Studies on enzymatic hydrolysis of thymidin-3′-yl thymidin-5′-yl phosphorofluoridates and the corresponding phosphorothiofluoridates

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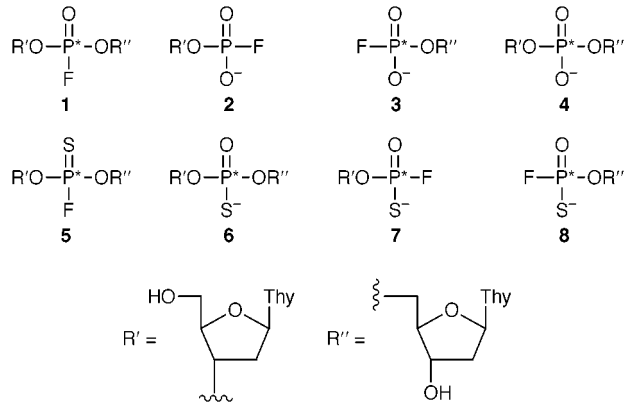
***Spleen* or *snake venom* nuclease-assisted cleavage of P–F bonds in nucleotides bearing a phosphorofluoridate moiety occurs at negatively charged phosphates only.**

Catalytic promiscuity of enzymes has attracted the attention of researchers studying the problem of a common binding site and common mechanistic features in catalysis involving substrates bearing functional groups different from those naturally cleaved by these enzymes.[1] Since such studies may shed light on the evolutionary diversification of enzymes, each example of 'unusual' activity of enzymes requires meticulous scrutiny.

In this respect, the cleavage of the P–O bond in dinucleoside 3′,5′-phosphorofluoridates ($N_{PF}N'$) by *spleen* phosphodiesterase (*spleen* PDE) and *snake venom* phosphodiesterase (*sv* PDE), as reported recently in several papers by Dabkowski *et al.*,[2] attracted our attention. Both enzymes are known as phosphodiester hydrolases that cleave oligonucleotides with natural 3′-5′-phosphodiester linkages to produce the corresponding nucleoside 3′- or 5′-phosphates.[3] These enzymes are known to require the presence of a negative charge at the cleavable phosphate moiety[4] and can also accept as substrate various charged oligonucleoside phosphate analogues. For example, dinucleoside 3′,5′-phosphorothioates ($N_{PS}N'$)[5] are recognised by *snake venom* phosphodiesterase albeit the rate of cleavage of the diastereomers differs significantly. This observed stereodifferentiation has been used for determination of absolute configuration of $N_{PS}N'$[6] and related compounds, *e.g.* ATPαS[7] and thymidine 5′-phosphorothiofluoridate,[8] and also as a practical method of preparing single diastereomers of $N_{PS}N'$ by selective degradation of a pool of chemically synthesised oligonucleoside phosphorothioates[9] In contrast to these, uncharged dinucleotide analogues, *e.g.* dinucleoside alkyl phosphates and dinucleoside methylphosphonates, are not substrates for *sv* and *spleen* PDE, most likely due to the lack of a negative charge at the cleavage site.[10]

In light of the above, the results of Dabkowski *et al.*[2] have to be viewed with caution, although a speculation that the strongly polarised P–F bond could mimic the P–O$^-$ bond cannot be rejected *a priori*. However, the reported data[2] (*viz.* presence of thymidine, thymidine 5′-phosphorofluoridate and unreacted $T_{PF}T$, after 3 h exposure to *sv* PDE in enzymatic buffer solution) seemed to be at odds with the hydrolytic instability of $N_{PF}N'$ in buffered media[11] and the fact that nucleoside 5′-phosphorofluoridate monoesters are known to be good substrates for *sv* PDE yielding nucleoside 5′-phosphates as products.[12] These factors prompted us to reinvestigate the problem of the susceptibility of uncharged nucleotide analogues containing the P–F bond towards *snake venom* and *spleen* phosphodiesterases, and as model compounds for our studies we chose a dinucleoside 3′,5′-phosphorofluoridate and the corresponding phosphorothiofluoridate.

