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 obention 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
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-dodecatymidylates. 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 purifcation except where otherwise stated. Bis(diisopropyl-amino)chlorophosphine, tetrazole (≥99%) and redistilled N,N-diisopropylethylamine were from Aldrich (St Quentin Fallavier, France). Methylene chloride was dried over P2O5 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 (5 g, 18.7 mmol) in dry CH2Cl2 (18 ml). The reaction mixture was allowed to warm to room temperature while stirring was maintained (30 min). To a cooled (ice-bath) and magnetically stirred solution of 5′-O-[(S-acetyl-2-thioethyl)N,N-diisoproplyphosphoramidite] (2a), a 0.5 A250 unit sample was mixed with 1.0 ml of an acetonitrile solution saturated with sinapinic acid and a 25 µl portion of this mixture was placed on a plate and dried at ambient temperature and pressure. MALDI-TOF mass 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 µm) column (150×4.6 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 5′-O-(S-acetyl-2-thioethyl) phosphoramidites (2)

5′-O-(4,4′-Dimethoxytrityl)-thymidine 3′-O-[S-(acetyl-2-thioethyl)N,N-diisoproplyphosphoramidite] (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 CH2Cl2 (100 ml), was added dropwise over 8 min, a solution of bis(N,N-diisopropylamino)-chlorophosphine (5 g, 18.7 mmol) in dry CH2Cl2 (18 ml). The reaction mixture was allowed to warm to room temperature while stirring was maintained (30 min). S-(2-Hydroxyethyl) thiocetate (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 CH2Cl2 (200 ml) was added and the reaction mixture was washed with saturated aqueous hydrogen carbonate (300 ml) and brine (2×300 ml) 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) ethyl acetate in cyclohexane and dried under vacuum. The residue was lyophilized from benzene affording a product which was of an improved purity and yield of 68% by 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]+. 31P-NMR (CD3CN): δ 148.9 and 148.6. 1H-NMR (CD3CN): δ 1.02–1.12 [m, 12 H, CH(CH3)2]; 1.48 and 1.5 [2 d, 3 H, CH3]; 2.26 and 2.28 [2 s, 3 H, CH2CO]; 2.32–2.4 [m, 2 H, H2′ and H2″]; 2.93–3.2 [m, 2 H, CH2–CH2–S]; 3.28–3.38 [m, 2 H, H5′ and H5″]; 3.51–3.62 [m, 4 H, CH2–O and CH2CH2]; 3.75 [s, 6 H, Ar-OCH3]; 4.05 and 4.1 [2 m, 1 H, H4′]; 4.61 [m, 1 H, H3′]; 5.00–5.04 [m, 2 H, CH2–S]; 5.79–5.81 [2 H, CH2]; 6.70–6.90 [m, 5 H, Ar]; 7.26–7.32 [m, 5 H, Ar]; 7.30–7.36 [m, 5 H, Ar]; 7.38–7.43 [m, 5 H, Ar]; 7.41–7.45 [m, 5 H, Ar]; 7.45–7.50 [m, 5 H, Ar].
Solid-phase synthesis of pro-dodecathymidylates

Photolabile CPG solid support (12) (65 µmol/g) was used and during each coupling step, a 10-fold molar excess was introduced in the column. A modified ‘10 µmol’ 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 (500 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 3H-1,2-benzodithiole-3-one-1,1-dioxide (0.05 M, Beaumage 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′-detyriylated 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-filtered output of a 125 W high-pressure Hg lamp for 20 min at 20 °C (2 mm thick) filtered output of a 125 W high-pressure Hg lamp for 20 min at 20 °C.

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.

RESULTS AND DISCUSSION

Since phosphotriesters and esters are sensitive to bases and nucleophiles, the efficient synthesis of pro-oligo deoxynucleotides bearing SATE phosphodiester backbone 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-oligo deoxynucleotides 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-[3-acetyl and pivaloyl-2-thioethy]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 eq.) in presence of N,N-diisopropylethylamine. The resulting nucleoside 3′-phosphorodiimidate 1 (15) was not isolated but rather reacted with S-(2-hydroxyethyl) thiocetate or S-(2-hydroxyethyl) thiopivalate (5) in presence of tetrazo (0.5 molar eq.). After purification by flash chromatography, thymidine 3′-O-phosphoramidites bearing
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, $^1$H and $^{31}$P-NMR.

Four different pro-dodecatrimetidines 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 $^{35}$S. 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.

$t$-Butyl hydroperoxide (16,17) and Beauchage 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 Rp and Sp isomers at each internucleoside linkage. $^{31}$P-NMR spectra of 3c and 3d exhibited two sets of peaks corresponding to internucleoside thionophosphate triesters ($\delta$ $\approx$ 68 p.p.m.) and 3'-terminal thiophosphate diester ($\delta$ $\approx$ 57 p.p.m.) in the expected ratio whereas spectra of 3a and 3b presented three sets of peaks corresponding to 5'-thionophosphate triester ($\delta$ $\approx$ 68 p.p.m.), internal phosphate triesters ($\delta$ $\approx$ 1 p.p.m.) and 3'-terminal phosphodiester ($\delta$ $\approx$ 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 Beauchage reagent as oxidizer. In addition, hydrolysis of 3a with 0.1 M NaOH for 1 h yielded only one product (Fig. 3E, lower profile) which corresponds to fully unmasked dodecatrimidylate. Hydrolysis of 3b required a longer time (24 h) due to the presence of a bulky tert-butyl group in the $\alpha$ 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) 10 T].

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 dodecatrimydylate 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 venon 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
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-live 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 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.

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

<table>
<thead>
<tr>
<th>Milieu</th>
<th>SVPa</th>
<th>CSPb</th>
<th>CEM cell extractsc</th>
<th>Mouse serum</th>
<th>Human serum</th>
<th>Human gastric juice</th>
<th>PLEd</th>
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<tr>
<td>3a</td>
<td>19e</td>
<td>28e</td>
<td>0.35 (22)</td>
<td>0.43</td>
<td>3</td>
<td>stablef</td>
<td>4.6</td>
</tr>
<tr>
<td>3c</td>
<td>ND</td>
<td>ND</td>
<td>9.7 (20)</td>
<td>stablef</td>
<td>stableh</td>
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a Snake venom phosphodiesterase (0.06 U/ml) in 0.01 M MgCl₂, 0.1 M Tris–HCl (pH 7.3).
b Calf spleen phosphodiesterase (0.08 U/ml) in 0.0025 M EDTA, 0.125 M ammonium acetate (pH 6.8).
c Value in brackets corresponds to the incubation time necessary for the formation of 50% corresponding fully-unmasked dodecathymidylate.
d Pig liver esterase (8 U/ml) in 0.2 M potassium phosphate (pH 7.36).
e The same value was obtained in absence of nuclease.
f No detectable degradation upon 7 day incubation.
g No detectable degradation upon 2 day incubation.
h No 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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