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Kinetics study of the biotransformation of an oligonucleotide prodrug in cells extract by matrix-assisted laser desorption–ionization time-of-flight mass spectrometry

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Abstract

The fate of a dodecathymidine prodrug in cell extract was monitored by MALDI-TOF MS. This technique allows a facile identification and a relative quantification of metabolites produced. We showed that the relative peak intensities were similar to the relative metabolite proportions that permitted the determination of their half-lives. We found a good fit between the calculated kinetics curves and the experimental points. The oligonucleotide prodrug was fully metabolized to yield the dodecathymidine phosphorothioate likely through a carboxyesterase mediated mechanism.

Keywords: Oligonucleotides; Dodecathymidine prodrug

1. Introduction

An oligonucleotide prodrug approach has been designed to overcome the hurdle of the poor uptake of oligonucleotides. Since the low uptake is due to the presence of negative charges on each phosphate [1] we decided to transitorily mask several of them with a carboxyesterase labile *S*-Acyl-2-ThioEthyl (SATE) group to gain more lipophilic oligos. We have shown that the resulting prooligos cross efficiently and rapidly the cellular membrane via a presumably passive mechanism and hence reach the nucleus [2,3]. The oligonucleotides bearing the enzymolabile SATE groups with acyl equal to acetyl were named MeSATE prooligonucleotides or MeSATE prooligos. Since our strategy involves an intracellular carboxyesterase activation to recover the unmasked oligonucleotide inside the cell, we studied the fate of these compounds in crude cell extracts as a model of the intracellular medium. The first model MeSATE-T_{12} prooligo was incubated in crude cell extract and its metabolization was monitored by MALDI-TOF mass spectrometry [4]. Thus we have shown that cell extract indeed possess carboxyesterase activities hence several prooligos being lost a different number of MeSATE groups were detected as well as the fully unmasked T_{12} phosphodiester. But the resulting prooligos bearing few MeSATE groups and several phosphodiester linkages were also substrates of nucleases and yield shortened prooligos and oligos T_n (n=11 to 4) after 10 h of incubation. This study showed that carboxyesterase and nuclease

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activities are competing hence the T_{12} phosphodiester was not obtained as the final product. To avoid such a problem we decided to synthesize MeSATE T_{12} prooligos that will release after hydrolysis of the MeSATE groups the nuclease resistant phosphorothioate T_{12} (PS-T_{12}). We already study the demasking of a fully thionophosphotriester MeSATE T_{12} by HPLC but due to the presence of diastereoisomers only broad peaks were obtained hence it was only possible to determine the half-life of the starting prooligo [5]. This study did not give any information about the nature of the formed metabolites.

In this paper, we report the kinetics study of the demasking of a MeSATE T_{12} prooligo **A** (Fig. 1) in cell extracts monitored by MALDI-TOF MS without

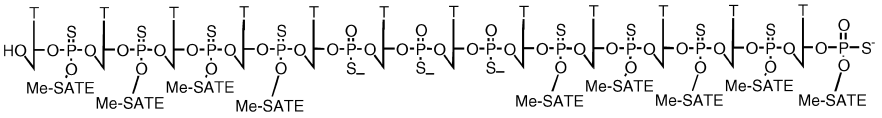
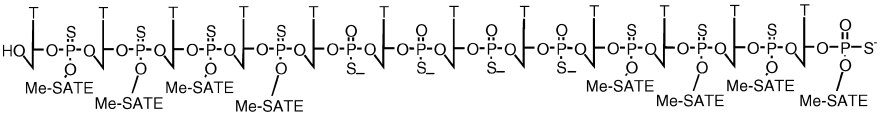
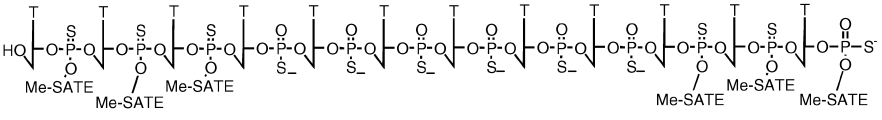
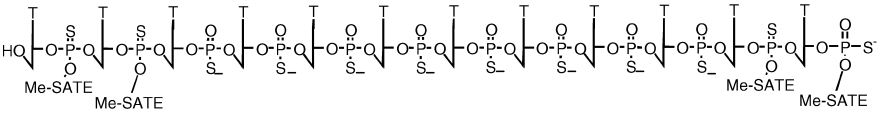
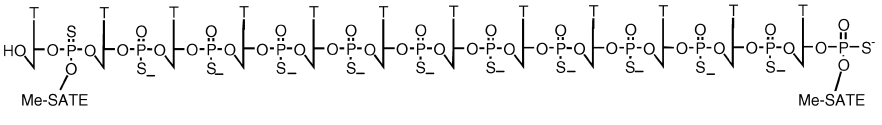
		Nbr of MeSATE	Nbr of PS^-
A		9	4
B		8	5
C		6	7
D		4	9
E		2	11

Fig. 1. Schematic structure of the prooligos **A** to **E**.

any sample pretreatment except drop dialysis to remove salts [6].

