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Retention of Configuration in the Action of Human Plasma 3′-Exonuclease on Oligo(deoxynucleoside phosphorothioate). A New Method for Assignment of Absolute Configuration at Phosphorus in Isotopomeric Deoxyadenosine 5′-*O*-[^{18}O]Phosphorothioate

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Abstract: A new method of analysis has allowed the exonucleolytic cleavage by human 3′-exonuclease to be determined. Hydrolysis by human plasma 3′-exonuclease proceeds with retention of configuration at phosphorus. The new method determines the sense of chirality at phosphorus in isotopomeric adenosine 5′-*O*-[^{18}O]phosphorothioates. This is based on stereospecific two-step conversion of the mono-thionucleotide into the corresponding deoxyadenosine 5′-*O*-α-[^{18}O]thiotriphosphate, followed by the use of terminal deoxyribonucleotidyl transferase and MALDI TOF mass spectrometry of the resulting elongated primer. Retention of configuration in the reaction of plasma 3′-exonuclease implies a two-step mechanism with two displacements on phosphorus. Inversion at each step leads to overall retention.

Introduction

Oligonucleotides possessing stereodefined P-chiral phosphorothioate internucleotide bonds became indispensable tools for studies of the stereoselectivity and the mechanism of cleavage of oligonucleotides by numerous endonucleases.[1] Enzymatic hydrolysis of the phosphorothioate linkage in [^{18}O]water provides either P-chiral mononucleotide 5′-[^{18}O]phosphorothioate or shortened oligonucleotides with 5′-*O*-[^{18}O]phosphorothioate at the 5′-end. In the latter case, the resulting oligonucleotide could be digested with nucleases such as nuclease P1[2] or phosphatase-free snake venom phosphodiesterase[3] to release a nucleoside 5′-*O*-[^{18}O]phosphorothioate. Assignment of absolute configuration at phosphorus in isolated mononucleotide 5′-*O*-phosphorothioates was possible by means of ^{31}P NMR. The analysis, which is based on precise measurement of isotopic chemical shift in appropriately derivatized compounds,[1c] allows for determination of the mode of action of nuclease under investigation. It is generally accepted that enzymatic hydrolysis of an internucleotide phosphorothioate with *inversion* of configuration at phosphorus speaks for a mechanism that does not involve a substrate−enzyme covalent intermediate. On the other hand, *retention* of configuration at phosphorus proves the two-step mechanism with participation of a covalent enzyme−substrate intermediate.[1] However, it must be pointed out that for a conclusive NMR experiment multimilligram quantities of nucleoside 5′-*O*-[^{18}O]phosphorothioates are required. In those cases when only nanomolar amounts of nucleoside 5′-*O*-[^{18}O]-phosphorothioates are accessible, the NMR methodology cannot be applied even when using the most sensitive spectrometers. This limitation was to a certain extent overcome by the method based on gas chromatographic−mass spectrometric analysis of chemically degraded adenosine 5′-*O*-α-[^{18}O]thiotriphosphate, which was obtained enzymatically from adenosine 5′-*O*-[^{18}O]-phosphorothioate with tandem adenylate kinase (AK)/pyruvate kinase (PK) in the presence of ATP. This chemical degradation allowed for determination of the stereochemical course of phosphorothioyl group transfer catalyzed by adenosine kinase[4] and T4 polynucleotide kinase.[5] Further improvement brought two new methods based on enzymatic ligation of oligomers bearing the 5′-*O*-[^{18}O]phosphorothioate group with an oligonucleotide template followed by MALDI TOF mass spectrometry analysis of the resulting oligonucleotide products.[6,7] However, these methods cannot be used for the analysis of either mononucleotides or oligonucleotides shorter than pentamers.

