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Capillary electrophoretic separation-based approach to determine the labeling kinetics of oligodeoxynucleotides

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Abstract

With the recent advances in electron microscopy (EM), computation, and nanofabrication, the original idea of reading DNA sequence directly from an image can now be tested. One approach is to develop heavy atom labels that can provide the contrast required for EM imaging. While evaluating tentative labels for the respective nucleobases in synthetic oligodeoxynucleotides (oligos), we developed a streamlined capillary electrophoresis (CE) protocol to assess the label stability, reactivity, and selectivity. We report our protocol using osmium tetroxide 2,2'-bipyridine (OsbiPy) as a thymidine (T) specific label. The observed rates show that the labeling process is kinetically independent of both the oligo length, and the base composition. The conditions, i.e. temperature, optimal OsbiPy concentration, and molar ratio of reagents, to promote 100% conversion of the starting oligo to labeled product were established. Hence the optimized conditions developed with the oligos could be leveraged to allow osmylation of effectively all Ts in single-stranded (ss) DNA, while achieving minimal mislabeling. In addition, the approach and methods employed here may be adapted to the evaluation of other prospective contrasting agents/labels to facilitate next-generation DNA sequencing by EM.

Keywords

ssDNA; oligodeoxynucleotides; osmium tetroxide; OsbiPy; thymidine; sequencing; kinetics

1 Introduction

It is anticipated that the advent of cheap and accurate DNA sequencing will usher in a revolution in health care. This thinking has prompted tremendous investment in developing sequencing technologies. Existing sequencing methods read the bases one after another, progressing along the chain. A far-reaching approach in sequencing has been to have a way to simply look at the molecule and obtain structural information directly [1–3]. As a step toward enabling it we sought to render the pyrimidine bases visible for single-atom resolution electron microscopy (EM) by forming their osmium addition products [3]. To this end one could label the DNA, stretch single strands of labeled DNA on suitable surfaces [4], image them by EM, and decipher their base sequence. Here we report our protocol using osmium tetroxide 2,2'-bipyridine (OsbiPy) as a thymidine (T) specific label, and describe using capillary electrophoresis (CE) to unravel the kinetics of its reactions. Although osmium tetroxide 2,2'-bipyridine (OsbiPy) is a well-known reagent for this derivatization [1–3, 5, 6], five aspects of its chemistry needed to be developed to ascertain that it could

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serve as a viable label. Specifically we needed to know: (i) How to analyze the reaction products to assess selectivity and quantify completeness of the reaction; (ii) How to purify labeled products from the excess reagent; (iii) Are the products sufficiently stable for our processing steps? (iv) Is there a labeling rate or selectivity dependence on DNA length? (v) Is there a rate or selectivity dependence on the nucleotide sequence? This report addresses each of these questions.

Osbipy is known to react preferentially with the C5–C6 double bond of exposed T in DNA. Osmium tetroxide (OsO_4) in the presence of tertiary amines or pyridines has been widely applied to probe damage, mismatches, mispairing, or insertions in double stranded DNA [5–9]. These osmylating systems were also shown to react selectively with Thymidine>Cytidine in ss DNA [10], and were exploited for labeling oligonucleotide reporter probes to assess DNA hybridization [11, 12]. Osbipy was reported as more selective in reacting with 5-Me-cytidine over C [13]. The electroactivity of Osbipy has enabled the use of the osmylation reaction for biosensor applications. In this context nanograms of osmium-labeled ssDNA were quantified by differential pulse stripping voltammetry [14, 15]. In addition to probing DNA structure, Osbipy has been used successfully in probing RNA species by efficiently labeling all pyrimidines [16], in dissecting accessibility of tryptophan in proteins [17], facilitating chemical characterization of proteins by peptide derivatization [18] and detecting modified polysaccharides at picomolar concentrations [19].

Our starting understanding was that OsO_4 (VIII) in the presence of 2,2'-bipyridine (bipyridine) is known to react readily with T mononucleotides and with T in oligos by addition to the C5–C6 double bond to yield an osmate (VI) ester while bipyridine remains stably coordinated to the final product (see Scheme 1). Our initial experiments were designed to confirm this understanding and typically were run at 25°C using a premixed stock solution of 12 mM OsO_4 and 12 mM bipyridine. Experiments between Osbipy and deoxythymidine 5'-monophosphate (dTMP) monitored by ^1H -NMR indicated a fast reaction with proton shifts consistent with T-osmylation. In order to extend our investigations to the oligos we selected CE as the main analytical tool. CE technique in its simplest form, free solution or Zone, is based on the separation of analytes in microcapillaries with 10 to 100 μm I.D. filled with an aqueous buffer under the influence of a high electric field. Typically the same capillary/method was used in order to monitor the reaction with mononucleotides, oligos, or ssDNA.

2 MATERIALS AND METHODS

2.1 Materials

Sodium phosphate 100 mM pH 7 (HPCE grade, 1N sodium hydroxide, thymidine, cytidine, the disodium salt of thymidine-5'-monophosphate (dTMP) purchased from Sigma-Aldrich. Solid osmium tetroxide was purchased from Ted Pella and a 4% aqueous osmium tetroxide solution from Spi-Chem; stock solutions prepared from either supplier exhibited comparable reactivity. A premixed solution of 50 mM sodium borate with 100 mM sodium dodecyl sulfate (SDS) pH 9.3 (HPCE grade) was purchased from Agilent Technologies. Deuterium oxide (99% D) was purchased from Cambridge Isotope Labs. J.T. Baker brand 2,2'-bipyridine (bipyridine) was purchased from VWR. Solutions of the triphosphates, dATP, dCTP, dGTP and dTTP, as well as an equimolar mixture of the four dNTPs were purchased from New England Biolabs and stored at –20°C. Oligos were purchased from IDT, they were diluted with water to 5mM and kept at –20°C. One group of oligos, identified as short, are 8 to 11 bases long and include only two bases; the other group, identified as long, are 80 to 90 bases long and include all four bases. Oligos used in this study, their abbreviation, and sequence are listed in Table 1.

