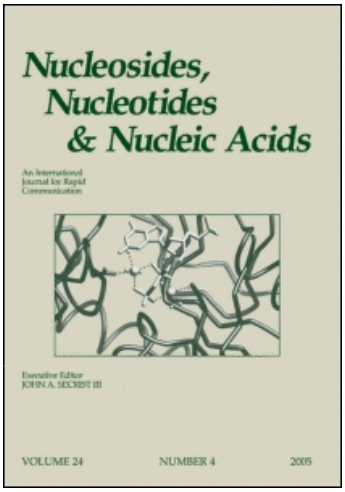

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KINETICS STUDY OF THE BIOTRANSFORMATION OF AN OLIGONUCLEOTIDE PRODRUG IN CELLS EXTRACT BY MATRIX-ASSISTED LASER DESORPTION/IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY

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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. The oligonucleotide prodrug was fully metabolized to yield the T_{12} phosphorothioate likely through a carboxyesterase mediated mechanism.

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 charge 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). Since our strategy involves an

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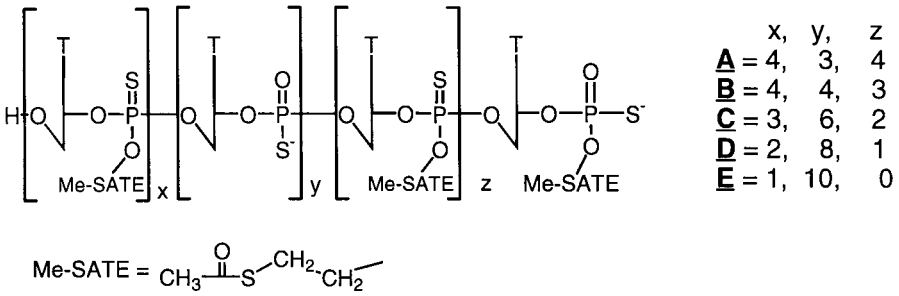

Figure 1. Schematic structure of the prooligos **A** to **E**.

intracellular carboxyesterase activation to recover the unmasked oligonucleotide inside the cell, we studied the fate of these compounds in crude cell extract as a model of the intracellular medium. In this paper, we report the kinetics study of the demasking of a MeSATE T_{12} prooligo **A** (Fig. 1) in cell extract monitored by MALDI-TOF MS without any sample pretreatment except drop dialysis to remove salts (4).

We studied the kinetics and the decomposition pathway of the prooligo **A** bearing nine thionophosphotriester MeSATE groups and four charged thionophosphodiesters (Fig. 1). 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** being 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 also studied the demasking kinetics of the four other related prooligos with respectively eight (**B**), six (**C**), four (**D**) and two (**E**) MeSATE groups (Fig. 1) to obtained more accurate half-life on the last metabolites. 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. Each prooligo was synthesized on solid support as already described in reference (5) 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 μM concentration in CEM SS cell extract. After a predetermined time, 5 μl of the resulting mixture was taken up and desalted by dialysis on a nitrocellulose membrane (Millipore) floating on a 0.1 M ammonium citrate solution for 30 min to remove salts (Na^+, K^+) able to form multiple adducts that will 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. Time-dependent mass spectra of prooligo **A** and its metabolites produced after incubation in cell extract is shown in Figure 2. It was easy to assign the attribution of each peak which were spaced out of 102 u corresponding to the loss of one MeSATE group (C_4H_6OS 102.2). Finally, the MALDI-TOF spectrum

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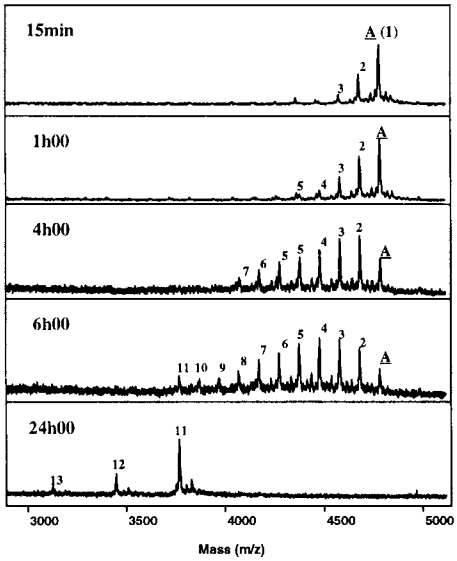

Figure 2. Time dependent MALDI-TOF mass spectra of prooligo **A** incubated in cell extract.

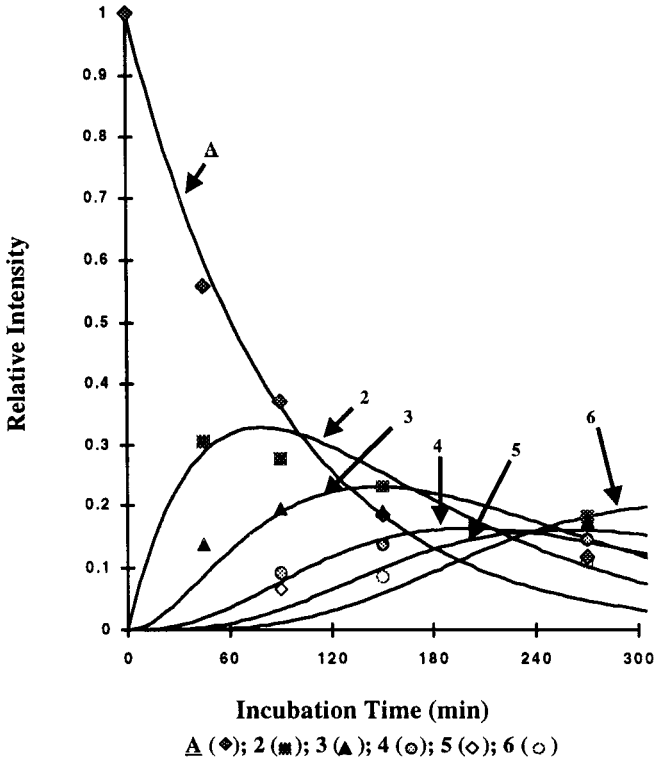

Figure 3. Kinetics curves of prooligos **A**.

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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).

From each MALDI spectrum, the height of each signal was measured and 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}). That allows a relative quantification of each metabolite. Quantification of oligonucleotides remains largely unexplored (6) and at 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, the relative proportion were treated according to a consecutive pseudo-first order kinetic model (7) and we found that experimental points of each analyte fit well with its calculated curve 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 were determined from the five kinetics. It appeared that the substrate capacity of prooligo for the carboxyesterases increased when the number of MeSATE group decreased ($t_{1/2}$ = 62 to 24 min).

In conclusion this method allows to visualize all the metabolites formed which correspond to the population of the prooligo being lost one to nine MeSATE groups and the 3′-end phosphorothioate monoester T_{12}. This method required only few material (0.25 OD_{260nm}, 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-TOMS spectra by means of a relative quantification. Finally, we showed that the expected oligonucleotide parent PS- T_{12} was obtained likely by means of a carboxyesterase mediated mechanism.

			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				44 ± 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
(min)	**10**	T12p				28	23	26[a] ± 3
	11	T 12				356	271	314[b] ± 43

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

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