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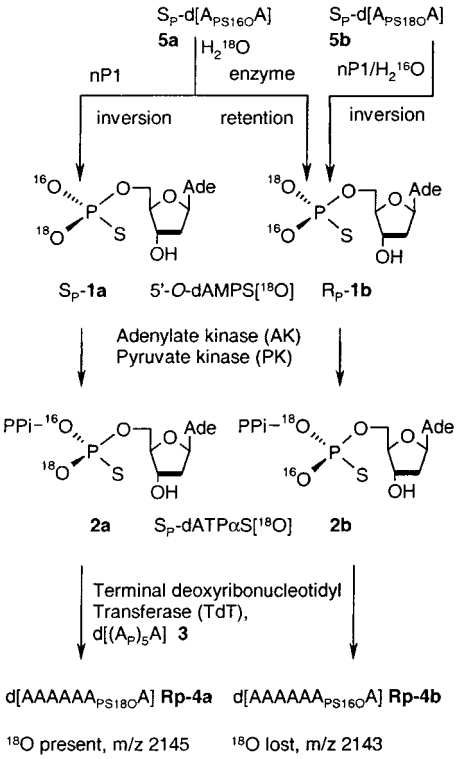
Scheme 1



In this report we present the new method employing MALDI TOF mass spectrometry for determining the sense of chirality at phosphorus in isotopomeric *mononucleotides*, namely adenosine 5′-*O*-[^{18}O]phosphorothioates. This method begins with the above-mentioned stereospecific two-step conversion of the mononucleoside 5′-*O*-[^{18}O]phosphorothioate into the corresponding adenosine 5′-*O*-α-[^{18}O]thiotriphosphate (dATPαS-[^{18}O]). Terminal deoxyribonucleotidyl transferase (TdT)-assisted stereospecific transfer of deoxyadenosine 5′-*O*-[^{18}O]phosphorothioate to the 3′-OH group of a short oligonucleotide follows, resulting in formation of internucleotide phosphorothioate linkage of the R_P-configuration.[8] Mass spectral analysis of the oligonucleotide reveals the stereochemistry.

Results and Discussion

Nucleolytic cleavage of the phosphorothioate internucleotide bond ...N_{PS}A... in $H_2{}^{18}O$ provides either S_P-deoxyadenosine 5′-*O*-[^{18}O]phosphorothioate (dAMPS[^{18}O], **1a**) or its R_P-counterpart (**1b**), depending upon the absolute configuration of the phosphorus atom in the scissile bond (predetermined by the enzyme substrate stereoselectivity) and the mechanism of enzymatic hydrolysis. For their configurational analysis, the following methodology was designed (Scheme 1): first, the isolated dAMPS[^{18}O] is converted in the presence of ATP with tandem adenylate kinase (AK)/pyruvate kinase (PK) into dATPαS[^{18}O] (**2**), with [^{18}O]oxygen at the bridging or the nonbridging position, respectively. The stereochemistry of both processes is well documented.[9] After HPLC isolation, compound **2** is used as the substrate for TdT. In this process, an oligonucleotide (i.e. dA_6, **3**) is elongated by TdT-assisted attack of its 3′-OH group on the phosphorus atom in position α of **2** with release of pyrophosphate. The heptamer [R_P]-d($A_5A_{PS}A$) (**4**) resulting from attack on dATPαS[^{18}O] with [^{18}O]oxygen at the bridging position should not contain the [^{18}O]-label, contrary to the heptamer formed from dATPαS[^{18}O] with [^{18}O]oxygen at the nonbridging position. In the case of a phosphorothioate substrate, the elongation is slow and under controlled conditions compound **4** is a predominant product. It should be noted that the presence of longer products does not affect the MALDI-TOF MS analysis with respect to the presence or absence of the isotope label. Alternatively, **4** can be degraded with an S_P-specific nuclease and the resulting d($_{PO}A_{PS}$A) can be studied by MS for [^{18}O]-label content. However, modern mass spectrometers working in a reflector mode have resolution good enough for unambiguous assignment of the presence of the isotope in a heptanucleotide.

We have checked the above-described method using nuclease P1, since it is well documented that this enzyme is Sp-stereoselective and hydrolyzes phosphorothioate diesters with inversion of configuration via the one-step mechanism.[10] The S_P isomer of the d(A_{PS}A) dinucleotide (**5a**) was hydrolyzed in the presence of $H_2{}^{18}O$ (65 atom %^{18}O) resulting in [^{18}O]-labeled S_P-dAMPS (**1a**). After HPLC isolation, compound **1a** was converted with tandem AK/PK into compound **2a**. The former enzyme is considered to be highly specific toward adenyl 5′-O-mononucleotides.[11] Nonetheless, because it has been reported that the enzyme is also able to phosphorylate CMP,[12] our attempts at pyrophosphorylation of dNMPS other than dAMPS and AMPS are in progress. In a crucial experiment, compound **2a** was used for the TdT-catalyzed elongation of hexamer **3**. This substrate was selected for two reasons. The enzyme can elongate oligomers as short as tetranucleotides, but for short substrates the efficiency of elongation is low. Also the enzyme has much higher affinity to homooligonucleotides containing only dG or dA residues, as compared with dT- or dC-rich oligomers.[8] We intended to elongate the primer only by one nucleotide and, therefore, we used an equimolar concentration of the primer and dATPαS and only 1 h of incubation of the reaction mixture. Under these conditions 35% of the primer was elongated by one nucleotide residue with the formation of the d($A_5A_{PS}A$) heptamer (**4a**) and 15% was elongated twice to form octamer d($A_5A_{PS}A_{PS}A$). These products were isolated by HPLC and analyzed by MALDI-TOF spectrometry without additional enzymatic degradation.