Aqueous solutions were prepared with ultra-pure water obtained from a Millipore purification system. Stock solutions of Osbipy at 4, or 12 mM (OsO_4 : bipyridine = 1:1) were prepared by mixing solid bipyridine powder into OsO_4 solution. Stock solution was aliquoted in small glass vials, sealed with parafilm, and kept at -20°C until use; no detectable change in reactivity was observed with solutions that were stored sealed at 4°C for two weeks. Experiments were initiated by mixing the Osbipy stock solution and the oligo stock solution directly in a CE vial filled with the appropriate amount of milli-Q water. The final concentration of Osbipy and oligo in the reaction mixture is reported with each experiment. No additional buffer was used because buffers are known to interact with Osbipy [23]. Unbuffered reaction mixtures gave routinely reproducible rates. The CE instrument carried out automatically the analyses. The aliquoting or quench time together with the product distribution, in the form of an electropherogram, were recorded by the CE. Typically experiments were carried out either at 25 ± 1 or at 44 ± 1 $^\circ\text{C}$ under temperature control.

2.2 ^1H NMR spectra and kinetic evidence for dTMP osmylation

^1H NMR spectra were recorded in D_2O on a Varian Inova spectrometer at 500 MHz. Chemical shifts were reported in ppm relative to the residual solvent peak at 4.79 ppm. A D_2O solution of dTMP was added to a vial containing solid bipyridine. The vial was heated at 60°C and the mixture stirred until homogeneous. The solution was cooled to room temperature and a 5 mm NMR tube was charged with $400\ \mu\text{L}$ of this solution. A solution of OsO_4 in D_2O was added to the NMR tube and spectra were acquired at defined time points over the course of the reaction. Reaction progress was measured by integration of the starting material peaks at 7.84 and 6.36 ppm, and the product peaks at 6.46, 6.27, 5.58 and 5.50 ppm.

2.3 CE analyses and methods

Analyses of the reaction mixtures were carried out using an Agilent 1600 CE instrument equipped with Diode Array Detector (DAD) and Chemstation software, Rev.B.04.02SP1 [212], for data acquisition and processing. The autosampler of the CE instrument was kept at a constant temperature using an external bath. Experiments conducted at 44°C were done in temperature-equilibrated vials. Only glass type CE vials were used, as Osbipy was found to react with polyurethane vials, lowering its effective concentration. CE vials are not completely sealed, and minor evaporation occurs with overnight analyses. This evaporation amounts to no more than a 5% sample concentration effect. In contrast to the other components, OsO_4 evaporates easily and this effect may prevent accurate rate determinations for the slower reactions. Untreated fused silica capillaries (short: $50\ \mu\text{m}$ x 40cm, long: $50\ \mu\text{m}$ x 72cm) with extended light path were purchased from Agilent Technologies. Capillaries were conditioned before use with 0.1N NaOH for 10 min, and reconditioned based on performance.

The CE methods (solution or zone) used for this work include 25 or 50 mM sodium phosphate buffer at pH 7 in conjunction with 20 to 30kV, or 80 mM TRIS acetate at pH 8 run with 30kV. The capillary temperature was maintained at 20°C for all the analyses. A typical method was 6 to 25 min long and included a 2 to 5 min automatic buffer wash ahead of sample analysis. Osbipy migrates early together with any other neutral compound present, nucleotides and oligos migrate last, and Osbipy-labeled products migrate in between. Resolution between starting materials and products was excellent for the short oligos. We also employed micellar electrokinetic chromatography (MEKC) using a 25 mM borate 50 mM SDS buffer at pH 9.3, in conjunction with the short capillary at 20kV. MEKC is the only electrophoretic technique that can be used for the separation of neutral solutes as well as charged ones. We applied the MEKC method to investigate the presence of the OsO_4 -

bipy complex (see Scheme 1) in the Osbipy stock solutions and its reactivity towards the nucleoside. This method achieved good resolution with peaks migrating in the order: OsO₄ first, bipy-OsO₄ complex, the nucleoside (thymidine), the product of the reaction (bipy-OsO₄-thymidine), and bipyridine last.

Peaks on CE were detected and identified using the DAD in the UV-vis region 200 to 450nm and the electropherograms were recorded at both 260 and 320nm. In contrast to mononucleotides and oligos that show no detectable absorbance at 320 nm, Osbipy-labeled products absorb at 320 nm [24]. We have exploited this feature to determine kinetics and to monitor extent of labeling.

2.4 Determinations of observed rates

Reactions were conducted under pseudo-first-order conditions and analyzed using the simplest form of CE, CZE in a phosphate buffer. A ratio of [Osbipy] : [T] = 12 led to complete consumption of starting material, but only when [Osbipy] = 3 mM. dTTP or dNTP were used at 0.08 or 0.16 mM, the short oligos in the range 0.025 to 0.075 mM, and the long oligos in the range 0.002 to 0.005 mM. Observed rate of dTTP or oligo disappearance, k_{obsd} (1/min), were obtained by plotting the natural logarithm (ln) of dTTP or oligo peak area as a function of incubation time (eq 1). Attention was given to obtain most of the data within the first two half-lives of the reaction to improve accuracy. Data were fitted linearly using Microsoft Excel with typical relative standard deviations at about 5%. The absolute value of the slope to the linear fit corresponds to k_{obsd} .

$$\text{Integrated first-order law: } \ln[\text{dTTP}] = -k_{\text{obsd}}t + \ln[\text{dTTP}]_0 \quad \text{eq 1}$$

In eq 1 [dTTP] is the concentration of dTTP or area under the dTTP peak, [dTTP]₀ is the initial concentration of dTTP, t is the incubation time or the time between mixing the reactants and the aliquoting time, as recorded by the CE. Values k_{obsd} represent the observed rate of the process which is independent of [dTTP]₀, but dependent on Osbipy concentration. When dTTP is replaced by an oligo, then k_{obsd} refers to the rate of oligo disappearance.

