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The pro-oligonucleotide approach: solid phase synthesis and preliminary evaluation of model pro-dodecathymidylates

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ABSTRACT

A modified phosphoramidite method has been designed for the solid-phase synthesis of two dodecathymidine phosphotriesters and two dodecathymidine thionophosphotriesters. In these analogs, each internucleoside link bears an S-acyl-2-thioethyl (Me-SATE or tBu-SATE) group removable upon esterase activation. Efficient synthesis of these lipophilic analogs was achieved thanks to the use of a photolabile linker anchored to the solid support in combination with thymidine-3'-O-phosphoramidites having a SATE group in place of the regular 2-cyanoethyl one. Both dodecathymidine phosphotriester and thionophosphotriester having S-acetyl-2-thioethyl groups were found to be stable in the presence of snake venom and calf spleen phosphodiesterases whereas, upon incubation in CEM cell extracts, they were selectively hydrolyzed to the anionic parent dodecathymidylate and dodecathymidine phosphorothioate, respectively. In addition, Me-SATE-protected dodecathymidine thionophosphotriester was stable in mouse and human sera as well as in human gastric juice. These results depict the potential of SATE-protected oligonucleotides as prodrugs of antisense oligonucleotides.

INTRODUCTION

Oligonucleotide (ON)-based therapy promises to be a highly specific tool for the treatment of numerous human diseases. However, to date the effectiveness of ONs has been limited due to several problems such as instability in serum, inability to reach their target site because of non-specific disposition, poor cell penetration and adverse pharmacokinetics (PK). Such disadvantages have stimulated efforts to prepare neutral or charged backbone modified oligonucleotides but the previous limitations have been only partly solved [for a general discussion, see (1) and other related chapters in the book].

The shortcomings in the delivery of ONs are likely to be connected with their anionic charges at physiological pH. The chemical derivatization of the internucleoside linkages with

enzymolabile protecting groups will conduct to neutral phosphotriester prodrugs, [the pro-oligonucleotides (pro-oligo)] which could be broken down in the body to release the parent drug (the ON). Application of this prodrug concept to various phosphorylated drugs is well established (2) but has never been envisaged for ONs before our preliminary presentation at the Cambridge Meeting on Synthetic Oligonucleotides and Analogues in 1993. But before releasing the ON, the lipophilic pro-oligo may present a completely different behaviour ranging from increased nuclease stability, low non-specific proteins interactions to more favourable pharmacokinetic/pharmacodynamic properties.

Therefore, to evaluate this concept we first had to select a suitable enzymolabile phosphodiester protecting group and to set the corresponding chemical approach in order to obtain some pro-oligo models, and then to gain information on their behaviour.

We previously introduced the enzymolabile SATE (3) phosphate protecting group on mononucleotide and this approach conducted to highly potent *in vitro* antiviral agents [for a review see (4)]. It was further shown that the SATE group is selectively removed *in vitro* (5) as well as *in vivo* (Somadossi *et al.*, unpublished results) upon esterase activation according to the mechanism shown in Figure 1. After preliminary comparative stability studies on various dinucleoside phosphotriesters, the SATE protecting group was also selected for the pro-oligo approach (6,7).

Then, to validate the pro-oligo concept, several oligomers (gapmers) were evaluated (8–11) for their stability in various biological media (culture medium, cell extract, human serum, gastric juice). These gapmers were obtained through a post-synthesis alkylation procedure (10). This approach was limited to the obtention of phosphorothiolate gapmer as phosphorothioate diesters are more nucleophilic than phosphate diesters. In addition to medium yields, the alkylation rate was slow, side reactions occurred and only a gapmer containing three SATE groups was obtained. Upon incubation in total cell extract, this compound was shown to selectively reverse back to the parent oligo (10).