Because nuclease P1 operates via the one-step mechanism with inversion of configuration, hydrolysis of S_P-**5a** in [^{18}O]-water furnishes S_p-**1a**, subsequently converted into S_P-**2** with [^{18}O]oxygen in the *nonbridging position*. Therefore, after elongation of **3**, the isotope label should be retained in **4a**. The relevant MALDI-TOF mass spectra (Figure 1A) showed peaks at *m/z* 2143 and 2145 of expected intensity, corresponding to unlabeled and labeled heptamers **4**. This result confirms the utility of our method for configurational analysis of isotopomerically labeled **1**.

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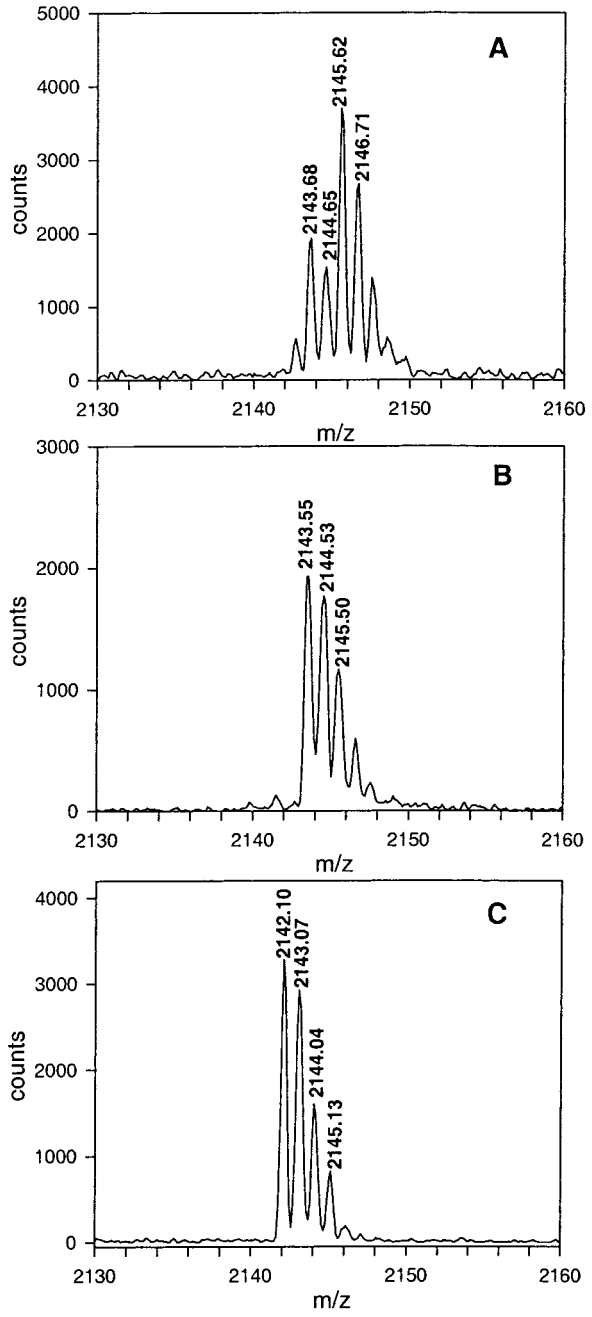

Figure 1. MALDI TOF mass spectrometry analysis for heptamers **4**: panel A, **5a** converted to **4a**; panel B, **5b** converted to **4b**; and panel C, **6** converted to **4b**. Spectral parameters are described in the Experimental Section.

