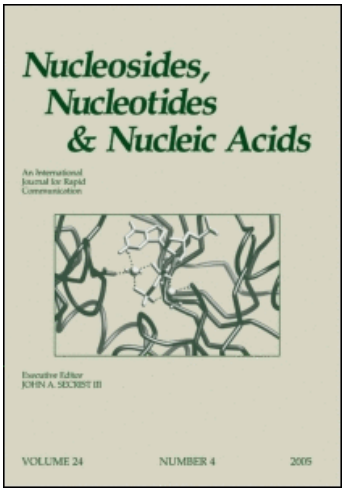

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Janina Baraniak[a]; Dariusz Korczynski[a]; Renata Kaczmarek[a]; Wojciech J. Stec[a]
[a] Department ofBioorganic Chemistry, Polish Academy of Sciences, Centre of Molecular and Macromolecular Studies, Lodz, Poland

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TETRA-THYMIDINE PHOSPHOROFLUORIDATES *VIA* TETRA-THYMIDINE PHOSPHOROSELENOATES: SYNTHESIS AND STABILITY

Janina Baraniak, Dariusz Korczynski, Renata Kaczmarek and Wojciech J.Stec[*†]
Polish Academy of Sciences, Centre of Molecular and Macromolecular Studies, Department of Bioorganic Chemistry, Sienkiewicza 112, 90-363 Łódź, Poland

ABSTRACT: Tetra-thymidine phosphoroselenoates obtained *via* phosphoramidite methodology with bis(di-*O,O*-isopropyl phosphinothioyl)diselenide as oxidising reagent, under treatment with iodine and triethylamine tris(hydrofluoride) (TAF) provide a diastereomeric mixture of tetra-thymidine phosphorofluoridates whose hydrolytic stability was studied by HPLC.

INTRODUCTION

Dinucleotides containing an internucleotide 3',5'-phosphorofluoridate linkage ($N_{PF}N'$) were obtained for the first time from the reaction of dithymidine 3',5'-phosphorothioates with 2,4-dinitrofluorobenzene.[1] Conversion of diastereomerically pure $T_{PS}T$ into $T_{PF}T$ appeared to be a non-stereospecific process. Dinucleoside phosphorofluoridates were insufficiently stable under the conditions of RP-HPLC isolation because of the rapid hydrolysis of the P-F bond in acetonitrile/TEAB buffer. Their reaction with ethanol in the presence of triethylamine provided a mixture of diastereomers of dithymidine *O*-ethyl phosphate ($T_{POEt}T$). Recently, several reports[2] aimed at the synthesis of oligonucleotide phosphofluoridates and limited to the synthesis of dinucleoside 3',5'-phosphorofluoridates have been published. The chemical properties of dinucleoside 3',5'-phosphorofluoridates, and especially their stability under solvolytic conditions, has become a matter of some controversy.[3,4] Moreover, evidence has been provided that dinucleoside 3',5'-