As the SATE pro-oligos could not be reasonably obtained through the post-oligo synthesis approach, we decided to synthesize them using an automated strategy. Pro-oligos, being phosphotriester (or thionophosphotriester) derivatives, are sensitive

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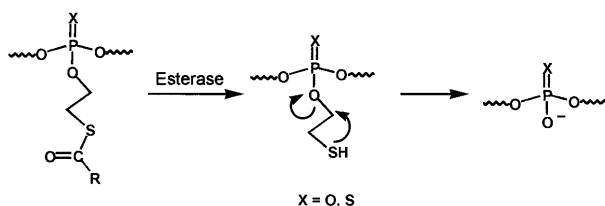


Figure 1. Esterase-mediated removal of internucleoside *S*-acyl-(2-thioethyl) masking group.

to base or nucleophile treatments, therefore a completely new strategy had to be set for their solid phase synthesis. More generally we had to consider: (i) the obtention of the necessary SATE phosphoramidite synthons; (ii) non-basic and non-nucleophilic cleavable solid support; (iii) nucleobase amino protecting groups which could be readily removed under non-basic and non-nucleophilic conditions. We recently described the synthesis of a new photolabile cleavable solid support (12) which is compatible with the above conditions (point ii). To rapidly evaluate the potentiality of the pro-oligo approach we decided, in a first step, to synthesize thymidine-containing oligomers, which avoids base protection (point iii).

This paper is therefore devoted to solid phase synthesis and to the preliminary evaluation of some model SATE pro-dodecathymidylates. We will thus show that this approach may represent an interesting alternative to overcome some of the above mentioned shortcomings.

MATERIALS AND METHODS

Reagents

All commercial chemicals were reagent grade and were used without further purification except where otherwise stated. Bis(diisopropyl-amino)chlorophosphine, tetrazole ($\geq 99\%$) and redistilled *N,N*-diisopropylethylamine were from Aldrich (St Quentin Fallavier, France). Methylene chloride was dried over P_2O_5 followed by distillation. DNA synthesis reagents, except oxidizers, were from Perseptive Biosystems Ltd (Voisins le Bretonneux, France). Anhydrous *tert*-butyl hydroperoxide (3 M in toluene) was from Fluka and was diluted with anhydrous methylene chloride (Aldrich). 3*H*-1,2-Benzodithiole-3-one-1,1-dioxide (Beaucage Reagent) was a gift from Isis Pharmaceuticals (Carlsbad, CA).

RPMI 1640 and inactivated fetal calf serum were purchased from Gibco-BRL (Cergy Pontoise, France). CEM cell extracts were kindly prepared by Dr A.-M. Aubertin (University of Strasbourg, France) (13). Human serum was a gift from the Centre Régional de Transfusion Sanguine (Montpellier, France). Pig liver esterase (200 U/mg) and calf intestine alkaline phosphatase (82 U/mg) were purchased from Aldrich whereas snake venom phosphodiesterase (1.5 U/mg) and calf spleen phosphodiesterase (2 U/mg) were from Boehringer Mannheim (Meylan, France). Human serum was obtained from a healthy volunteer and mouse serum was a gift from Isis Pharmaceuticals. Human gastric juice was a gift from Dr J.-C. Cuber (INSERM U-45, Lyon, France).

Analytical and preparative methods

Thin-layer chromatography was run on precoated silica gel plates (Kieselgel 60F₂₅₄, Merck) and preparative silica gel flash

chromatography was performed using Kieselgel 60 (Merck, art. 9385). 1H -NMR spectra were recorded on a Bruker DRX 400 at 400 MHz in CD_3CN or CD_2Cl_2 . Chemical shifts (δ) are expressed in parts per million relative to the central line of CHD_2CN or $CHDCl_2$ set at 1.94 and 5.32 p.p.m., respectively. The signals are described as: s, singlet; d, doublet; t, triplet; m, multiplet. ^{31}P -NMR spectra were recorded on either a Bruker AC 250 or a Bruker DRX 400 relative to 85% phosphoric acid as an external standard. Fast-atom bombardment (FAB) and mass spectra (MS) were recorded on a Jeol DX 300 spectrometer. MALDI-TOF mass spectra were recorded on a Voyager (Perseptive Biosystems, Framingham, MA, USA) mass spectrometer equipped with an N_2 laser. A 0.5 A₂₆₀ unit sample was mixed with 1.0 ml of an acetonitrile solution saturated with sinapinic acid and a 25 μ l portion of this mixture was placed on a plate and dried at ambient temperature and pressure. MALDI-TOF spectra were obtained by averaging data from 5–15 laser shots.

High-performance liquid chromatography (HPLC) analyses were performed on a Waters-Millipore instrument equipped with a Model 600E solvent delivery system, a Model U6K injector and a Model 486 absorbance detector. A reverse-phase C₁₈ Nucleosil (5 μ m) column (150 \times 4.6 mm, Macherey-Nagel, Germany) was used at a flow rate of 1 ml/min. Products formed upon incubation in biological media were analysed using an improved 'on-line cleaning' technique (5).