In a control cross-experiment we used a dinucleotide S_P-d($A_{PS(^{18}O)}$A) (**5b**) containing the [^{18}O]oxygen (47 atom % ^{18}O) at the nonbridging position, synthesized by methodology developed recently in our laboratory.[13] **5b** was degraded in unlabeled water by the nuclease P1. Resulting [^{18}O]-labeled dAMPS (**1b**) possessing the 5′-*O*-[^{18}O]phosphorothioate moiety of the absolute configuration opposite to that in **1a** was isolated by RP-HPLC and converted into the corresponding dATPαS (**2b**) as described for **2a**. MALDI-TOF analysis of **4b** (obtained by the use of the **2b** in the TdT-catalyzed elongation of **3**) showed a peak at *m*/*z* 2143, which, as expected, was not accompanied by a peak at *m*/*z* 2145 (Figure 1B). Table 1 summarizes results obtained for the nuclease P1 with the use of these two substrates.

Taking into account the mechanisms of enzymatic phosphoryl transfer reactions used for designing the presented protocol, some general rules can be defined. Stereoinvertive hydrolysis of internucleotide phosphorothioate of S_P absolute configuration in $H_2{}^{18}O$ furnishes **1a**, which under enzymatic treatment provides **4** carrying the [^{18}O]label. In the case of stereoretentive hydrolysis of the S_P phosphorothioate bond in $H_2{}^{18}O$ the formation of **4** leads to the loss of the [^{18}O]oxygen atom. An oligonucleotide substrate with R_P phosphorothioate at the scissile bonds yields the opposite result: loss of the [^{18}O]oxygen atom for a one-step hydrolysis and its retention for a two-step reaction.

The present methodology has been applied to the determination of the stereochemistry of nucleolytic cleavage by human plasma 3′-exonuclease.[14] The enzyme is responsible for the degradation of oligonucleotides and their phosphorothioate analogues used as antisense therapeutics against viral, cardiovascular, cancer, and other diseases.[15] Previous studies have shown that the enzyme degrades phosphorothioate internucleotide bonds of R_P configuration, while S_P bonds are resistant to its action.[16] This enzyme has not been purified, cloned, or sequenced and was not available in pure form. The oligonucleotide degradation had to be performed with 50% human plasma in the ratio 2 mL per 1 OD unit of the oligonucleotide substrate. To avoid the consumption of a few milliliters of labeled water, we used as the substrate for exonucleolytic activity the hexamer d($A_{PS}A_{PS}A_{PS-SP}A_{PS(^{18}O)-RP}A_{PS(^{18}O)-RP}$A) (**6**) containing at the 3′-end two consecutive phosphorothioate internucleotide bonds of R_P configuration labeled in the nonbridging positions with the [^{18}O]oxygen (Scheme 2). The phosphorothioate linkage of S_P configuration in the middle of this compound was introduced to prevent hydrolysis of the nonlabeled part of the oligonucleotide. Oligonucleotide **6** was incubated with plasma at 37 °C for 4 h. The resulting dAMPS[^{18}O] was isolated by HPLC and converted to **4** as described above. MALDI-TOF mass spectra of this heptamer (Figure 1C) contained a signal at *m*/*z* 2143 corresponding to the unlabeled oligomer. Taking into account that the enzyme is R_P-selective and the [^{18}O]oxygen was chemically incorporated into the substrate, the loss of [^{18}O]-label proved that the 3′-exonuclease-assisted hydrolysis proceeds by a double displacement mechanism with the participation of an enzyme−substrate intermediate.

The procedures presented above allow for the determination of the stereochemistry of exonucleases degrading DNA. However, terminal transferase is able to use as the substrates not only dNTPαS but also NTPαS. In the presence of NTP, the enzyme can elongate a DNA primer by 1−4 ribonucleotide units forming chimeric DNA-RNA product.[17] In the same way the enzyme elongates the primer using ATPαS and the efficiency of this reaction is comparable to that observed for dATPαS (unpublished observation). Therefore, the TdT-based method