When the oligo has one T only, the rate of oligo disappearance is the same as the rate of labeled product appearance. When the oligo has more than one T, the rate of oligo disappearance is for statistical reasons faster compared to the appearance of the final, fully labeled, product. With the long oligos that contain 19 to 29 Ts our methods were not fast enough to measure the rate of oligo disappearance. Nevertheless the rate of product appearance was determined by three different methods, as described below.

Rates of product appearance, k_{obsd} , for long oligos were obtained by plotting (i) the exponential decrease of the migration time (mt) shift and/or (ii) the exponential increase of the absorbance at 320nm as a function of incubation time. These two methods suffer from the following limitations: Migration time (mt) peak shifts due to increased labeling are dependent on peak shape, and prone to some uncertainty due to small mt shifts associated with typical run to run variation. The use of a standard is recommended to ensure that mt shifts are within acceptable limits. Comparable uncertainty is also associated with CE peak area measurements at a certain wavelength that may vary from run to run due to instrument sampling. The third method is free of the above uncertainties. It relies on the ratio of peak area at 320nm over the peak area at 260nm, identified as Ratio 320/260 (method iii). This measure is independent on mt shift and instrument injection/sampling. Because Ratio 320/260 is independent of the resolution between starting materials and products, it is especially useful for measuring relatively fast rates that require short analysis time.

To determine observed rate for product appearance it is important to establish infinity (6 to 7 half-lives) of the process, so that the difference between the specific property at a given time (property at t) to the infinity (property at infinity) is correlated to the incubation time (t). For example, in Figure 9b the average value of the raw data at incubation time longer than 75 min provide the infinity value (mt at infinity). To apply method (i) above, one may plot $\ln\{(mt \text{ at time } t) - (mt \text{ at infinity})\}$ with time t (see Figure 9b). To apply method (ii) above, one may plot $\ln\{(\text{peak area at 320nm at infinity}) - (\text{peak area at 320nm at time } t)\}$ with time t (not shown). To apply method (iii), one plots $\ln\{(\text{infinity Ratio 320/260}) - (\text{Ratio 320/260 at time } t)\}$ with time t (see Figure 8). Rates obtained by either one of the three methods were typically in excellent agreement with each other.

In biphasic kinetics the infinity of the fast process depends on the slow process and hence depends on time t . An example is presented in Figure 10a, where the fast process refers to T-labeling and the slow process refers to C-labeling (see Results and Discussion under long oligos). The solid line in Figure 10a represents the time-dependent infinity of the fast process and was used to obtain k_{obsd} . One may define infinity Ratio 320/260 (T) as the theoretical complete T-labeling of this oligo in the absence of C-labeling interference. In Figure 10a infinity Ratio 320/260 (T)=0.3751 is given by the 0 time value of the extrapolated line of the slow process. Similarly, one may define infinity Ratio 320/260 (T+C) the 320/260 ratio once both T and C are fully labeled. Infinity Ratio 320/260 (T+C) values for the long oligos were determined from experiments conducted at 35°C for 50 hours in the presence of 11.6 mM Osbipy. Rate k_{obsd} for the slow C-labeling was obtained by normalizing the slope of the solid line (slope=0.0003 in Figure 10a) against the difference of the two infinities {infinity Ratio 320/260 (T+C)-infinity Ratio 320/260 (T)}. Values of k_{obsd} and infinities are listed in Table 1.

2.5 MALDI TOF determinations

Analyses were performed on a Bruker MALDI-TOF instrument in the positive or negative linear mode, externally calibrated with an oligonucleotide mix. Labeled oligos were analyzed after removal of unreacted Osbipy. The matrix used was purchased from Bruker and is 10mg/mL diammonium hydrogen citrate in 1:1 water-acetonitrile saturated with 3-hydroxy picolinic acid. Observed molecular ion mass was in satisfactory agreement to the theoretical one for several of the labeled oligos and clearly showed that bipyridine is quantitatively present in the labeled product. For example, Osbipy-oligoT1 determined at 3150 (theor. 3161), (Osbipy)2-oligoT2 determined at 3246 (theor. 3253) and (Osbipy)3-oligoT3 determined at 4538 (theor. 4597).

2.6 Purification /removal of excess Osbipy label

TrimGen spin columns TC-100 were used to remove excess Osbipy at the conclusion of the labeling reactions according to the manufacturer's instructions. Practically 100% recovery of labeled oligo and removal of up to 3 mM Osbipy to detectability levels after just one round was achieved.

3 Results and Discussion

3.1 Protocol for evaluation of the reactivity and selectivity of a prospective DNA Label

The following attributes are desired for a molecule to be considered as a viable single strand oligonucleotide/DNA label: 1) Reactivity in the mM range and completion of labeling within hours; 2) High selectivity for one of the four nucleobases, so that the labeled DNA includes a relatively small percentage of mislabeled bases; 3) Negligible DNA degradation under the labeling conditions; 4) Labeled products with good stability and solubility in aqueous media.

The protocol we established for tentative label evaluation and described here for Osbipy starts by assessing reactivity and selectivity with an equimolar mixture of dNTPs, continues with short oligos that contain T or C within a series of Adenosines, and ends with long oligos that contain all four nucleobases (see Table 1 for the list of oligos). This step-wise approach of increased complexity is necessitated by the absence of analytical tools to dissect sites of reactivity in biopolymers and presumably results in a detailed and thorough understanding of the labeling process.