Synthesis of thymidine 3'-*O*-(*S*-acyl-2-thioethyl) phosphoramidites (2)

5'-*O*-(4,4'-Dimethoxytrityl)-thymidine 3'-*O*-[(*S*-acetyl-2-thioethyl) *N,N*-diisopropylphosphoramidite] (2a). To a cooled (ice-bath) and magnetically stirred solution of *5'*-*O*-(4,4'-dimethoxytrityl)-thymidine (8.49 g, 15.6 mmol) and *N,N*-diisopropylethylamine (3.26 ml, 20.3 mmol) in dry CH_2Cl_2 (100 ml), was added dropwise over 8 min, a solution of bis(*N,N*-diisopropylamino)-chlorophosphine (5 g, 18.7 mmol) in dry CH_2Cl_2 (18 ml). The reaction mixture was allowed to warm to room temperature while stirring was maintained (30 min). *S*-(2-Hydroxyethyl) thioacetate (5) (2.195 g, 18.17 mmol) was added followed by (portionwise) the addition, over 15 min, of solid tetrazole (0.546 g, 7.8 mmol) and the reaction mixture was stirred for 15 h. Then CH_2Cl_2 (200 ml) was added and the reaction mixture was washed with saturated aqueous sodium hydrogen carbonate (300 ml) and brine (2 \times 300ml) and dried over anhydrous sodium sulfate. The residue obtained after evaporation under reduced pressure of the organic layer was purified on a silica gel column prepared with 1% (v/v) triethylamine in cyclohexane and washed with cyclohexane (1 bed volume) before the products were deposited. Elution was performed with a stepwise gradient of ethyl acetate (10–40%, v/v) in cyclohexane, and fractions containing the desired product were combined and evaporated to dryness. The residue was lyophilized from benzene affording a colourless powder (8.4 g). Yield 68% as a diastereoisomeric mixture. FAB-MS (3-nitrobenzyl alcohol): m/z 690 [M-[CH₃-C(O)S-C₂H₄]⁻], 792 [M-H]⁻, 946 [M+NBA-H]⁻, 1585 [2 M-H]⁻. ^{31}P -NMR (CD_3CN): δ 148.9 and 148.6. 1H -NMR (CD_3CN): δ 1.02–1.2 [m, 12 H, CH(CH₃)₂]; 1.48 and 1.5 (2 d, 3 H, CH₃-5); 2.26 and 2.28 (2 s, 3 H, CH₃CO); 2.32–2.4 (m, 2 H, H2' and H2''); 2.93–3 (m, 2 H, CH₂-CH₂-S); 3.28–3.38 (m, 2 H, H5' and H5''); 3.51–3.62 [m, 4 H, CH₂-CH₂-O and CH(CH₃)₂]; 3.75 (s, 6 H, Ar-OCH₃); 4.05 and 4.1 (2 m, 1 H, H4'); 4.61 (m, 1 H, H3');

6.24 (m, 1 H, H1'); 6.8–7.49 (m, 14 H, ArH and H6); 9.08 (broad s, 1 H, NH).

5'-O-(4,4'-Dimethoxytrityl)-thymidine 3'-O-[(S-pivaloyl-2-thioethyl)N,N-diisopropylphosphoramidite] (2b). This compound was obtained as described above by substituting *S*-(2-hydroxyethyl) thiopivaloate (5) (3.034 g, 10.2 mmol) for *S*-(2-hydroxyethyl) thioacetate. Yield (7.2 g) 55% as a diastereoisomeric mixture. FAB-MS (3-nitrobenzyl alcohol): m/z 690 [M-(*tert*Bu-C(O)S-C₂H₄)⁻], 834 [M-H]⁻, 988 [M+NBA-H]⁻, 1669 [2 M-H]⁻. ³¹P-NMR (CD₃CN): δ 148.9 and 148.4. ¹H-NMR (CD₃CN): δ 1.02–1.23 [m, 21 H, CH(CH₃)₂ and C(CH₃)₃]; 1.48 and 1.5 (2 d, 3 H, CH₃-5); 2.19 and 2.37 (2 m, 2 H, H2' and H2''); 2.92 and 3.03 (2 m, 2H, CH₂-CH₂-S); 3.31 (m, 2 H, H5' and H5''); 3.48–3.69 [m, 4 H, CH₂-CH₂-O and CH(CH₃)₂]; 3.75 (s, 6H, Ar-OCH₃); 4.07 (m, 1 H, H4'); 4.6 (m, 1 H, H3'); 6.24 (m, 1 H, H1'); 6.84–7.5 (m, 14 H, ArH and H6); 9.38 (broad s, 1 H, NH).

