Dithymidylyl-3′,5′-phosphorofluoridates: New Synthesis and Stability under Solvolytic Conditions

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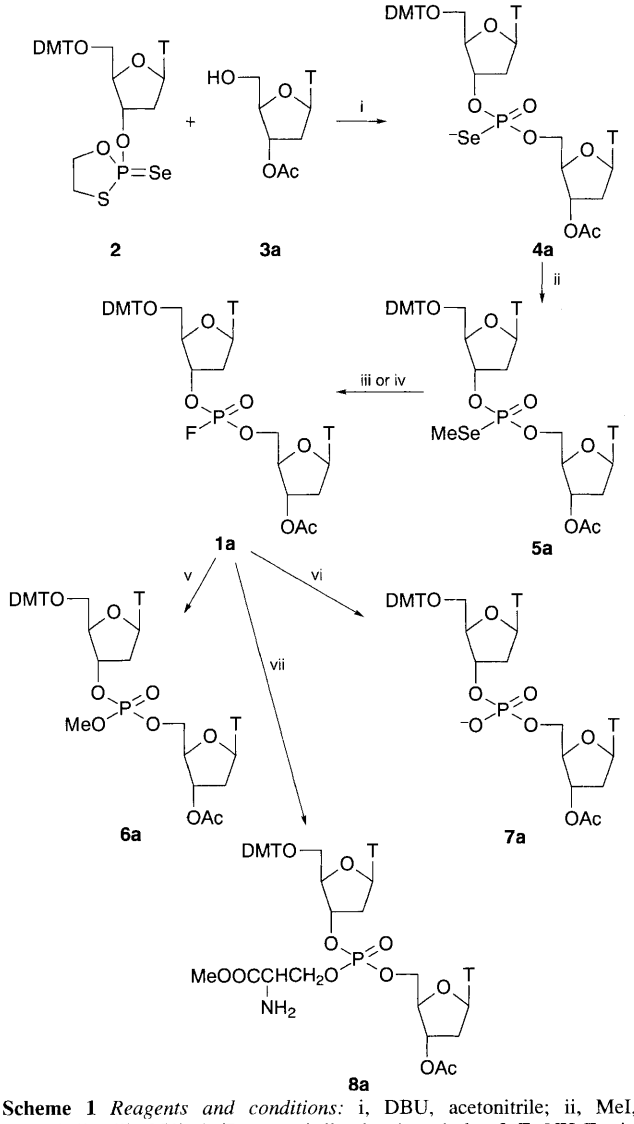
Dithymidylyl-3′,5′-phosphorofluoridates, prepared by nucleophilic substitution of methylselenyl by fluoride ion, are relatively unstable under solvolytic conditions and undergo rapid base-catalysed hydrolysis/methanolysis.

The recent reports[1,2] on the synthesis of dinucleoside-3′,5′-phosphorofluoridates **1** and their phosphodiesterase-assisted hydrolyses leading to nucleoside-3′-*O*- or 5′-*O*-phosphorofluoridates prompted us to publish our results on a new synthesis of **1**. We have found that the DBU-assisted reaction of 5′-*O*-DMT-thymidine-3′-*O*-(2-seleno-1,3,2-oxathiaphospholane) **2** (DMT = 4,4′-dimethoxytrityl),† with 3′-*O*-acetylthymidine **3a** in acetonitrile gives 3′-*O*-acetyl-5′-*O*-DMT-dithymidylyl-3′,5′-phosphoroselenoate **4a**‡ which, without isolation, under treatment with methyl iodide is converted into the 3′-*O*-acetyl-5′-*O*-DMT-dithymidylyl-3′-5′-Se-methylphosphoroselenoate **5a**§ (Scheme 1). A solution of **5a** in acetonitrile reacts with a 25% aqueous solution of silver fluoride or, independently, with triethylammonium fluoride to give 3′-*O*-acetyl-5′-*O*-DMT-dithymidylyl-3′,5′-phosphorofluoridate **1a**.¶ The reaction with AgF is fast, and ^{31}P NMR analysis indicates the disappearance of **5a** within 5 min. The reaction of **5a** with $Et_3NH^+F^-$ is much slower, reaching completion only after 12 h. Attempts to purify **1a** on silica gel failed. When ethyl acetate is used as eluent, only 24% of product **1a** is eluted (calculated on raw **1a** after extraction and evaporation of solvents; ^{31}P NMR analysis indicates the presence of **1a** as the only phosphorus-containing compound). If a mixture of dichloromethane with methanol (9:1 *v*/*v*) is used as the developing system during attempted isolation on preparative TLC plates, 3′-*O*-acetyl-5′-*O*-DMT-dithymidylyl-3′,5′-*O*-methylphosphate **6a**‖ is obtained.