3.2 ^1H NMR spectra of the reaction between dTMP and Osbipy

^1H NMR provided evidence for the osmylation reaction with mononucleotides. A 10 mM solution of Na_2dTMP (dTMP) containing 10 mM of bipyridine was treated with 20 mM OsO_4 at 25 °C. As the intensity of the dTMP peaks decreased, the intensity of the product peaks increased proportionally (see Figure 1). The resonance at 7.84 ppm (C-6 of the pyrimidine) was converted to two resonances at 5.50 and 5.58 ppm. The resonance at 6.36 (1'-H of the deoxyribose) was converted to two resonances at 6.46 and 6.27 ppm. For both sets of signals, the product peaks appeared in a 3.5:1 ratio throughout the course of the reaction indicative of the kinetically controlled formation of two diastereomeric products (more on this later). In the absence of bipyridine, no reaction was observed between dTMP and OsO_4 over the same time. As the reaction proceeded, signals for coordinated bipyridine grew in intensity as well (9.38 and 9.16 ppm for the major diastereomer, 9.37 and 9.27 ppm for the minor diastereomer, not shown). As control a solution containing bipyridine and dTMP (10 mM each) gives the same chemical shifts as the individual components. The rate of the osmylation reaction showed a strong dependence on concentration. At a concentration of 20 mM OsO_4 , 10 mM dTMP, and 10 mM bipyridine, the reaction showed an apparent half-life of less than one minute. Reducing the overall concentration by a factor of two gave a half-life of six minutes.

3.3 Evidence for OsO_4 -bipy complex obtained by MEKC

Based on the high reactivity of Osbipy for T, it was initially anticipated that a 1:1 solution of OsO_4 and bipyridine at 12 mM each contains substantial amounts of the complex bipy- OsO_4 (Scheme 1). However the addition of OsO_4 to a D_2O solution of bipyridine showed no evidence of coordination in the NMR spectrum. CZE does not discriminate between neutral solutes. Therefore OsO_4 , bipyridine, as well as their complex appear as one peak. To achieve resolution of the neutral solutes we exploited micellar electrokinetic chromatography (MEKC), as described in the Experimental Section. Using the MEKC method we detected a small peak that migrates right after the OsO_4 peak and exhibits an increase as a function of OsO_4 and bipyridine concentrations. This new peak, assigned to the bipy- OsO_4 complex, exhibits a substantially different UV-vis spectrum compared to either OsO_4 , or bipyridine, or the sum of both. In contrast to the observed slow evaporation of the OsO_4 peak overnight, the new peak exhibits a slight concentration effect over time, consistent with its assignment as a bipyridine complex. From these experiments the conjecture was made that bipy- OsO_4 accumulates at no more than 10% under the tested conditions. This estimate is in accordance with equilibrium constants reported for the complex formed between OsO_4 and tertiary amines in various solvents at 25°C [21, 23].

3.4 Reactivity of Osbipy with mononucleotide triphosphates

Figure 2a shows the reaction of 0.16 mM dTTP with 2.2 mM Osbipy in water at 25°C as monitored by CE at 260 nm. At these conditions dTTP is practically consumed in two hours. The product appears by CE as a doublet, consistent with the observations by ^1H -NMR, and suggestive of Osbipy addition across the C5–C6 double bond from either side of the T-ring resulting in two diastereomers represented as (5S,6R) and (5R,6S) [25]. Reactions with dNTPs were carried out at 25°C with a large excess of label to dNTP. The minimum

concentration of dNTP that gives reliable rates is 0.08 mM. In the presence of 1.3 mM Osbipy dTTP did not completely disappear even after 1 day, so we deliberately exploited higher concentrations of Osbipy up to 3.6 mM. Reactions carried out in the presence of all four nucleotides were kinetically comparable with reactions conducted in the presence of one nucleotide only, suggesting that Osbipy does not form any unproductive association complexes with the other three nucleobases at these conditions.

Figure 2b shows the UV-vis spectra of the peaks of Osbipy, dTTP, and Osbipy-dTTP illustrating that while Osbipy and dTTP have no detectable absorbance at 320 nm, Osbipy-dTTP absorbs significantly at 320nm. This feature, validated for all the Osbipy-T, as well as for the Osbipy-C products, was routinely exploited for rate of product formation and extent of labeling determinations.

Reaction of 3.3 mM Osbipy with an equimolar mixture of dNTPs, at 0.16mM each, was monitored for over 3 hours at 25°C (see Supporting information Material 1, SM1a). The data suggest no detectable reactivity with the triphosphates of the purine-nucleosides, a high reactivity for dTTP ($k_{\text{obsd}} = 0.046$ per min) and a low reactivity for dCTP ($k_{\text{obsd}} = 0.0016$ per min) leading to an intrinsic selectivity = 29 for T over C. Reaction rates obtained for dTTP in the presence of 1.3 to 3.6 mM Osbipy correlate linearly with label concentration (see Supporting information Material 1, SM1b), but because this range is rather narrow broader mechanistic conclusions were not drawn.

To confirm the observed high selectivity we conducted competition experiments in mixtures of dTTP and dCTP, with $[\text{dCTP}] \gg [\text{dTTP}]$. The two nucleotides were premixed, Osbipy was added, and the mixture was analyzed by CE after a few minutes incubation at 25°C. Starting materials and products resolve well from each other by CE. The length of incubation actually does not matter as long as most of the dTTP remains intact. Table 2 summarizes the observed CE areas at 260nm as well as the calculated total areas and percent conversion for each. The ratio of conversion to product is a measure of selectivity and the average of three such competition experiments provided 25 ± 2 -fold selectivity, in agreement with the 29-fold selectivity determined from the corresponding reactivities of dTTP and dCTP.