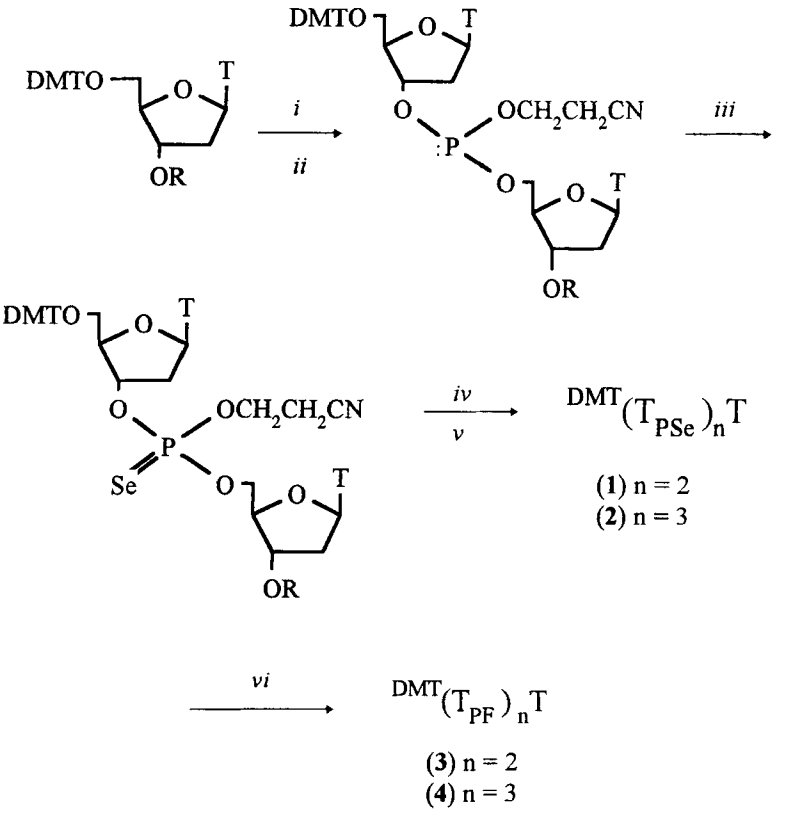
† E-mail: wjstec@bio.cbmm.lodz.pl

phosphorofluoridates undergo cleavage in the presence of phosphodiesterases furnishing the corresponding nucleoside and a nucleoside phosphorofluoridate,[5] notwithstanding earlier reports from two laboratories indicating that nucleoside phosphorofluoridates are good substrates for these enzymes.[6] Most recently, Stawiński reported a new and simple method of synthesis of dinucleoside phosphorofluoridates relying upon the reaction of a dinucleoside phosphorothioate (or phosphoroselenoate) with iodine in the presence of NEt3x3HF (TAF).[7] This elegant methodology permits the introduction of the fluorine atom into an unprotected oligonucleotide and in this way the problem of degradation of a modified oligonucleotide during the deprotection step seems to be avoided. However, only dithymidine phosphorofluoridates isolated from the reaction mixture by silica gel chromatography were described by Stawiński *et al.* and attempts at the synthesis of longmers were not reported.

RESULTS AND DISCUSSION

Because of our interest in oligonucleotide analogues of potential biological application we decided to prepare oligonucleotide phosphorofluoridates longer than $T_{PF}T$ and to check their hydrolytic stability by HPLC.

In this paper we describe the synthesis of tri- and tetrathymidines containing a phosphorofluoridate linkage at each internucleotide bond using as substrates the corresponding tri- and tetra-thymidine phosphoroselenoates (**1,2**). Phosphoroselenoate oligonucleotides themselves are not promising compounds with respect to antisense strategy because of their toxicity to cells.[8] However, phosphoroselenoates offer a possibility of replacing selenium with other functionalities. Tri- and tetra-thymidine phosphoroselenoates (**1, 2**) were prepared *via* phosphoramidite coupling followed by selenisation by means of a new selenium-transfer reagent, namely bis(di-*O,O*-isopropyl phosphinothioyl)diselenide (**5**).[9] So far, elemental selenium,[10] potassium selenocyanate,[11] or 3H-1,2-benzothiaselenol-3-one[12] have been reported as a source of electrophilic selenium for selenisation of P(III) derivatives. As presented here, reagent (**5**) was readily obtained after oxidation of di-*O,O*-isopropyl phosphoroselenothioate by means of an aqueous solution of iodine/potassium iodide. This process was fast and provided (**5**) in quantitative yield. After crystallisation from ethanol, (**5**) (m.p. 86-88°C) can be stored for months in closed, dark containers without any decomposition and is soluble in acetonitrile,



SCHEME 1. *Reagents and conditions:* R = succinyl CPG; *i*, 3% $CHCl_2COOH$ in CH_2Cl_2; *ii*, 5'-*O*-DMT-T-3'-*O*-P(N*i*Pr$_2$) (OCH_2CH_2CN), 1-H-tetrazole in CH_3CN; *iii*, [(*i*PrO)$_2$P(S)Se]$_2$ (**5**) in pyridine; *iv*, repetition of steps *i-iii*; *v*, NH_4OH; *vi*, I_2, NEt_3x3HF.

acetone, isopropanol, or pyridine. A pyridine solution of (**5**) (0.5 M) has been used for the selenisation process, which is complete within 3 min. The 5'-*O*-DMT-$T_{PSe}T_{PSe}T$ (**1**) and 5'-*O*-DMT-$T_{PSe}T_{PSe}T_{PSe}T$ (**2**) were obtained in 96% stepwise yields (DMT cation assay) and were cleaved from the solid support by treatment with ammonia (SCHEME 1).

