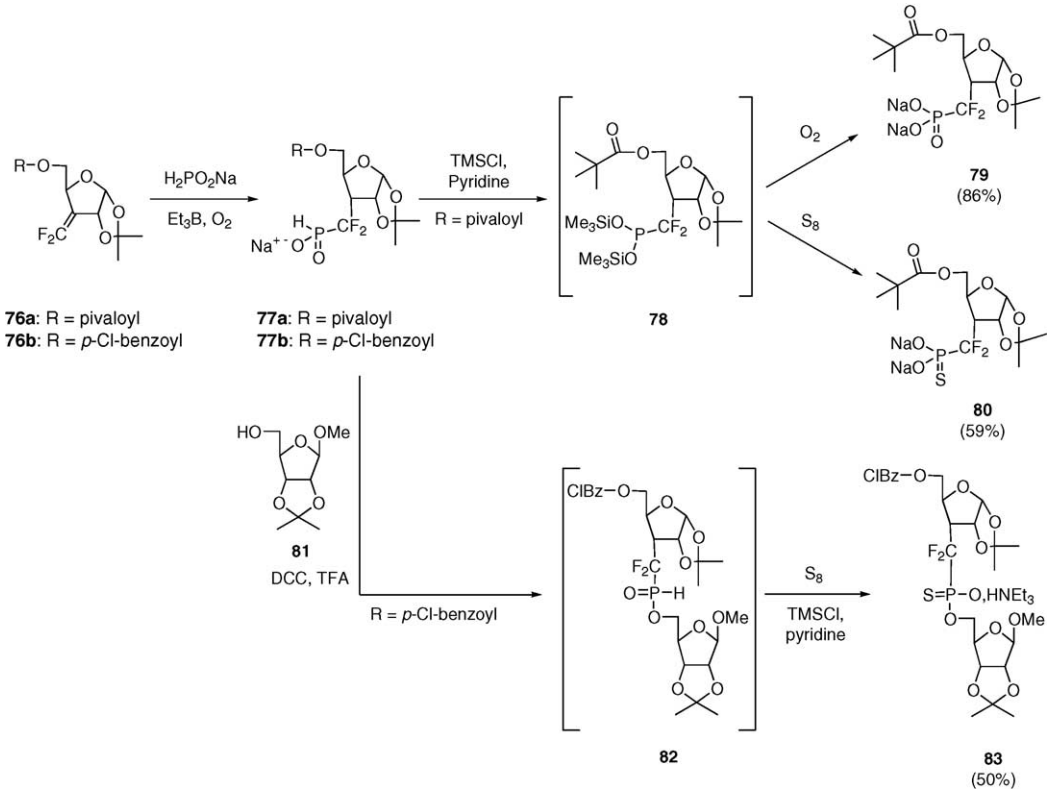
Scheme 14.



Scheme 15.



Scheme 16.



Scheme 17.

phosphonyl and phosphonothioyl radicals), the stereodirecting effect of the 1,2-acetonide unit proved efficient in blocking the concave face of the ribofuranose [15,38]. Transforming adduct **77a** into phosphite **78** and interaction with oxygen (air) or sulfur afforded protected phosphonic acid **79** and phosphonothioic acid **80**, respectively, in the form of their solid, disodium salts (86% and 59% isolated yield, respectively) [39]. Adduct **77b** could be esterified by reaction with alcohol **81** in the presence of DCC and trifluoroacetic acid; the intermediate, α,α-difluoro-*H*-phosphinic acid monoester **82** was treated with trimethylsilyl chloride, pyridine and sulfur to deliver difluorophosphonothioic acid monoester, isolated as its triethylammonium salt **83** in 50% yield.

3. Conclusion

The work described in this paper reports on the successful development of new functional groups isostere to the phosphate. In particular, two approaches, based on the ionic introduction of a phosphonothiodifluoromethyl unit, or on a radical-based construction of the same functional group, resulted in the efficient synthesis of 3-deoxyribofuranose derivatives bearing this phosphate mimic in position 3 of the tetrahydrofurane ring. These furanose derivatives have been transformed into analogues of nucleoside-3′-phosphates, and may also be used to obtain intermediates of the type **83**, featuring two ribofuranosyl units linked by a difluorophosphonothioyl moiety in positions 3 and 5′ of the oxacycles.

Additionally, the ground methodology for the preparation of α,α-difluorophosphinates from the hiterto unreported α,α-difluoro-*H*-phosphinates has been established. Work is now in progress to use these various functional groups in the design and preparation of modified dinucleotides and trinucleotides, and evaluate their impact in the context of antisense and antigene strategies.

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