Cotton and coworkers [5] reported the use of OsO_4 for labeling mismatches in double stranded DNA for both T and C, but pointed out the higher selectivity for T over C. Jelen and coworkers [10] reacted OsO_4 for 20 hours in the presence of bipyridine with each DNA homopolymer, dT, dC, dG, and dA. They measured content of modified bases by stripping voltametry and absorption spectrophotometry and reported that modification of the base was the highest for poly(dT). Poly(dC) modification was about 10-times lower compared to poly(dT). The observation of 10-fold selectivity for T over C with the homopolymers [10] is not in disagreement with the data presented here. Our results approximate intrinsic selectivity, whereas Jelen's data refer to the apparent selectivity observed after most of the Ts in poly(dT) were labeled. More on apparent selectivity will be discussed later.

3.5 Reactivity of Osbipy with oligos 8 to 11 bases long

The reaction of Osbipy with short oligos, T1, T2, T3, C1, C2, C3 (see Table 1) was explored next. Figure 3 shows consecutive CE profiles from a reaction of 0.08 mM oligoT1 with 1.3 mM Osbipy, a ratio of $[\text{label}]:[\text{T}] = 16$. The reaction forms one product, assigned as Osbipy-oligoT1 or P1 in the figure, seen as a doublet, and rationalized by the facial selectivity of the reagent as described earlier for dTTP. The reaction reaches infinity overnight, and, most importantly, Osbipy-labeled oligoT1 remains unchanged over the next two days at 25°C and in the presence of the excess label. Experiments at higher Osbipy concentration, [Osbipy] 1.3 mM, lead quickly to complete oligoT1 peak disappearance. In contrast to oligoT1,

oligoC1 reaction yields couple of minor peaks that appear to increase slightly over the next few days, while the oligoC1 peak appears practically intact (see Supporting information Material 2).

Figure 4 illustrates three time points of the osmylation reaction with oligoT2. This oligo quickly disappears leading to the formation of a group of peaks identified as P1, which stands for the mono-labeled-oligo, and P2, which stands for the di-labeled-oligo. P1 gradually appears and then gradually disappears, while P2 accumulates. The flow of material monitored every 23 min (not shown here), clearly shows that P2 is formed from P1 that is formed from oligoT2. Figure 5 compares the product distribution of two reaction mixtures, one with oligoT2 (top) and the other with oligoC2 (bottom), under otherwise identical conditions. Specifically, Figure 5 illustrates that after 19 hours oligoT2 has disappeared and replaced by approximately 90% P2 and 10% P1. In contrast, oligoC2 is still the major peak after 19 hours; only a small amount of material, that exhibits an earlier mt shift compared to the starting oligo, is observed and assigned to Osbipy-oligoC2 products.

Why are multiple peaks observed for both P1 and P2? Based on the facial selectivity of Osbipy for the C5–C6 double bond in T, one may assign “a” to the isomer with Osbipy located over the T-ring and “b” to the isomer where Osbipy is located under the T- ring (arbitrarily set: over and under in 5' to 3' strand direction). There are four theoretical diastereomers: AAA T^aTA AA, AAA T^bTA AA, AAA TT^aA AA, and AAA TT^bA AA. CE analysis provided evidence for three out of the plausible four and the issue was not pursued any further. Similarly, there are four plausible diastereomers predicted for the di-labeled oligoT2: AAA T^aT^aA AA, AAA T^aT^bA AA, AAA T^bT^aA AA, and AAA T^bT^bA AA. CE analysis provided evidence of all four, even though base resolution was not achieved (see Figure 5, top profile). Evidence for the presence of all four plausible diastereomers and specifically for accumulation of the isomer, AAA T^bT^aA AA, has interesting implications in discussing steric hindrance to labeling tandem Ts. Isomer AAA T^bT^aA AA includes two Osbipy molecules positioned “under-over”, i.e., in the space between two tandem Ts. The presence of this isomer implies no detectable steric hindrance to formation of the most sterically hindered isomer.

Figure 6a illustrates the corresponding reaction of Osbipy with oligoT3 initially monitored in 20min intervals. These profiles clearly illustrate the disappearance of oligoT3 with concomitant appearance of a group of peaks identified as P1 (stands for mono-labeled-oligo), followed by the appearance of another group of peaks identified as P2 (stands for di-labeled-oligo), followed in turn by the appearance of the last group of peaks, identified as P3 (stands for tri-labeled-oligo). After 23 hours oligoT3 is completely consumed with most of the material accumulating under P3 and the rest under P2. Stability of P3 is excellent under the reaction conditions, as shown by comparing the bottom two profiles in Figure 6a obtained 48 hours apart. Additional experiments at 2.6 or 3.0 mM Osbipy quickly consume oligoT3 and produce P3 quantitatively. Attempt to better resolve the multiple peaks observed under P3, led us to analyze a TrimGen purified P3 with a longer capillary (see Experimental) and obtain a set of 6 partially resolved peaks (Figure 6b), in place of the expected 8 peaks corresponding to 8 plausible isomers (see earlier discussion and caption of Figure 6b). In analogy to the observations with oligoC1 and oligoC2, oligoC3 shows negligible reactivity within the time period required for complete labeling of oligoT3 (profile not shown).

Instability of the short oligo osmylated-products in the presence/absence of Osbipy could be detected by CE analysis to an estimated 1% level of degradation by the appearance of new minor peaks as a function of storage time. Actually we were somewhat surprised that the purified osmylated products do not release any bipy-OsO₄ upon storage.

In the presence of 1.3 mM Osbipy, the rates of oligo disappearance for T1, T2 and T3 are $k_{\text{obsd}} = 0.0081, 0.0256, \text{ and } 0.0374$ per min, respectively. Figure 7 shows that these rates correlate well with the number of Ts in each oligo. The observed linear correlation is consistent with statistical considerations. The observed linearity is also consistent with the absence of steric hindrance, or acceleration for that matter, for labeling the two tandem Ts in oligoT2. In the case of steric hindrance the oligoT2 rate would deviate negatively, and in the case of acceleration this rate would deviate positively from the line defined by oligoT1 and oligoT3.