Scheme 1 *Reagents and conditions:* i, DBU, acetonitrile; ii, MeI, acetonitrile; iii, 25% AgF, acetonitrile; iv, 1 mol dm^{-3} $Et_3NH^+F^-$ in acetonitrile; v, MeOH, Et_3N; vi, 1 mol dm^{-3} NH_4OH; vii, $HOCH_2CH(NH_2xHCl)COOMe$, Et_3N, acetonitrile

The known phosphorylating properties of neutral phosphorofluoridates reported elsewhere prompted us to study the hydrolytic stability of **1a**. ^{31}P NMR monitoring of **1a** in aqueous media at pH 5.0 (triethylammonium acetate buffer), pH 7.5 (triethylammonium bicarbonate buffer) and pH 10.5 (aq. NEt_3) indicates that within this pH range, **1a** undergoes 32% hydrolysis during 2.5 h, giving 3′-*O*-acetyl-5′-*O*-DMT-dithymidylyl-3′,5′-phosphate **7a**.** At higher pH (1 mol dm^{-3} NH_4OH), the total disappearance of **1a** is observed after 15 min.

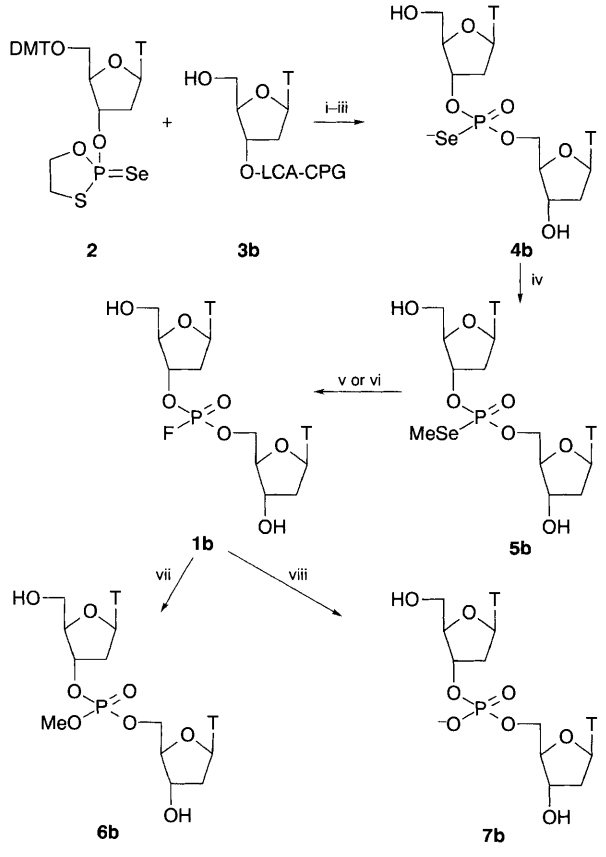
Phosphorofluoridate **1a** reacts smoothly with methanol in the presence of triethylamine or pyridine to give methyl ester **6a**. Compound **1a** also reacts with L-serine methyl ester in the presence of triethylamine giving 3′-*O*-acetyl-5′-*O*-DMT-dithymidyl-3′,5′-*O*-(serinyl methylester)phosphate **8a**†† in 87% yield. The diastereoisomeric ratio of **8a** was 90:10. The stereoselectivity of formation of **8a** is probably caused by kinetic preference of one diastereoisomer of **1a** to react with L-serine methylester. The fluoride ion released in the process of nucleophilic substitution at phosphorus causes fast epimerisation[3] of the slow-reacting diastereoisomer of **1a**. In good agreement with previous observations of Horner *et al.*[4a,b] that phosphorofluoridates possess poor phosphorylating activity towards amines, we found that **1a** does not form an amide when mixed with an excess of *n*-hexylamine.