Solid-phase synthesis of pro-dodecathymidylates

Photolabile CPG solid support (12) (65 μ mol/g) was used and pro-oligonucleotides were assembled on a 10 μ mol scale on a Model 381A (Applied Biosystems Inc.) DNA synthesizer. Nucleoside amidite **1a** and **1b** solutions (0.1 M) in anhydrous acetonitrile were used and during each coupling step, a 10-fold molar excess was introduced in the column. A modified '10 μ mol' cycle was applied and modifications included an extended coupling time (180 s 'wait' for the first four coupling steps and thereafter 30 s 'wait' for the next ones) as well as an extended capping time (300 s 'wait') for the first capping step. A solution of *tert*-butyl hydroperoxide (1.1 M, obtained from commercially available anhydrous solution in toluene and diluted with the appropriate volume of anhydrous methylene chloride) and a solution of 3*H*-1,2-benzodithiole-3-one-1,1-dioxide (0.05 M, Beaucage Reagent) in acetonitrile were used as oxidizers for the formation of phosphate triester (60 s 'wait') and thionophosphate triester (30 s 'wait') internucleoside linkages, respectively. When the required number of cycles were completed, the column was disassembled and the supported 5'-detritylated pro-oligo was divided into three equal portions. Each portion was suspended in CH₃CN-H₂O (4/1, v/v; 3 ml) within a 1 cm path length quartz cell. The magnetically stirred suspensions were exposed to the Pyrex (2 mm thick) filtered output of a 125 W high-pressure Hg lamp (HPK 125, Philips) for 20 min at 20°C. Glass beads were filtered off and washed with the same solvent mixture (2 \times 1 ml). The combined filtrates were concentrated under reduced pressure and the residue was dissolved in dioxane (5 ml) and lyophilized to afford a colourless powder.

3a. Yield 26.7 mg. ³¹P-NMR (dioxane-D₂O, 5/3, v/v) δ 68.0 (1 P); 0.3 (1 P); -1.09 (10 P). MALDI-TOF MS m/z 4906.1 (calc. 4910.3).

3b. Yield 43 mg. ³¹P-NMR (dioxane-D₂O, 5/3, v/v) δ 68.2 (1 P); 0.14 (1 P); -0.78 (10 P). MALDI-TOF MS m/z 5416.8 (calc. 5415.3).

3c. Yield 27.5 mg. ³¹P-NMR (dioxane-D₂O, 5/3, v/v) δ 68.0 (11 P); 57.3 (1 P). MALDI-TOF MS m/z 5089.7 (calc. 5087.0).

3d. Yield 31.1 mg. ³¹P-NMR (dioxane-D₂O, 5/3, v/v) δ 68.4 (11 P); 57.5 (1 P). MALDI-TOF MS m/z 5586.3 (calc. 5592.0).

Hydrolysis of pro-dodecathymidylates 3a–d in basic media

(i) *In aqueous ammonia*. **3a–d** (2–3 A₂₆₀ U) was dissolved in 30% aqueous ammonia (0.5 ml) and stirred for 15 min at room temperature. Volatile matters were removed under reduced

pressure and the residue was redissolved in water (0.1 ml). To a fraction (0.03 ml) of the resulting solution were added, 0.2 M phosphate buffer (pH 7.3, 0.03 ml) and alkaline phosphatase (1 U). After 15 min incubation at room temperature, the mixture was heated (boiling water bath) for 5 min and products were analysed by reverse-phase HPLC using a linear gradient of acetonitrile (0–25%) in 0.05 M aqueous triethylammonium acetate (pH 7.0) over 35 min.