2. Experimental

2.1. Chemicals

2,4,6-trihydroxyacetophenone monohydrate (THAP) was from Fluka, and ammonium citrate (98%) was from Aldrich.

2.2. Prooligonucleotides synthesis

Prooligonucleotides **A** to **E** (Fig. 1) were synthesized on solid support with a photolabile linker [7] on a DNA synthesizer (ABI 381A). The SATE phosphotriester linkages were introduced using a thymidine MeSATE phosphoramidite building block by means of phosphoramidite chemistry, whereas the phosphodiester linkages were introduced using an H-phosphonate monoester thymidine building block by means of H-phosphonate chemistry as described in Ref. [8].

2.3. Cell extracts preparation

The CEM cell extracts were prepared by A.-M. Aubertin (Université Strasbourg I, France) according to the following procedure: CEM cells in logarithmic growth were separated from their culture medium by centrifugation ($10^4\times$g, 4 min, 4°C). The residue (about 100 μl, 5×10^7 cells) was resuspended in 2 ml of buffer (10 mM Tris HCl, 140 mM KCl, pH 7.4) and sonicated. The lysate was centrifuged ($10^5\times$g, 1 h, 4°C) to remove membranes, organelles, and chromatin. The upper layer was filtered (0.22 μm Millex GV, Millipore) and stored in a sterile container at −80°C.

2.4. Metabolism studies

The prooligonucleotide (0.25 A_{260} units, about 2.5 nmol in 25 μl of pure water) was diluted to 250 μl with cell extract. The solution was vortex-mixed and then was incubated at 37°C, after the required duration the solution was vortex-mixed and 5 μl was taken for direct drop dialysis.

2.5. MALDI-TOF sample preparation

After incubation the samples (5 μl) were desalted using the drop dialysis technique through a membrane filter WSWP 0.025 μm, 13 mm (Millipore) floating on 0.1 *M* ammonium citrate solution. After 30 min dialysis, 1 μl of the sample was withdrawn and mixed with 10 μl of matrix (45 mg 2,4,6-trihydroyacetphenone (THAP) and 10 mg ammonium citrate in 500 μl of acetonitrile/water, 1:1, v/v). The mixture (1 μl) was directly spotted on the stainless steel MALDI target and dried under air.

2.6. MALDI-TOF mass spectrometry

MALDI-TOF mass spectra were recorded on a Voyager DE mass spectrometer (Perseptive Biosystems, Framingham, MA, USA) equipped with an N_2 laser (337 nm). MALDI conditions are as follow: laser power, 2800 (arbitrary units); accelerating voltage, 24 000 V; guide wire, 0.05% of accelerating voltage; grid voltage, 94% of accelerating voltage; delay extraction time, 550 ns and 256 scans averaged. All reported spectra here were obtained in negative ion mode and were not smoothed.

2.7. Kinetics study

For each Maldi spectrum, we calculated from the height of each signal, the relative proportion of the starting compound and their metabolites. These relative proportions were treated according to a ''consecutive pseudo-first order'' kinetic model corresponding to the general decomposition pattern [9]:

T12p+9MeSATE→T12p+8MeSATE→T12p+7MeSATE→ . . . . →T12p+4MeSATE

This model calculates the rate constants of each step and the deduced half-lives of the starting materials and the first five metabolites (Tables 1–3).