Our conclusion that rate of osmylation is independent of the neighboring base, be it T or otherwise, is not in agreement with the observations reported by Nomura and Okamoto [26]. They reported higher reactivity of oxidation for tandem Ts compared to isolated ones. Nevertheless their study and ours are not directly comparable. We routinely exploited mild conditions with 3 mM Osbipy, whereas they used a combination of 100 mM of potassium osmate and 100 mM of potassium hexacyanoferrate. After oxidation, Nomura and Okamoto [26] treated the samples with piperidine at 90°C for 20 min in order to cleave the phosphodiester bond at the oxidized T sites. It is plausible that the harsh oxidizing and cleaving conditions [26] resulted in the apparent difference of oxidation between isolated and tandem Ts.

Several experiments were conducted with the aim to determine intrinsic selectivity of Osbipy for T vs. C with oligos. For example, in the presence of 2.6 mM Osbipy in water at 25°C, the rate of oligo disappearance for oligoT1 and oligoC1, was determined to be $k_{\text{obsd}} = 0.044$ and 0.0014 per min, respectively. The ratio of these rates provides 31-fold selectivity, in good agreement with the $25(\pm 2)$ -fold selectivity determined using dNTPs (see above). Alternatively, in the presence of 1.3 mM Osbipy in water at 25°C the rate of oligo disappearance for oligoT3 and oligoC3 was determined to be $k_{\text{obsd}} = 0.0374$ and 0.0081 per min, respectively. The ratio of these rates provides 47-fold selectivity for T over C. To improve upon these measurements we conducted experiments with the long oligos, as will be described below.

In contrast to the oligos with a small number of Ts, oligoT8 (Table 1) reaction with Osbipy yielded poorly resolved products by CE. Instead we observed peak broadening, a shift in the mt of the starting oligo peak with incubation time towards earlier times, and the formation of a rather sharp peak with stable mt at the end of the process. In addition there was an increase of the peak area at 320nm with incubation time, while the peak area at 260nm remained the same within experimental error. Upon closer inspection the oligoT8 peak's mt shift exhibited an exponential decrease with incubation time. Similarly, the peak area at 320nm exhibited an exponential increase with time. Both changes were successfully used to determine observed rates of product appearance as described in detail in the Experimental Section. A third measure, proved to be more accurate compared to mt shift and 320nm increased absorbance (see Experimental Section). This measure is the increase in the ratio of the peak area at 320nm over the peak area at 260nm, abbreviated Ratio 320/260, with time. Figure 8 illustrates the use of Ratio 320/260 to plot the data obtained from a series of experiments that evaluated the effect of oligoT8 concentration on the rate of labeled product formation. As seen in Figure 8, all three different concentrations of oligoT8 (0.025, 0.050 and 0.075) when reacted with 3 mM Osbipy exhibited comparable values of Ratio 320/260 over a 300 min incubation time. This observation suggests no dependence of k_{obsd} on oligoT8 concentration, as expected for a reaction where oligoT8 is the minor component. The resulting plateau obtained from the data between 130 and 300 min has an average value of $\text{Ratio } 320/260 = 1.51 \pm 0.02$. This value, associated with complete labeling of all Ts, is abbreviated Infinity Ratio 320/260 (T), and listed in Table 1. Such values determined for the oligos in this study are listed in the penultimate column in Table 1.

To investigate the ensuing absorbance changes for C-labeling we used oligos TC1, TC2, and TC3 (Table 1) and allowed the osmylation reaction to proceed in the presence of 11.6 mM Osbipy at 35°C for 50 hours, conditions developed to label essentially all Ts and all Cs. Values for Ratio 320/260 for these three oligos after complete labeling, abbreviated infinity Ratio 320/260 (T+C), are also listed in Table 1. These values are somewhat lower compared to infinity Ratio 320/260 (T) determined for oligoT8 and, perhaps, suggest a somewhat smaller absorbance change associated with C-labeling compared to T-labeling (more on the extent of labeling later).

3.6 Reactivity of Osbipy with oligos 80 to 90 bases long

Initially we anticipated that labeling long oligos would take much longer compared to labeling short oligos, so we conducted experiments with the CE autosampler at 44°C to speed up osmylation. Figure 9a, trace #1, shows the CE profile of a reaction between oligo L1 (Table 1) and 2.6 mM Osbipy conducted in water at 44°C obtained 3min after initiating the reaction by adding Osbipy. Even after only 3min L1 has reacted as evidenced by a peak shift to an earlier mt compared to the mt of L1 in the absence of label. The more extensive the labeling, the earlier the peak migrates. Since mt shifts can also be due to instrument instability, dCTP was included in the reaction mixture as an internal standard. Figure 9a, traces # 2 to 5, show consecutive analyses of the reaction mixture L1 with 2.6 mM Osbipy in the presence of dCTP. In contrast to the dCTP peak that shows no detectable mt drift and no reaction during this short incubation, the L1 peak exhibits a dramatic mt shift with time, strongly suggestive of labeling.

Analyses of the above reaction mixture, one after 77 min and the other after 106 min exhibit excellent overlap of these two traces suggesting an apparent infinity (see Supporting information Material 3). Figure 9b shows a plot of the observed mt shifts. These mt shifts decrease exponentially with time, consistent with a kinetically well-behaved pseudo-first order process. We also noticed that the L1 peak areas observed at 260nm during reaction were comparable within experimental error. On the contrary, peak areas at 320nm started from zero and ended up at a maximum value providing a plot (not shown) that exhibits an exponential increase. We determined the rate of L1 labeling by mt shift as well as by peak area at 320nm, for a number of conditions with excellent agreement. In analogy with the experiments conducted with oligoT8, experiments with the long oligos were also evaluated using the third measure, i.e., Ratio 320/260, as a function of incubation time.