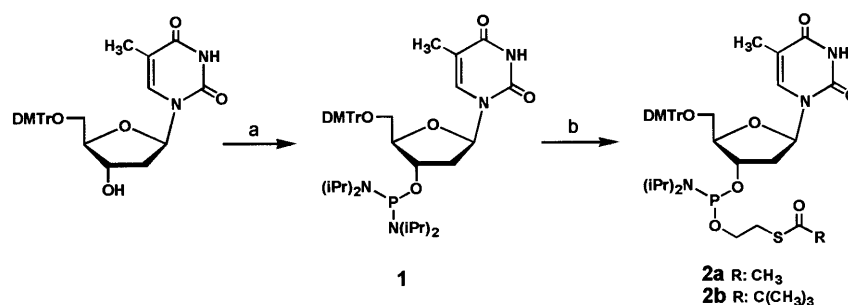
(ii) *In diluted sodium hydroxide*. To a solution of **3a–d** (2–3 A₂₆₀ U) in dioxane (0.05 ml), was added 0.1 M aqueous NaOH (0.5 ml). The reaction mixture was stirred for 1 h (**3a** and **3b**) or for 24 h (**3c** and **3d**) at room temperature before being neutralized by addition of 0.1 M AcOH and analyzed by HPLC as described above.

Hydrolysis of pro-dodecathymidylates 3a–d in presence of purified enzyme or in biological media

Pro-dodecathymidylate **3a–c** (1 A₂₆₀ U in 5% dimethylsulfoxide aqueous solution, 0.1 ml) or **3d** (1 A₂₆₀ U in 10% dimethylsulfoxide aqueous solution, 0.1 ml) was added to a solution of either pig liver esterase (8 U) in 0.2 M potassium phosphate (pH 7.36, 0.9 ml), snake venom phosphodiesterase (0.06 U) in 0.01 M MgCl₂, 0.1 M Tris-HCl (pH 7.3, 0.9 ml) or calf spleen phosphodiesterase (0.08 U) in 0.0025 M EDTA, 0.125 M ammonium acetate (pH 6.8, 0.9 ml) or to biological medium (human serum, mouse serum, human gastric juice or CEM cell extract, 0.9 ml) and incubated at 37°C. At different periods of incubation, aliquots (0.08 ml) were analysed by reverse-phase HPLC.

RESULTS AND DISCUSSION

Since phosphotriesters and esters are sensitive to bases and nucleophiles, the efficient synthesis of pro-oligodeoxynucleotides bearing SATE phosphodiester masking groups requires milder conditions as compared to those used during standard oligonucleotide synthesis. This precludes the use of any solid-support linker or nucleobase protecting group, the cleavage of which necessitates nucleophilic and/or basic conditions. In this regard, we recently developed a photolabile linker, constituted of 1-*o*-nitrophenyl-1,3-propanediol, which was successfully applied to the solid-phase synthesis of dodecathymidine methylphosphotriester (12). A supported oligonucleotide analog in suspension with aqueous methanol, was cleaved upon exposition to the Pyrex-filtered output of a high-pressure Hg lamp for 15 min at 20°C. We are also currently investigating base protecting groups compatible with SATE-phosphotriesters but until now none of the examined protecting groups fulfills the conditions which allow the efficient synthesis of pro-oligodeoxynucleotides by the phosphoramidite method (14). The last requirement was the availability of deoxynucleoside 3'-*O*-phosphoramidites bearing the SATE group in place of the regular 2-cyanoethyl group. The synthesis of 5'-*O*-dimethoxytrityl thymidine 3'-*O*-(*S*-acetyl and pivaloyl-2-thioethyl)phosphoramidites **2a** and **2b** respectively was achieved in two steps from 5'-*O*-dimethoxytrityl thymidine (Scheme 1). This latter compound was reacted first with bis(*N,N*-diisopropylamino)chlorophosphine (1.2 molar equ.) in presence of *N,N*-diisopropylethylamine. The resulting nucleoside 3'-phosphorodiamidite **1** (15) was not isolated but rather reacted with *S*-(2-hydroxyethyl) thioacetate or *S*-(2-hydroxyethyl) thiopivaloate (5) in presence of tetrazole (0.5 molar equ.). After purification by flash chromatography, thymidine 3'-*O*-phosphoramidites bearing



Scheme 1. Synthesis of 5' DMT-thymidine 3'-(SATE)phosphoramidites. (a) Bis(*N,N*-diisopropylamino)chlorophosphine, *N,N*-diisopropylethylamine. (b) *S*-(2-hydroxyethyl) thioacetate or pivaloate, tetrazole.

either a Me-SATE (**2a**) or a tBu-SATE (**2b**) were obtained in 68 and 55% overall yield, respectively. These compounds were fully characterised by FAB-mass spectrometry, ¹H and ³¹P-NMR.