Table 1
Masses and assignments of observed metabolites from prooligo **A**

	Metabolites	Observed mass	Mass difference	Calculated mass
A (1)	T12p+9 MeSATE	4779.6		4779.6
2	T12p+8 MeSATE	4677.4	102.2	4677.4
3	T12p+7 MeSATE	4575.2	102.2	4575.3
4	T12p+6 MeSATE	4473.0	102.2	4473.1
5	T12p+5 MeSATE	4371.0	102	4370.9
6	T12p+4 MeSATE	4269.2	101.8	4268.8
7	T12p+3 MeSATE	4167.3	101.9	4166.6
8	T12p+2 MeSATE	4065.7	101.6	4064.5
9	T12+2 MeSATE	3968.8	96.9	3968.4
10	T12+1 MeSATE	3867.7	101.1	3866.3
11	T12	3765.4	102.3	3766.1
12	T11	3445.7	319.7	3444.9
13	T10	3125.2	320.5	3123.6

Table 2
Masses and assignments of observed metabolites from prooligo **E**

	Metabolites	Observed mass	Mass difference	Calculated mass
E (8′)	T12p+2 MeSATE	4063.1		4064.5
9′	T12p+1 MeSATE	3961.3	101.8	3962.3
10′	T12p	3861.0	100.3	3860.2
11′	T12	3763.6	97.4	3766.1

3. Results and discussion

We chose to study the kinetics and the decomposition pathway of the prooligo T_{12} **A** bearing nine thionophosphotriester MeSATE groups and four charged thionophosphodiesters (Fig. 1). Indeed fully thionophosphotriester MeSATE T_{12} was found poorly soluble in water due to the hydrophobicity brought by replacement of oxygen atoms by sulfur atoms [5]. In addition we have shown that the introduction of

Table 3
Half-lives of prooligos **A** to **E** and their metabolites

		Metabolites	**A**	**B**	**C**	**D**	**E**	Average
H	**1**	T12p+9MeSATE	62*					62
A	**2**	T12p+8MeSATE	49	50*				49
L	**3**	T12p+7MeSATE	44	37				41±4
F	**4**	T12p+6MeSATE	35	33	26*			34±1
–	**5**	T12p+5MeSATE	33	30	25			32±2
L	**6**	T12p+4MeSATE	29	26	28	15*		28±2
I	**7**	T12p+3MeSATE		24	28	15		28±2
F	**8**	T12p+2MeSATE			27	16	14*	22±6
E	**9**	T12p+1MeSATE			30	22	19	24±5
(m	**10**	T12p				28	23	26[a]±3
in)	**11**	T12				356	271	314[b]±43

[a] 3′-phosphatase activity.
[b] 3′-exonuclease activity.
* corresponds to an unique substrate.

few charges [3–5] in a prooligo is enough to recover a good hydrosolubility and keeping a high overall lipophilicity to allow good cellular uptake [2].

The hydrolysis of the prooligo **A** should yield the nuclease resistant $T_{12}p$ phosphorothioate (PS-$T_{12}p$) and the expected metabolites should be the prooligo **A** having lost one to nine MeSATE groups. Furthermore, as cell extract displays also phosphatase activities the 3′-end phosphorothioate will be hydrolyzed to yield the parent phosphorothioate dodecathymidine (PS-T_{12}).

We decided to study the demasking kinetics of the prooligo **A** and also the four other related prooligos with eight (**B**), six (**C**), four (**D**) and two (**E**) MeSATE groups respectively (Fig. 1). Firstly, because our kinetics model allows the half-life determination of only six compounds and secondly because the determination of the half-life for the last metabolites could be less accurate than for the first ones. Each prooligo **B** to **E** is a possible metabolite of **A**. Thus, we assumed that they are representative compounds of the prooligo population bearing the same number of MeSATE groups. Thus, for each prooligo the half-life of the starting prooligo and its first five metabolites was determined and some half-lives were confirmed during the next kinetics.

Each prooligo was synthesized on solid support as already described in Ref. [8] using the both phosphoramidite and H-phosphonate chemistries. Then, to study the kinetics of hydrolysis, each prooligo (**A** to **E**) was incubated at 37°C at a 10 μ*M* concentration in CEM SS cell extract. After a predetermined time, 5 μl of the resulting mixture was taken up and the sample was desalted by dialysis on a nitrocellulose membrane (Millipore) floating on a 0.1 *M* ammonium citrate solution for 30 min. Indeed the salts (Na^+, K^+) should be removed absolutely because the multiple adducts formed with the analytes compromise the mass analysis. Then 1 μl was mixed with 10 μl of 2,4,6-trihydroxyacetophenone (THAP) matrix and 1 μl was loaded on the MALDI target to crystallize and subjected to MALDI-TOF MS analysis. The THAP matrix is especially well suited for the desorption of oligonucleotides of molecular mass below 5000 [10] and does not allow the desorption of proteins of higher molecular mass [11]. The ratio analyte:matrix 1:10 was found to provide better desorption than the 1:1 and the 1:20 ratio also tested. This corresponds to an amount of 910 fmol of the starting prooligo and it was possible to visualize metabolites around 10% of the total signal (i.e. around 90 fmol). Furthermore MALDI-TOF MS analyses were done at two laser intensity (2800 and 3000 arbitrary unit) and similar results were obtained. Time-dependent mass spectra of prooligo **A** and **E** and their metabolites produced after incubation in cell extract are shown in Figs. 2 and 3 (The spectra corresponding to prooligos **B** to **C** are not shown but displayed the same pattern). It was easy to assign the attribution of each peak (Tables 1 and 2) which were spaced out of 102 u corresponding to the loss of one MeSATE group (C_4H_6OS 102.2) according to the mechanism depicted in Scheme 1. A first hydrolysis of the acetyl group by carboxyesterase led to an unstable compound which rearranges by a spontaneous elimination of episulfide to yield the phosphorothioate diester. The mass difference observed between metabolite 8 and 9 for **A** (96.9u, Table 1) and between the metabolites 10′ and 11′ for **E** (97.4u, Table 2)