Since the work with the dNTPs established some reactivity of Osbipy for C, we used the four long oligos (Table 1) and extended the incubation time to 12 or more half-lives of the T-labeling. Indeed we observed a much slower process attributed it to C-labeling. Figure 10a illustrates the experimentally determined Ratio 320/260 with incubation time for the reaction of 2 μ M L1 with 3 mM Osbipy at 25°C. Two processes can be clearly seen, a fast one associated with T-labeling, and a slow one associated with C-labeling. Comparable plots to Fig 10a were obtained for the reaction of the other three long oligos in the presence of 3 mM Osbipy. Experiments of these four long oligos with 6 mM Osbipy exhibited a faster T-labeling, and a faster C-labeling with an approximate doubling of the observed rates compared to the one with the 3 mM Osbipy. Experiments with each of the long oligos in the presence of 11.6 mM Osbipy conducted at 35°C for 50 hours, resulted in labeling essentially all Ts and Cs, and provided values of infinity Ratio 320/260 (T+C), analogous to the data obtained for short oligos, TC1, TC2, and TC3.

Values k_{obsd} for T-labeling at 3 mM Osbipy were obtained by taking into consideration the time-dependent infinity as described in the Experimental section; $k_{\text{obsd}} = 0.042$ per min for L1 (Figure 10b); $k_{\text{obsd}} = 0.045, 0.039, 0.043$ per min for L2, L3 and L4, respectively (not shown, listed in Table 1). Rates for the four long oligos are the same within experimental

error, and allow for an average $k_{\text{obsd}} = 0.042 \pm 0.003$ per min at 3 mM Osbipy and at 25°C. Slopes corresponding to C-labeling with 3 mM Osbipy are 0.00033, 0.00044, 0.00021 and 0.00026 per min for L1, L2, L3 and L4, respectively. These values were normalized with respect to the total change observed for the C-process, {Ratio 320/260 (T+C) – Ratio 320/260 (T)}, and provided $k_{\text{obsd}} = 0.0013, 0.0014, 0.0009$, and 0.0008 per min for C-labeling of L1, L2, L3, and L4, respectively. These k_{obsd} values are associated with a relatively high standard deviation and hence presumed comparable. The average $k_{\text{obsd}} = 0.0011 \pm 0.0003$ per min is considered a representative rate of the C-labeling process in the long oligos. A 38-fold intrinsic selectivity for T over C in long oligos is obtained from the ratio between the average rate for T-labeling and the average rate for C-labeling ($0.042/0.0011=38$). The 38-times selectivity is in acceptable agreement with the 31- and 47-fold higher selectivity determined for the short oligos, and somewhat higher compared to the 25-fold selectivity measured with dNTPs. For the purpose of this work, the 38-fold selectivity, obtained as the average from four long oligos, is considered our best estimate in labeling ssDNA with Osbipy.

It is important to assess how the 38-fold intrinsic selectivity of T over C affects the labeled product distribution over time. Table 3 lists selected calculated values of % labeled-T (T^*/T) and % labeled-C (C^*/C) under the typical conditions used in this study (25°C and 3 mM Osbipy) as a function of time. For convenience calculations were done for every half-life of the T-process, but can be easily extended to any time. The last column in this table lists apparent selectivity of T over C calculated directly from the values $(T^*/T)/(C^*/C)$. It is not surprising that even after only one half-life, the apparent selectivity is equal to 27, which is markedly lower than the intrinsic selectivity. Furthermore the apparent selectivity decreases steadily with increasing incubation time and reaches values of 11 or 9 after 5 or 6 half-lives, respectively. Table 3 exemplifies the agreement between our results and the reported 10-fold apparent selectivity for T over C in the homopolymer series [5, 10]. Table 3 presents a convenient way to select a certain incubation time for a labeling reaction based on the desired product distribution and/or the acceptable degree of mislabeling.

Inspection of values k_{obsd} (T) listed in Table 1 (3rd column) shows that rates of fully labeled product formation are approximately the same for dTTP, for short oligos, and for long oligos. One calculates a global average k_{obsd} (T) = 0.042 ± 0.005 per min, or a half-life of 17 min at 25°C with 3 mM Osbipy. This result strongly suggests that T-labeling with Osbipy is independent of oligo length, and is contrary to the initial expectation that longer oligos may take a longer time to label. Incidentally, the oligos tested in this study were designed with sequences that minimize secondary structure. Hence it is plausible that random oligos and long ssDNA, subject to partial hybridization, may exhibit slower labeling kinetics. Still the observed independence with oligo length, begs the question whether or not ssDNA, tested under denaturing conditions, behaves just like another oligo.

3.7 A simple equation to assess extent of labeling

Figure 11 presents a correlation of the values Infinity Ratio 320/260 (T), reported in Table 1, with the number of Ts over the total number of nucleobases (N_{total}) in the tested oligo. The data are well fitted with a line that goes through the origin and exhibits slope=1.53. This slope is, within experimental error, the same as the value for infinity Ratio 320/260 for dTTP (1.53 ± 0.05) and for oligoT8 (1.51 ± 0.03). The correlation in Figure 3, described by eq 2, is consistent with the hypothesis that for every osmylated-T in a sequence of nucleobases, there is an added chromophore with 1.53-fold higher absorbance at 320nm compared to the intact T absorbance at 260nm. The simplicity of eq 2 results from the selection of the two wavelengths: 320nm where only Osbipy-T absorbs and 260nm where T and Osbipy-T have comparable extinction coefficients (isosbestic point).