Four different pro-dodecathymidines **3a–d** were synthesized which correspond to two different SATE masking groups i.e., Me-SATE or tBu-SATE and phosphate or thionophosphate triester internucleoside linkages (Fig. 2). It is noteworthy that **3a–d** contained a thionophosphotriester internucleoside link at their 5'-end in order to be eventually labeled with ³⁵S. Since these oligonucleotide analogs were designed for numerous *in vitro* and eventually *in vivo* experiments, relatively large amounts were necessary. Consequently, syntheses were carried out on a 10 μmol scale on a DNA synthesizer using highly loaded photolabile CPG solid support (12). A modified '10 μmol' cycle was applied which included an extended coupling time (180 s) for the first four coupling steps to ensure high coupling yields. *tert*-Butyl hydroperoxide (16,17) and Beaucage reagent (18) were used as oxidizers for the formation of phosphate and thionophosphate triester internucleoside linkages, respectively. After photolysis, reverse-phase HPLC analysis of the crude materials (26–43 mg) indicated satisfactorily homogeneous products. Among the four pro-oligos **3a–d**, the less hydrophobic one was the dodecanucleotide **3a**, containing Me-SATE phosphate triesters and the more hydrophobic one was **3d**, made of tBu-SATE thionophosphate linkages (Fig. 3). In each case a broad peak was obtained which reflects the presence of a diastereoisomeric mixture due to R_P and S_P isomers at each internucleoside linkage. ³¹P-NMR spectra of **3c** and **3d** exhibited two sets of peaks corresponding to internucleoside thionophosphate triesters (δ ~68 p.p.m.) and 3'-terminal thiophosphate diester (δ ~57 p.p.m.) in the expected ratio whereas spectra of **3a** and **3b** presented three sets of peaks corresponding to 5'-thionophosphate triester (δ ~68 p.p.m.), internal phosphate triesters (δ ~1 p.p.m.) and 3'-terminal phosphodiester (δ ~0 p.p.m.). MALDI-TOF mass spectra of crude **3a–d** were in agreement with the expected structure. For compound **3a**, a small peak corresponding to an additional product with a mass 103 U lower than that of **3a** was observed (data not shown). This was assigned to the partial loss of one of the SATE masking groups during the synthesis. A similar result was observed with **3b**, and could be correlated with the presence of a minor peak with a shorter retention time in the HPLC profiles of **3a** and **3b** (Fig. 3A and B). Formation of these minor products was tentatively associated with the use of *tert*-butyl hydroperoxide during the oxidation steps of the solid-phase synthesis of **3a** and **3b** since no corresponding by-products were detected in the mass spectra of **3c** and **3d** the synthesis of which was carried out with Beaucage reagent as oxidizer. In addition, hydrolysis of **3a** with 0.1 M NaOH for 1 h

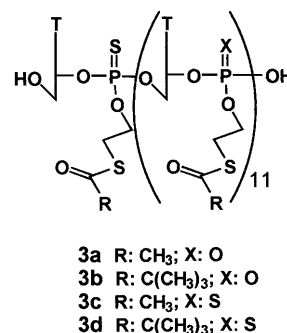


Figure 2. Structure of pro-dodecathymidylates **3a–d**.

yielded only one product (Fig. 3E, lower profile) which corresponds to fully unmasked dodecathymidylate. Hydrolysis of **3b** required a longer time (24 h) due to the presence of a bulky *tert*-butyl group in α position to the carbonyl group. Formation of several minor by-products with shorter retention times was assigned to side reactions occurring during the alkaline treatment and not to the presence of failure sequences in crude **3b** since no such by-products were formed when **3b** was treated with concentrated aqueous ammonia (Fig. 3F, lower profile). Furthermore, a subsequent treatment with alkaline phosphatase afforded a compound (Fig. 3F, upper profile) which co-migrated with an authentic sample of d[Tps(Tp)₁₀T].