The identity of both compounds, obtained as the mixture of diastereomers, was confirmed by MS-MALDI. It is important to note that besides the selenium-transfer process a sulfur-transfer also occurs. Although we were unable to detect any product with an internucleotide phosphorothioate function in the HPLC-profile, analysis of MS-MALDI spectra of crude compounds (**1**) and (**2**) showed a very low intensity of ions derived from compounds containing one P-S bond (e.g. DMT-$T_{PSe}T_{PS}T$ - MW 1232 and DMT-

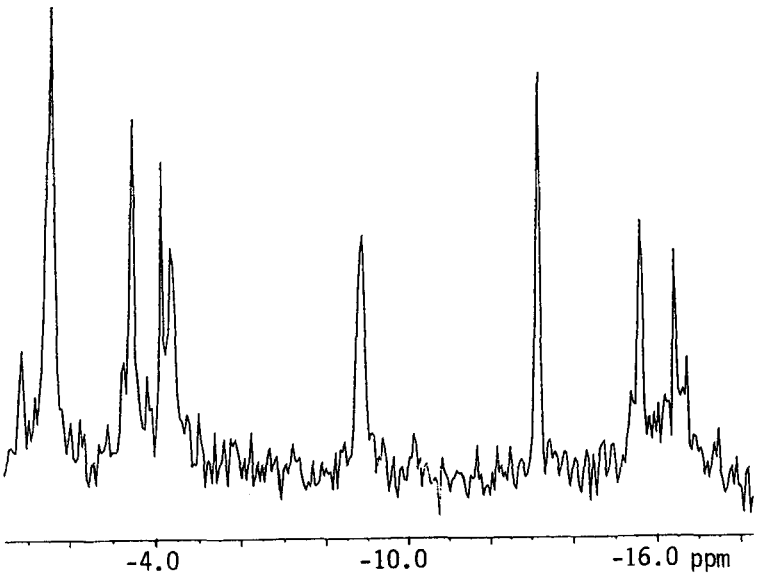


FIG. 1. ^{31}P NMR of 5'-*O*-DMT-$T_{PF}T_{PF}T$ (3)

$T_{PSe}T_{PSe}T_{PS}T$ - MW 1599, respectively).[13] However, those compounds did not interfere with formation of the target oligonucleotide phosphorofluoridates, since under the experimental conditions the internucleotide P-S bond also undergoes conversion into the phosphorofluoridate.[7]

Without isolation, compounds (1) and (2) were converted into the corresponding phosphorofluoridates (3) and (4) by a treatment with NEt_3 (2 eq) and TAF (1 eq) followed by iodine (2.5 eq), respectively.[7] After 10 min the reaction mixtures were diluted with $CHCl_3$ and washed with a 10% solution of $Na_2S_2O_3$. The organic phase was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The 5'-*O*-DMT-$T_{PF}T_{PF}T$ (3) was studied by ^{31}P NMR (FIG. 1).

As expected compound (3) consists of a mixture of four P-diastereomers as seen by the ^{31}P NMR doublets: δ ($CDCl_3$) -9.00 ($^1J_{PF}$ 937 Hz), -9.54 ($^1J_{PF}$ 982 Hz), -10.28 ($^1J_{PF}$ 994 Hz), -10.51 ($^1J_{PF}$ 1000 Hz).

Attempts were made to isolate compounds (3) and (4) by means of RP-HPLC with TEAB (pH 7.5) or NH_4OAc (pH 7.2) buffers in acetonitrile gradient. Although compound (3) was isolated as a single peak (TEAB as the buffer: R_T 24.1 min), RP-HPLC trace of collected

fractions after lyophilization has shown the presence of $T_{PO}T_{PO}T$ (co injection with a genuine sample, R_T 10.7 min; 67%) and unhydrolysed (3) (13.4%), as was additionally proved by MS-MALDI analysis (m/z 849 and m/z 1155, respectively). Apparently, during lyophilization and repeated RP-HPLC analysis and acidification of the sample, some DMT-protecting group removal had occurred.

Similarly, (4) was obtained from (2) and was subjected to RP-HPLC analysis. In this case we chose NH_4OAc as a buffer and, after lyophilization, (4) was rechromatographed in the same buffer gradient showing the presence of $T_{PO}T_{PO}T_{PO}T$ (R_T 12.1 min; 54.6%), $T_{PF}T_{PF}T_{PF}T$ (R_T 22.5 min; 11.4%) and DMT-$T_{PF}T_{PF}T_{PF}T$ (R_T 29.7 min; 10%). Attempts at stabilisation of DMT (3) and (4) *via* addition of triethylamine to a buffered solution of (3) or (4) caused accelerated hydrolysis of the P-F bond.