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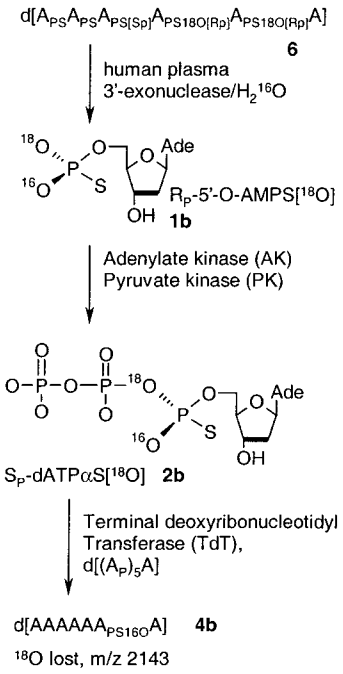
Table 1. TdT-Based Analysis of the Stereochemical Course of Hydrolysis by Nuclease P1

substrate	water	dAMPS	dATPαS	$d(A_5A_{PS}A)$
S_P-**5a**	$H_2{}^{18}O$	dAMP [^{16}O, ^{18}O, S] (MW: 345 + 347)	dATPα [^{16}O, ^{18}O, S] (MW: 505 + 507)	$d(A_5A_{PS(18O)}A)$ (MW: 2143 + 2145); ^{18}O present
S_P-**5b**	$H_2{}^{16}O$	dAMP[^{16}O, ^{18}O, S] (MW: 345 + 347)	dATPα[^{16}O, ^{18}O, S] (MW: 505 + 507)	$d(A_5A_{PS(16O)}A)$ (MW: 2143); ^{18}O lost

Scheme 2



also can be applied to stereochemical studies of ribonucleases. Moreover, it also can be used for determining the stereochemistry of endonucleases, but in this case the resulting 5′-phosphorothioylated oligonucleotide has to be degraded by nuclease P1 (or other nuclease) to yield 5′-phosphorothioylated mononucleotide, which is subsequently converted to dATPαS, as described above.

In summary, here we present a novel method for assignment of absolute configuration at the phosphorus atom in isotopomeric deoxyadenosine 5′-*O*-[^{18}O]posphorothioates. So far, the only limitation of this method is caused by high substrate specificity of adenylate kinase, and the presence of deoxyadenosine or adenosine located 3′- to the scissile bond is required. Further studies on the extension of this method to other nucleoside 5′-*O*-phosphorothioates are in progress.

Experimental Section

Enzymes and Chemicals. Adenylate kinase and pyruvate kinase were purchased from Sigma (St.Louis, MO). The adenylate kinase was supplied as an $(NH_4)_2SO_4$ suspension and was dialyzed extensively against 50 mM Tris (pH 7.5), 0.1 mM EDTA, and 0.1 mM dithioerytreitol prior to use. Terminal deoxynucleotidyl transferase was obtained from Amersham (Buckinghamshire, UK). Nuclease P1 was obtained from Pharmacia LKB (Uppsala, Sweden). [^{18}O]Water was purchased from ICON Isotopes (Summit, NJ). Plasma was isolated from human blood containing 0.38% sodium citrate by centrifugation at 16 000 g for 5 min. Plasma aliquots were carefully withdrawn, leaving the packed cells untouched.

Chemical Synthesis of Oligonucleotides. The synthesis of unmodified oligonucleotides and [Mix]-d($A_{PS}A$) dinucleotide (**5a**) was performed on an ABI 394 DNA synthesizer (Applied Biosystems, Inc., Foster City, CA) at a 1 μmol scale using a standard phosphoroamidite or phosphoroamidite/sulfurization protocol, respectively. The diastereomers of the dinucleotide **5a** were separated by RP-HPLC (ODS Hypersil, 5 μm, 4.6 × 220 mm column) using the linear gradient 0−30% CH_3CN/0.1 M TEAB (triethylammonium bicarbonate), pH 7.4, 0.85%/min at a flow-rate of 1 mL/min.

The synthesis of stereodefined [PS]-dinucleotide **5b** and [PS]-oligonucleotide **6** was performed manually. The first nucleoside units were anchored to the solid support by a sarcosinyl linker. 5′-*O*-DMT-*N*^{6}-Bz-deoxyadenosinyl monomers possessing 3′-O-(2-thio-"spiro"-4,4-pentamethylene-1,3,2-oxathiaphospholane) moiety were synthesized and separated chromatographically into pure diastereomers. Isotopically labeled deoxyadenosyl monomer, necessary for synthesis of the stereodefined PS^{18}O internucleotide bond, was obtained using phosphitylating reagent 2-chloro-"spiro"-4,4-pentamethylene-1,3,2-[^{18}O]-oxathiaphospholane (47 atom % ^{18}O) carrying an isotope label in the endocyclic position. The protocol of the synthesis has been published elsewhere.[18] All oligomers were purified either by two-step RP-HPLC (DMT-on and DMT-off, an ODS Hypersil column) or by ion-exchange chromatography (a DNAPack PA 100 column, Dionex, Austin, TX) and their purity and identity was assessed by polyacrylamide gel electrophoresis and MALDI TOF mass spectrometry.