We therefore had synthesized four different SATE pro-oligo models having in common one negative charge at their 3'-end and a thionophosphotriester link at their 5'-end. Compounds **3a**, **3b** and **3c**, **3d** differ on the nature of the SATE protecting groups, the MeSATE being more easily removed by esterases than the bulkier tBuSATE one (5). In addition, **3a** and **3b** are expected to be decomposed to dodecathymidylate containing one internucleoside phosphorothioate linkage at the 5'-end whereas hydrolysis of **3c** and **3d** would give rise to the corresponding fully phosphorothioate analog. In addition, the lipophilicity order of the four pro-oligos is expected to be **3d** > **3b** > **3c** > **3a** as reflected by their HPLC retention time under the same conditions (19).

Stability of **3a** and **3c** was investigated towards the action of purified pig liver esterase (PLE), calf spleen phosphodiesterase (CSP) and snake venom phosphodiesterase (SVP). Only **3a** is a substrate for PLE, the thiono derivative **3c** being resistant to hydrolysis (Table 1). The thiono derivative **3c** being more lipophilic than **3a** (*vide supra*) one can suspect that **3c** is too lipophilic to be a good substrate for esterase. In addition **3a** was not degraded by

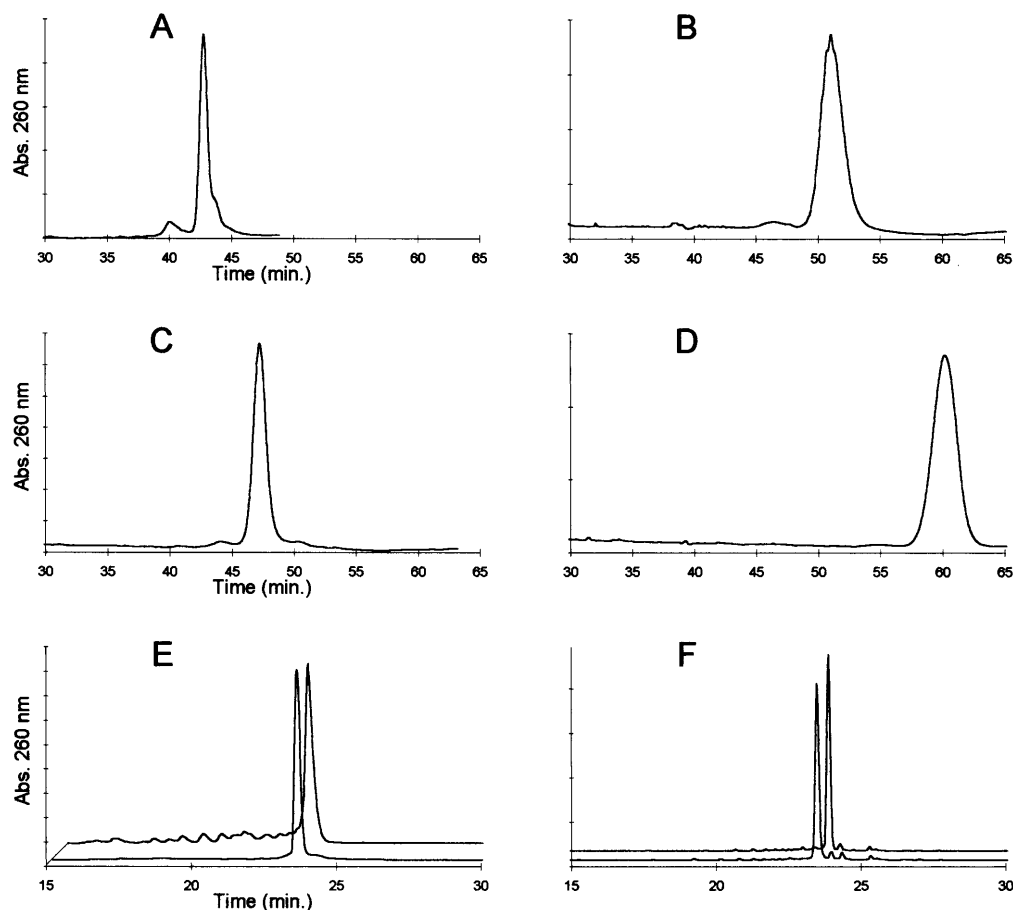


Figure 3. Reverse-phase HPLC analysis of crude pro-dodecathymidylates. (A) **3a**; (B) **3b**; (C) **3c**; (D) **3d**; (E) **3a** (lower profile) and **3b** (upper profile) upon treatment with 0.1 M NaOH; (F) **3b** upon treatment with conc. aq. NH_3 (lower profile) and alkaline phosphatase (upper profile). For experimental conditions, see Materials and Methods.