The above results clearly indicate that internucleotide phosphorofluoridate linkage undergoe fast hydrolysis under the conditions of purification *via* RP-HPLC. This precludes the use of oligonucleotide phosphorofluoridates in an antisense strategy.[14] where integrity of the oligonucleotide construct and its stability under physiological conditions are essential conditions for reliable biological experiments.[15]

EXPERIMENTAL

General. Nuclear magnetic resonance spectra were recorded on a Bruker AC-200 instrument (81 MHz, 85% H_3PO_4 as the external standard for ^{31}P). MALDI mass spectra were recorded on a Voyager-Elite instrument (PerSeptive Biosystems Inc.). Acetonitrile, DBU, and triethylamine trihydrofluoride (TAF) were supplied by Aldrich. Acetonitrile for solid phase synthesis was dried over P_2O_5 (5 g L^{-1}) and distilled through a Vigreux column. HPLC analyses were performed on a Waters Millenium liquid chromatography system with a Supelco LC-18 column (2.1 x 250 mm), buffers TEAB (pH 7.5) and NH_4OAc (pH 7.2).

Bis(di-*O,O*-isopropyl phosphinothioyl)diselenide (5)

Aniline (9.3g; 100 mmol) was added to a benzene solution of di-*O,O*-isopropyl phosphorochloridite (9.2g; 50 mmol) and elemental selenium (2g; 25 mmol). The reaction mixture was left at r.t. for 1 h then an excess of selenium and aniline hydrochloride were filtered off and solvent evaporated. The crude product, di-*O,O*-isopropyl phosphoroseleno-

anilidate [δ ^{31}P (CD_3CN) 63.5 ppm, $^1J_{PSe}$ 895 Hz] was purified by column chromatography on silica gel (0→10% methanol in chloroform) in 75% yield. Then, the phosphoroseleno-anilidate (3.2g; 10 mmol) was dissolved in anhydrous acetonitrile under argon atmosphere and DBU (2.1g; 14 mmol) followed by CS_2 (5 ml) were added. Stirring was continued for 3 h, volatile solvents were evaporated under reduced pressure, and the crude DBU salt of di-*O,O*-isopropyl phosphoroselenothioate was dissolved in benzene (50 ml). Iodine (1.2g; 5 mmol) in aqueous potassium iodide was added dropwise to this solution at 45°C. Stirring was continued for 2 h and the mixture was left overnight at r.t. The organic phase was separated, washed with aqueous sodium thiosulfate and water, and dried over anhydrous $MgSO_4$. After evaporation of solvent, diselenide (**5**) was isolated in 80% yield (2.1 g) by crystallisation from ethanol [m.p. 86-88°C, δ ^{31}P (C_6D_6) 69.7 ppm, $^1J_{PSe}$ 501 Hz].

Single cycle for the solid-phase synthesis of 5'-*O*-DMTT$_{PSe}$T$_{PSe}$T (1) and 5'-*O*-DMT-T$_{PSe}$T$_{PSe}$T$_{PSe}$T (2). Reactions were carried out manually by syringe technique using LCA-CPG support loaded with 1 mmol of nucleoside placed in commercially available columns. A single cycle of chain elongation was as follows:

a) detritylation; 3% dichloroacetic acid in methylene chloride (5 ml);

b) wash; acetonitrile (5 ml);

c) coupling step; 0.3 M 1-H tetrazole solution in acetonitrile (20-fold molar excess) and 0.3 M 5'-*O*-DMT-thymidine 3'-[*O*-(2-cyanoethyl) *N,N*-diisopropylphosphoramidite] solution in acetonitrile; coupling time 15 min;

d) wash; acetonitrile (5 ml);

e) capping; acetic anhydride (DMAP) 2,6-lutidine/tetrahydrofuran (0.15 ml, 2 min);

f) wash; acetonitrile (5 ml);

g) selenisation step; 0.5 M solution of (**5**) in pyridine (8-fold molar excess); time 3 min;

h) wash; acetonitrile (5 ml).

Cleavage from the support was performed by treatment with ammonia at 55°C for 2 h. MS-MALDI: compound (**1**) m/z 1279; compound (**2**) m/z 1646.

Conversion of (1) and (2) into corresponding phosphorofluoridates (3) and (4). Compound (**1**) (1 mmol) was rendered anhydrous by repeated evaporation with dry acetonitrile and dissolved in the same solvent (100 ml) containing triethylamine (9 ml) and

TAF (5 ml). Iodine in acetonitrile (15 ml of 0.2 M solution) was then added. After 10 min the reaction mixture was diluted with chloroform (100 ml) and extracted with aq. 10% $Na_2S_2O_3$ (50 ml). The organic phase was dried over Na_2SO_4, then concentrated *in vacuo* and the residue (**3**) was analysed by HPLC chromatography. In the same way compound (**2**) was transformed into (**4**).

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