Solid-support-anchored thymidine **3b** (1 μmol) was detritylated and treated with a 0.2 mol dm^{-3} solution of **2** and 0.5 mol dm^{-3} solution of DBU (both in acetonitrile) (Scheme 2). After 10 min the column was washed with acetonitrile and, after detritylation, dithymidylyl-3′,5′-phosphoroselenoate **4b** was released from the solid support by ammonolysis. After concentration under reduced pressure, the resultant solid residue was dissolved in 0.1 mol dm^{-3} Tris-HCl, pH 7.2, and treated with MeI. Alkylation was maintained for 2 h and then dithymidylyl-3′,5′-Se-methyl phosphoroselenoate **5b** was purified by reversed-phase HPLC to yield 38% **5b**, relative to the starting amount of **3b**. An acetonitrile solution of **5b** was subjected to AgF or $Et_3NH^+F^-$. The reaction progress was followed by reversed-phase HPLC. The disappearance of the peaks corresponding to diastereoisomers of **5b** was accompanied by transient formation of dithymidylyl-3′,5′-phosphorofluoridates **1b** and final dithymidylyl-3′,5′-phosphate **7b**.‡‡ The maximum concentration of **1b** was observed after 15 min. Pure **1b** was collected by HPLC and left for 1 h at room temperature in an HPLC buffer (0.1 mol dm^{-3} NH_4OAc, pH 7.0). The only product, detected by repeated HPLC analysis, was identified as

7b. In the light of this observed hydrolytic instability of dithymidylyl phosphorofluoridates, further attempts to synthesise longer sequences of oligo(nucleoside phosphoroselenoate)s, and their conversion into oligo(nucleoside phosphorofluoridate)s were abandoned.

A solution of compound **5b** in a mixture of acetonitrile and methanol was also treated with AgF and, as expected, formation of dithymidylyl-3′,5′-*O*-methyl phosphate **6b**§§ was observed. To exclude the direct hydrolysis or methanolysis of methylselenyl ester **5b**, the solution of this compound in a mixture of acetonitrile–water with added silver nitrate was left at room temperature and after 2 h only 5% of **7b** was observed. In a similar experiment, when the reaction was performed in a mixture of acetonitrile–methanol, after 12 h only traces of **6b** were observed. The poor ability of the MeSe group to act as a leaving group in $AgNO_3$-catalysed methanolysis was also observed in our earlier studies[5] and was also confirmed using **5a** as a substrate.

The observed limited hydrolytic stability of **1** is consistent with the commonly accepted good phosphorylating properties of *O*,*O*-dialkyl phosphorofluoridates and with our original observation[6] made during attempts to convert dinucleoside-3′,5′-phosphorothioates into the corresponding phosphorofluoridates by means of 2,4-dinitrophenyl fluoride and facile solvolysis of the resulting phosphorofluoridates in the presence of EtOH–NEt_3, leading to dinucleoside-3′,5′-*O*-ethyl phosphates. Moreover, the results of our studies have thrown additional light on the mechanistic aspects of the CsF-catalysed transesterification of 2,2,2-trichloroethyl phosphates.[7]



Scheme 2 *Reagents and conditions:* i, DBU, acetonitrile; ii, $CHCl_2COOH$, CH_2Cl_2; iii, conc. NH_4OH; iv, MeI, 1 mol dm^{-3} Tris-HCl pH 7.2; v, 25% AgF, acetonitrile; vi, 1 mol dm^{-3} $Et_3NH^+F^-$ in acetonitrile; vii, MeOH; viii, 0.1 mol dm^{-3} NH_4OAc (LCA–CPG = long chain aminoalkyl controlled pore glass)

In conclusion, although phosphorofluoridate modification of oligonucleotides can be potentially useful for the generation of new molecular probes able to covalently bind to proteins, their hydrolytic instability may limit their more general application.

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Footnotes

† Compound **2** was obtained in the reaction of 5′-*O*-DMT-thymidine with *N*,*N*-diisopropylamino-1,3,2-oxathiaphospholane in the presence of tetrazole[8] and subsequent oxidation by elemental selenium. **2** Consists of a mixture of diastereoisomers, δ_{31P} (C_6D_6, 81 MHz) 98.66, 98.68, $^1J_{31P-77Se}$ 960 Hz (for both diastereoisomers). Mass spectrum (+ FAB), *m*/*z* 730.1 (M^+ + 1).