SVP and CSP (under the experimental conditions) as the same half-life was observed in presence or in absence of nucleases.

The behavior of compounds **3a** and **3c** was then evaluated in various biological media namely total CEM cell extracts (TCE), human serum and human gastric juice. Their stability was determined using an on-line cleaning HPLC procedure which allows direct injection of the incubated media without any pretreatment (5). No degradation was observed upon incubation in gastric juice (Table 1) which is in agreement with the expected stability of phosphotriesters in acidic media. In TCE, the half-life for monodeprotection of **3a** and **3c** were 0.35 and 9.7 h, respectively. Such differences may reflect the capacity of pro-oligos to be intracellular esterase substrates. It is noteworthy that both compounds are fully deprotected to the parent oligo with a half-life of 22 and 20 h, respectively.

In human serum, the half-life for monodeprotection of **3a** was 3 h, i.e. eight times higher than in TCE. The tBu SATE pro-oligos **3b** and **3d** were not deprotected in PLE, gastric juice and TCE. The fact that **3c** is a substrate for esterases present in TCE and not for PLE is not surprising since a large variety of carboxyesterases from various origins but with different substrate specificity have been isolated and characterized (20–23). That may be also the reason why pro-oligos, as well as pronucleotides (3–5), are more stable in sera than in TCE (Table 1).

In addition, the *ex-vivo* stability studies herewith presented

should not fully reflect the *in vivo* behavior of the pro-oligos. We have further observed that the esterase activity present in total cell extracts decreases after 3–4 h at 37°C (data not shown). Therefore, the fact that **3b** and **3d** are not decomposed in TCE or in presence of PLE does not preclude that it could be the same in intact cells or *in vivo*.

In conclusion, we have shown that SATE-pro-dodecathymidylates can be efficiently synthesized on solid support. As expected these compounds are not degraded by nucleases and present lower affinity to serum proteins as compared to the parent oligonucleotides (Vlassov *et al.*, unpublished results). In addition, compounds **3a** and **3c** are selectively hydrolysed to the parent oligonucleoside phosphodiester or phosphorothioate in total cell extract but are much more stable in human serum. One can thus hypothesise that the bioavailability of such neutral and lipophilic pro-oligos will be different from the one of the parent oligos. Furthermore their pharmacokinetics may be modulated according to the nature of the enzymolabile protecting group i.e. Me-SATE versus tBu-SATE. The validation of the pro-oligo approach must be envisaged *in vivo* and experiments along this line are in progress. The use of such depot form for oligos delivery could be of the greatest interest for their use as therapeutics. However, much more work has to be done in this area before reaching any definitive conclusion and we hope that the pro-oligo approach herewith presented could be the premise for the development of a new generation of antisense oligonucleotides.

Table 1. Half-lives (h) of pro-dodecathymidylates **3a** and **3c** in presence of purified enzymes or in biological media

Milieu	SVP ^a	CSP ^b	CEM cell extracts ^c	Mouse serum	Human serum	Human gastric juice	PLE ^d
3a	19 ^e	28 ^e	0.35 (22)	0.43	3	stable ^f	4.6
3c	ND	ND	9.7 (20)	stable ^g	stable ^h	stable ^f	stable ^g

^aSnake venom phosphodiesterase (0.06 U/ml) in 0.01 M MgCl₂, 0.1 M Tris-HCl (pH 7.3).

^bCalf spleen phosphodiesterase (0.08 U/ml) in 0.0025 M EDTA, 0.125 M ammonium acetate (pH 6.8).

^cValue in brackets corresponds to the incubation time necessary for the formation of 50% corresponding fully-unmasked dodecathymidylate.

^dPig liver esterase (8 U/ml) in 0.2 M potassium phosphate (pH 7.36).

^eThe same value was obtained in absence of nuclease.

^fNo detectable degradation upon 7 day incubation.

^gNo detectable degradation upon 2 day incubation.

^hNo detectable degradation upon 24 h incubation. Then a precipitation was observed and the signal corresponding to initial compound in the supernatant progressively decreased without any detectable formation of hydrolysis products. This was tentatively assigned to coprecipitation of **3c** with serum proteins.

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