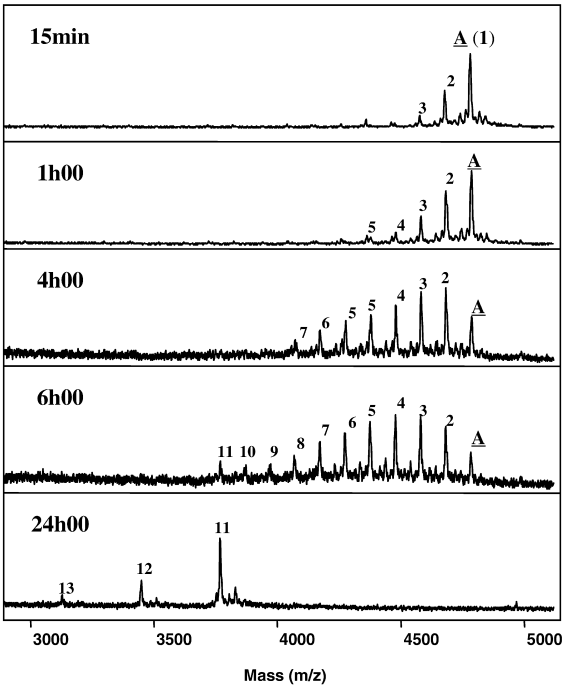


Fig. 2. Time dependent MALDI-TOF mass spectra of prooligo **A** incubated in cell extract.

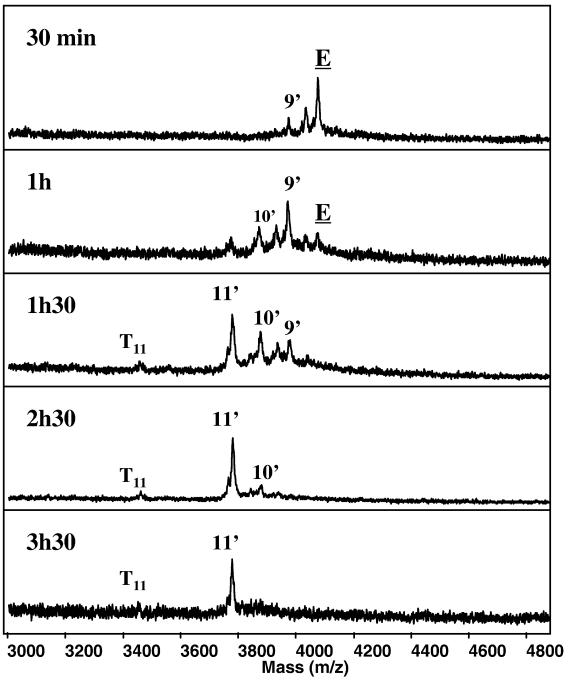
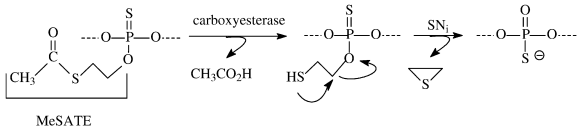


Fig. 3. Time dependent MALDI-TOF mass spectra of prooligo **E** incubated in cell extract.

corresponds to the loss of the 3′-thiophosphomonoester (HPO_2S, 96.0) due to phosphatase activity of the cell extract. Finally, the MALDI-TOF spectrum obtained after 24 h incubation shows that the parent PS-T_{12} fully unmasked was reached and then was slowly degraded by a 3′-exonuclease activity which corresponds to a loss of a 5′-monophosphorothioate thymidine ($C_{10}H_{13}N_2O_6PS$, 320.3) (Fig. 2 and Table 1). From each MALDI spectrum the height of each signal was measured and it was converted in relative proportion, assuming that each metabolite flew in a similar extent than the others. This hypothesis was done since the formed metabolites exhibit a similar structure (all T_{12}) and it allows a relative quantification of each metabolite. In paral-



Scheme 1. Mechanism of MeSATE hydrolysis mediated by carboxyesterases.