‡ **4a** Consists of a mixture of diastereoisomers, δ_{31P} (CD_3CN, 81 MHz) 50.0, 50.4, $^1J_{31P-77Se}$ 810 Hz (for both diastereoisomers), ratio 46:54.

§ **5a** Was obtained in 73% overall yield after its purification on silica gel column. **5a** Consists of a mixture of diastereoisomers, δ_{31P} (CD_3CN, 81 MHz) 22.4, 22.7, $^1J_{31P-77Se}$ 502 Hz, ratio 58:42, respectively. Mass spectrum (+FAB), *m*/*z* 968.4 ($M^{+\cdot}$ + 1).

¶ **1a** Was isolated from the reaction mixture by the addition of water, evaporation of acetonitrile, and extraction of aqueous emulsion with dichloromethane. The yield of **1a** is 75% (AgF) and 71% ($Et_3NH^+F^-$). **1a** Consists of a mixture of diastereoisomers, δ_{31P} (CD_3CN, 81 MHz) −9.0 ($^1J_{31P-19F}$ 977 Hz), −9.4 ($^1J_{31P-19F}$ = 985 Hz), ratio 1:1. Mass spectrum (+FAB), *m*/*z* 892.3 ($M^{+\cdot}$).

‖ **6a** Was isolated as a mixture of diastereoisomers, δ_{31P} (CD_3CN, 81 MHz) 0.46, 0.30, ratio 1:1. Mass spectrum (+ FAB), *m*/*z* 904.3 ($M^{+\cdot}$).

** **7a** Was isolated and characterized by ^{31}P NMR [δ_{31P} (CD_3CN, 81 MHz) 0.08] and mass spectrometry: (−FAB), *m*/*z* 889.2 ($M^{+\cdot}$).

†† **8a** Was isolated as a mixture of diastereoisomers, δ_{31P}(CD_3CN, 81 MHz) −0.87, −1.04, ratio 10:90. Mass spectrum (+ FAB), *m*/*z* 992.6 ($M^{+\cdot}$ + 1).

‡‡ The presence of **7b** was confirmed by co-injection with the sample made by the phosphoramidite method.

§§ The presence of **6b** was confirmed by co-injection with the sample prepared by the phosphoramidite method.[9]

References

1 W. Dąbkowski, J. Michalski and F. Cramer, *Protocols for Oligonucleotides and Analogs*, ed. S. Agrawal, Humana Press, Totowa, NJ, 1993, p. 245.

2 W. Dąbkowski, J. Michalski, J. Wasiak and F. Cramer, *J. Chem. Soc., Perkin Trans. 1*, 1994, 817; (*b*) W. Dąbkowski, F. Cramer and J. Michalski, *J. Chem. Soc., Perkin Trans. 1*, 1992, 1447; J. Michalski, W. Dąbkowski, A. Łopusiński and F. Cramer, *Nucleosides Nucleotides*, 1991, **10**, 283; W. Dąbkowski, F. Cramer and J. Michalski, *Tetrahedron Lett.*, 1988, **29**, 3301.

3 R. J. P. Corriu, J.-P. Dutheil and G. F. Lanneau, *J. Chem. Soc., Chem. Commun.*, 1981, 101.

4 (*a*) L. Horner and H.-W. Flemming, *Phosphorus Sulfur Relat. Elem.*, 1984, **19**, 345; (*b*) L. Horner and R. Gehring, *Phosphorus Sulfur Relat. Elem.*, 1982, **12**, 295.

5 L. A. Woźniak, B. Krzyżanowska and W. J. Stec, *J. Org. Chem.*, 1992, **57**, 6057.

6 W. J. Stec, G. Zon and B. Uznański, *J. Chromatogr.*, 1985, **326**, 263.

7 K. K. Ogilvie, S. L. Beaucage, N. Theriault and D. W. Entwistle, *J. Am. Chem. Soc.*, 1977, **99**, 1277.

8 W. J. Stec, A. Grajkowski, M. Koziołkiewicz and B. Uznański, *Nucleic Acids Res.*, 1991, **19**, 5883.

9 R. H. Alul, C. N. Singman, G. Zhang and R. L. Letsinger, *Nucleic Acids Res.*, 1991, **19**, 1527.