Enzymatic Reactions. Degradation of di- and oligonucleotides and the corresponding phosphorothioate congeners with nuclease P1 was carried out in 40 μL of buffer containing 100 mM Tris-Cl (pH 7.2) and 1 mM $ZnCl_2$ in the presence of 2 μg of the enzyme. The reaction mixtures were incubated at 20 °C for 3 h. After quenching the reactions by heating at 95 °C for 2 min, the samples were evaporated and the resulting dAMPS[^{18}O] was isolated by RP-HPLC (ODS Hypersil 5 μm column) using the linear gradient 0−30% CH_3CN/0.1 M TEAB (triethylammonium bicarbonate), pH 7.4, 0.85%/min at a flow-rate of 1 mL/min. Under these conditions, dAMPS was eluted at 21.2 min.

The human plasma-catalyzed hydrolysis of the oligonucleotide **6** was carried out using 30 nmol of the substrate dissolved in a mixture of 5 mL of human plasma and 5 mL of PBS (phosphate-buffered saline) divided into 20 samples, each of them containing 500 μL of the reaction mixture. The samples were incubated for 4 h at 37 °C, and then immersed in a boiling water bath for 5 min. Subsequently, 500 μL of water was added to each denatured sample. After vigorous shaking, the protein precipitates were spun down. The washing of the precipitates was repeated and the resulting aqueous solutions were concentrated to dryness in a SpeedVac rotary evaporator. Then, the resulting dAMPS-[^{18}O] (10 nmol) was isolated by RP-HPLC as described above.

Conversion of dAMPS[^{18}O] to S_P-dATPαS[^{18}O]. The dAMPS-[^{18}O] (10−15 nmol), produced by nuclease P1 or 3′-exonuclease-assisted cleavage of oligo(nucleoside phosphorothioate)s, was dissolved in 100 μL of 50 mM Tris (pH 7.5) containing 50 mM KCl, 10 mM $MgCl_2$, 0.8 mM ATP, and 10 mM phosphoenolpyruvate. Adenylate kinase (18 units) and pyruvate kinase (40 μg) were added, and the mixture was incubated at 37 °C for 6 h. Then, after quenching the reactions by heating at 95 °C for 2 min, the samples were analyzed by RP-HPLC and the resulting S_P-dATPαS[^{18}O] was isolated under conditions described above with a retention time 25.75 min.

Terminal Deoxyribonucleotidyl Transferase Assay. The assay mixture (volume 30 μL) containing 1−2 nmol of the dA_6 primer, S_P-

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dATPαS[^{18}O] (1−2 nmol), and 20 units of TdT in 100 mM sodium cacodylate, pH 7.2, 10 mM $MgCl_2$, and 1 mM $CoCl_2$, was incubated at 37 °C for 1 h. Then, after quenching the reaction by heating at 95 °C for 3 min, the resulting products were isolated under conditions specified above (retention times for dA_6, $d(A_5A_{PS}A)$, and $d(A_5A_{PS}A_{PS})$ were 32.1, 35.5, and 38.1 min, respectively).

Mass Spectrometry. Mass spectrometry analyses have been performed using a Voyager-Elite MALDI-TOF mass spectrometer (PerSeptive Biosystems Inc., Framingham, MA) equipped with delayed extraction. Typical conditions included 20 kV acceleration voltage and nitrogen laser pulse (wavelength 337 nm). High-resolution negative-ion spectra were recorded in reflector mode. Two-point external calibration has been made with dT_3 and dT_6 as standards. The matrix used consisted of 2,4,6-trihydroxyacetophenone (10 mg/mL in ethanol) and diammonium hydrogen citrate (50 mg/mL in acetonitrile/water (1:1)), mixed with matrix in a 1:1 ratio on the surface of a sample plate, and left for crystallization at room temperature. The spectra have been obtained with mass spectrometer resolution about 3000 (defined as the following relation: mass/width at half-height of the mass peak), allowing for observation of isotope peaks distribution.

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