Dithymidine 3′,5′-phosphorofluoridate **1** (mixture of two diastereomers, *ca.* 1:1 ratio) was obtained by two independent methods[11,13] and isolated by means of silica gel chromatography.† The reference compounds, thymidine 3′-phosphorofluoridate **2** and thymidine 5′-phosphorofluoridate **3** were obtained either in DBU-assisted reactions of protected thymidine 3′- or 5′-(2-oxo-3-oxa-1-thia-2-phosphaspiro[4.5]decane)s[14] with triethylamine trishydrofluoride,‡ or independently, in reactions of pyridine solutions of suitably protected thymidine hydrogen 3′- or 5′-phosphonate with trimethylsilyl chloride, followed by treatment with iodine and triethylamine trishydrofluoride.[15]



The hydrolytic stability of phosphorofluoridate **1** was checked in various buffer solutions using an RP-HPLC assay.§ It was found that compound **1** (0.1 mM) dissolved in 0.05 M acetate buffer (pH 5.0) (*spleen* PDE buffer) at 37 °C was quantitatively hydrolysed within 5 min providing dithymidine 3′,5′-phosphate **4**. The same results were obtained using 0.1 M Tris-HCl (pH 8.0) containing 20 mM $MgCl_2$ (*sv* PDE buffer) as medium. Incubations performed in a presence of *spleen* PDE¶ (0.15 U ml^{-1}) and *sv* PDE¶ (0.005 U ml^{-1}), using the same buffer systems as for the blank experiments, in both instances resulted in formation of phosphodiester **4** only (5 min, no starting material present) without detectable amounts of phosphorofluoridate **2** and **3**, respectively (RP-HPLC analysis using genuine samples of **2**, **3** and **4**). Prolonged incubation times (20, 40 and 60 min) led to a gradual hydrolysis of the initially formed phosphodiester **4** to produce thymidine and thymidine 3′- or 5′-phosphate, respectively. In separate experiments we found that rates of enzymatic hydrolysis (*sv* and *spleen* PDE) of pure phosphodiester **4** and that produced *in situ* from phosphorofluoridate **1** were identical, indicating that fluoride anion neither accelerated nor inhibited the activity of these phosphodiesterases.

We also confirmed earlier observations by Wittmann[12] and others[12] that products of a putative enzymatic hydrolysis of phosphorofluoridate **1** claimed by Dabkowski *et al.*,[2] *viz.* compounds **2** or **3**, are substrates for phosphodiesterases. We found that phosphorofluoridate **2** was hydrolysed by *spleen* PDE slower than phosphodiester **4**, and thus if **1** were a substrate for this enzyme (cleavage of the P–O bond), transient formation of **2** would be anticipated. In the instance of *sv* PDE, we could not exclude a remote possibility that phosphorofluoridate diester **1** underwent slow enzymatic hydrolysis with

P–O bond cleavage to produce thymidine 5′-phosphorofluoridate **3**, since the latter was found to be a better substrate for the enzyme than phosphodiester **4**.

A diastereomeric mixture of dithymidine 3′,5′-phosphorothiofluoridate **5** (ratio *ca.* 1:1)[8,13] was also examined as a potential substrate for *spleen* and *snake venom* phosphodiesterases. Compound **5**‖ was found to be significantly more hydrolytically stable than its oxo congener **1** in the buffer solutions used (*vide supra*) and required *ca.* 1 h at 37 °C for complete hydrolysis to produce dithymidine 3′,5′-phosphorothioate **6**. Attempted enzymatic hydrolysis of **5** was performed using a 10× higher concentration of *spleen* and *sv* PDE than those used in experiments with phosphorofluoridate **1**. It was found that during incubations of **5** with *spleen* and *sv* PDE no trace of products resulting from P–O bond cleavage, *i.e.* thymidine 3′-*O*-phosphorothiofluoridate **7**[8,15] or 5′-phosphorothiofluoridate **8**,[8,15] respectively, could be detected (RP-HPLC assay). After 1 h, the only product present was phosphorothioate diester **6** (diastereomeric ratio *ca.* 1:1) that upon prolonged enzymatic incubation (samples analysed after 2, 4 and 8 h) yielded gradually thymidine and thymidine 3′- or 5′-phosphorothioate, respectively, as the sole products. For the reactions performed in the presence of *sv* PDE, as expected, a stereoselective digestion of (R_P)-**6** was observed.[6] Since in our earlier studies[8] we demonstrated that (R_P)-phosphorothiofluoridate **8** is a good substrate for *sv* PDE one should expect the accumulation of (S_P)-phosphorothiofluoridate **8** in the reaction mixture, if an enzymatic cleavage of the P–O bond in **5** produced both diastereomers of **8**.