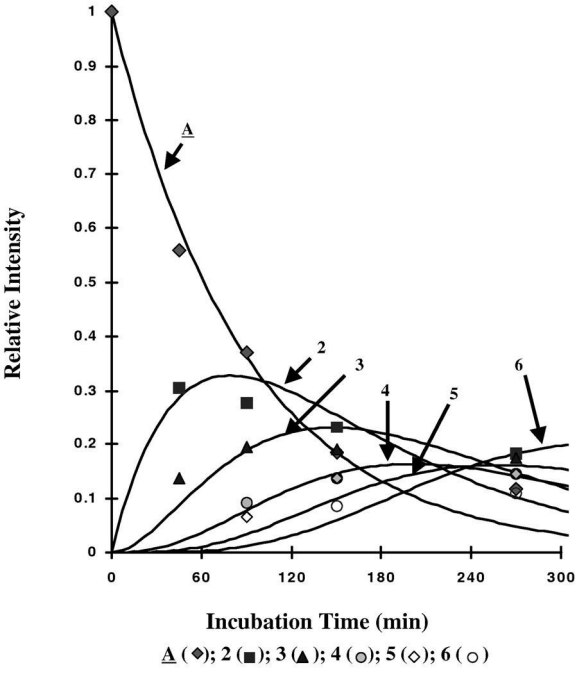


Fig. 4. Kinetics curves of prooligos **A**.

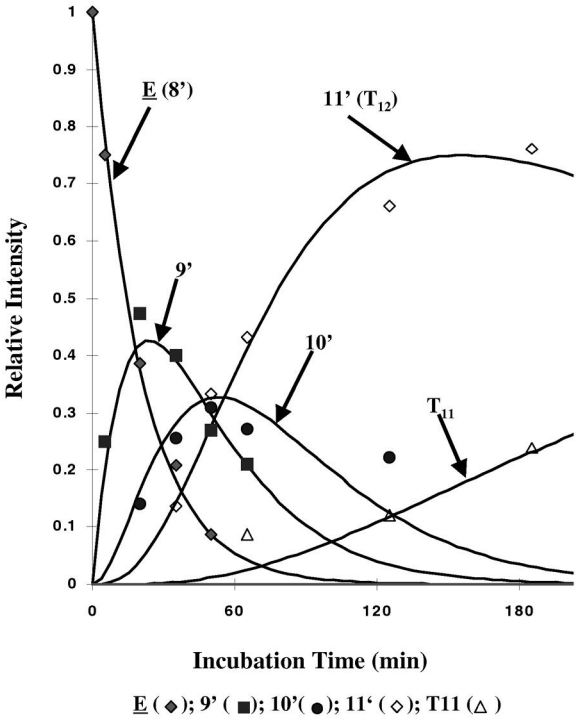


Fig. 5. Kinetics curves of prooligos **E**.

lel, to verify this hypothesis, we mixed two to three prooligos in various proportions and analyzed them by MALDI-TOF MS. We found that the relative peak intensities were similar to the relative prooligo proportion. MALDI-TOF MS was already used to quantify several kinds of biological molecules [12]. In particular, it was shown for proteins that their concentrations could be estimated from the relative signal intensities [13]. On the other hand, quantification of oligonucleotides remains largely unexplored [14] and to our knowledge this is the first time that a relative quantification of oligonucleotide metabolites in a biological medium by MALDI-TOF MS is reported.