In conclusion, the presented results on enzymatic hydrolysis of dithymidine 3′,5′-phosphorofluoridate **1** and dithymidine 3′,5′-phosphorothiofluoridate **5** by *spleen* and *snake venom* phosphodiesterases indicate that these compounds first undergo chemical hydrolysis to dinucleotides **4** and **5**, respectively, which are subsequently hydrolysed enzymatically to the corresponding nucleoside and nucleotide derivatives. The released fluoride anion under the reaction conditions did not inhibit the nucleolytic activity of the investigated enzymes.

These studies support the earlier findings that *sv* and *spleen* PDE accept as substrates negatively ionised phosphate derivatives and thus the enzymatic hydrolysis of phosphorofluoridate diesters by these enzymes reported by Dabkowski *et al.*[2] could not be confirmed.

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Notes and references

† *Selected data* for **1**: δ_P(CD_3OD, 121 MHz) − 9.5 and −10.1 ($^1J_{PF}$ 971, 980); δ_F(CD_3OD, 282 MHz) −80.6 and −81.2 ($^1J_{FP}$ 973, 984); δ_H (CD_3OD, 300 MHz) 6.27 (2H, m, H-1′, two anomeric protons), 5.24 (2H, m, H-3′).

‡ *Syntheses and selected data* for **2** and **3**: 5′-*O*-dimethoxytritylthymidine 3′-*O*-(2-oxo-3-oxa-1-thia-2-phosphaspiro[4.5]decane) (ref. 14) was dissolved in dry MeCN. To this solution a 1 M solution of triethylamine trishydrofluoride in dry THF (1 equiv.) and DBU (2 equiv.) were added. After 15 min the reaction mixture was concentrated and redissolved in 80% AcOH and left for 1 h. Crude **2** was purified by DEAE-Sephadex chromatography using 0.05 to 0.5 M $Et_3NH^+HCO_3^-$ buffer (pH 7.5) as an eluent. Compound **2** was obtained in 65% yield: δ_P(D_2O, 81 MHz) −5.60 ($^1J_{PF}$ 932); δ_F(D_2O, 188 MHz) −80.7 ($^1J_{PF}$ 932); *m/z* (−FAB) 323.1 (M − H). Synthesis of **3** was performed in the same way starting from 3′-*O*-methoxyacetylthymidine 5′-*O*-(2-oxo-3-oxa-1-thia-2-phosphaspiro[4.5]-decane) and using conc. ammonia for deprotection (yield, 20%): δ_P(D_2O, 81 MHz) −6.29 ($^1J_{PF}$ 933); δ_F(D_2O, 188 MHz) −77.7 ($^1J_{PF}$ 934); *m/z* (−FAB) 323.1 (M − H).

§ Conditions for HPLC analysis: Econosphere C18 column; buffer A, 0.1 M NH_4OAc; buffer B, 0.04 M NH_4OAc in 80% MeCN; gradient up to 60% buffer B in 15 min; flow 1 ml min^{-1}.

¶ *Spleen* and *sv* PDE were from Sigma (P-6897 and P-6877, respectively).

‖ *Selected data* for **5**: δ_P(CD_3OD, 121 MHz) 61.9 and 61.2 ($^1J_{PF}$ 1071, 1082); δ_F(CD_3OD, 282 MHz) −44.9 and −45.0 ($^1J_{F\text{-}P}$ 1072, 1084); δ_H(CD_3OD, 300 MHz) 6.27 (2H, m, H-1′, two anomeric protons), 5.31 (2H, m, H-3′).

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