For the five kinetics studies, we found that experimental points of each analyte fits well with its calculated curve (Figs. 4 and 5) which substantiates the reliability of the method. These data suggests that the hydrolysis of MeSATE group proceeded according to a mechanism of pseudo-first order and confirms our hypothesis that each analyte displays similar desorption ionization properties. The half-lives determined from the five kinetics are reported in Table 3 and in Fig. 6. We distinguished the half-lives corresponding to a defined substrate **A**, **B**, **C**, **D** and **E** and the half-lives of the generated metabolites since these latter correspond to an average half-life of each possible metabolite. From the first half-life of prooligos **A** to **E**, it appeared that the substrate capacity of prooligo for the carboxyesterases increased when the number of MeSATE group decreased (62–14 min, Table 3). All the other half-lives determined correspond to an average half-life of the possible metabolites formed and bearing the same number of MeSATE groups. Thus we considered the average of these half-lives as reported in Table 3. The discrepancy between the half-life of **C**, **D** and **E** and the corresponding average half-life of the population bearing the same number of MeSATE group comes from the fact that each possible metabolite displayed its own half-life in a function of its substrate capacity. This substrate capacity could be quite different to another metabolite according to its structure since carboxyesterases are sensitive to hydrophilic and lipophilic environment [15]. Finally the half-life of the metabolite 10 corresponds to a

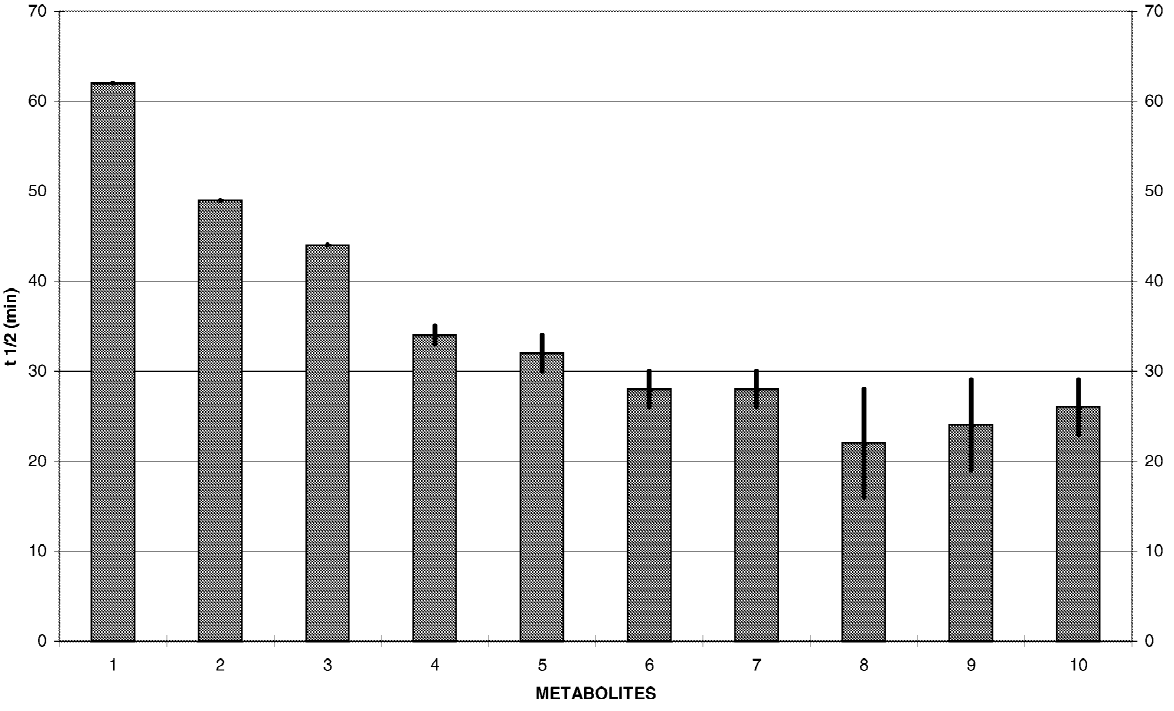


Fig. 6. Histogram of the average half-life with standard deviation.

phosphatase activity ($t_{1/2}$ 26 min, hydrolysis of the 3′-end monoester phosphorothioate) to yield the fully unmasked PS-T_{12} (metabolite 11, Table 3). Additional studies are in progress with other possible metabolites to gain more information about the biotransformation of our prooligos to define if a rational could be found between the structure of the prooligo and the enzyme activities.

In conclusion this method allows us to visualize all the metabolites formed which correspond to the population of the prooligo which lose one to nine MeSATE groups and the 3′-end phosphorothioate moiety. This method required only few materials (0.25 $O.D._{260\ nm}$, 2.5 nmol), was rapid and did not necessitate a sample preparation except a drop dialysis. It was possible to determine the half-life of each metabolite from the data obtained on MALDI-TOF MS spectra by means of a relative quantification. Finally, we showed that the expected oligonucleotide parent PS-T_{12} was likely obtained by means of a carboxyesterase mediated mechanism.

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