The data plotted in Figure 11 are obtained after complete labeling of all the Ts. These data can be looked upon as a training set, with the expectation that additional T-containing oligos also fit the correlation. Hence for a given new oligo Infinity Ratio 320/260 (T) can be calculated from eq 2 when the sequence, or at least the fraction T/N_{total} , is known. Eq 3 describes conditions where the oligo is under labeled or slightly, let us say 110%, over labeled. Under these conditions, C-labeling is minimal and so is its slightly lower contribution to the total absorbance at 320nm (see earlier discussion with TC1-3 oligos). Assuming conditions delineated by eq 3, eq 2 can be extended to eq 4. Equation 4 describes a correlation that is not limited to complete labeling. In eq 4 Ratio 320/260 is the experimentally obtained parameter at time t during labeling, following conditions defined by eq 3, and N_{labeled} refers to any labeled nucleobases during time t. Values for Ratio 320/260 can be obtained either by CE analysis, or by UV-Vis spectrophotometric measurement following label purification, as described in the Experimental Section. By dividing eq 4 with eq 2, eq 5 is derived. Eq 5 estimates extent of labeling, defined as fraction of labeled bases over the total number of Ts. Since the properties included in the right side of eq 5 are spectrophotometric properties of the final product, the labeling conditions (such as temperature, incubation time, or Osbipy concentration) may vary from the ones used here. Eqs 4 and 5 contain N_{labeled} instead of T_{labeled} as a reminder that an oligo containing all four bases is subject to T-labeling and C-labeling occurring independently of each other. Therefore eq 6 is applicable to Osbipy labeling, and is used to modify eq 5 to eq 7. Furthermore assuming that the number of Ts in a specific oligo is equal or comparable to the number of Cs (eq 8), eq 9 follows. Eq 9 then modifies eq 5 to give eq 10.

$$\text{Infinity Ratio 320/260 (T)} = 1.53 \times T / N_{\text{total}} \quad \text{eq 2}$$

Assuming that the oligo is under labeled or less than 10% over labeled (eq 3),

$$\{\text{Ratio 320/260}\} / \{\text{Infinity Ratio 320/260 (T)}\} < 1.1 \quad \text{eq 3}$$

eq 2 can be extended to eq 4

$$\{\text{Ratio 320/260}\} = 1.53 \times N_{\text{labeled}} / N_{\text{total}} \quad \text{eq 4}$$

$$N_{\text{labeled}} / T = \{\text{Ratio 320/260}\} / \{\text{Infinity Ratio 320/260 (T)}\} \quad \text{eq 5}$$

$$\text{In the presence of Osbipy: } N_{\text{labeled}} = T_{\text{labeled}} + C_{\text{labeled}} \quad \text{eq 6}$$

By substituting eq 6 in eq 5, one obtains eq 7

$$\{T_{\text{labeled}} + C_{\text{labeled}}\} / T = \{\text{Ratio 320/260}\} / \{\text{Infinity Ratio 320/260 (T)}\} \quad \text{eq 7}$$

With the added assumption that # of Ts = # of Cs or, at least comparable

$$\# \text{ of Ts} \approx \# \text{ of Cs} \quad \text{eq 8}$$

Then eq 9 is a good approximation

$$\{T_{\text{labeled}} + C_{\text{labeled}}\} / T \approx (T_{\text{labeled}} / T) + (C_{\text{labeled}} / C) \quad \text{eq 9}$$

Using Eq 9 one modifies eq 5 to yield eq 10

$$(T_{\text{labeled}} / T) + (C_{\text{labeled}} / C) \approx \{\text{Ratio 320/260}\} / \text{Infinity Ratio 320/260 (T)} \quad \text{eq 10}$$

Assuming that the above conditions (eqs 3, 6 and 8) are met, Eq 10 provides the means to estimate extent of labeling as follows: A value for $\{(T_{\text{labeled}} / T) + (C_{\text{labeled}} / C)\}$ is obtained from eq 10. This value is compared to the values listed in the 5th column of Table 3, or an extended version of it (see Table caption). A match allows one to read the corresponding values of % labeled T and % labeled C in the 3rd and 4th columns of Table 3. Since eq 10 is independent of the actual T-labeling rate, Table 3 can be exploited to obtain % labeled T and % labeled C for reactions conducted under different conditions, as long as these conditions do not affect selectivity. Selected experiments with long oligos validated the successful use of eq 10. Specifically, in experiments conducted under different conditions than the ones reported here, the extent of labeling was determined (i) from the kinetics of the actual process, as monitored by CE, and (ii) using eq 10 in conjunction with Table 3, with excellent agreement between the two approaches.

4 Concluding remarks

Our objectives included establishing conditions for efficient T-labeling of oligodeoxynucleotides. To this end CE, specifically CZE, was successfully used to determine the kinetics of osmylation with mononucleotides, with oligos 9 to 11 bases long, and also with oligos 80 to 90 bases long. The oligos that were 8 to 11 bases long contained 1, 2, or 3 Ts within a series of adenosines. These were treated with Osbipy and their reactions monitored by CE. CE analysis achieved baseline resolution of the starting oligos, intermediates, and of the final products. For oligos, 80 to 90 bases long, two kinetically well resolved processes were observed. The fast process was demonstrated to be T-labeling and the slow process to be cytidine (C)-labeling.

Osbipy-labeled oligos can be readily purified from unreacted Osbipy by a simple spin filtration. The products are water-soluble and are stable in solution at 4°C. Reactions conducted under pseudo-first order conditions with 3 mM Osbipy in water at 25°C afford practically 100% T-labeling, with no detectable oligo degradation. The observed rates of oligo disappearance increase linearly with the number of Ts in the oligos tested. This observation indicates that the kinetics are statistically driven. This linear trend includes oligoT2 with two consecutive Ts, indicating neither steric hindrance nor acceleration in the labeling of tandem Ts. Observed rates for the formation of the fully labeled product appear to be independent of oligo length, and indeed closely resemble the kinetics of dTTP osmylation with a 17 min half-life. Under the tested conditions the intrinsic selectivity of Osbipy for T over C is 38-fold, but the apparent selectivity approaches ten-fold as the T-labeling approaches completion. The oligos investigated here served as a training set to deduce a relationship between the absorbance of the labeled product and the fraction of Ts in a sequence (eq 2). Based on this correlation, a simple equation (eq 10) is proposed and qualified for a much broader set of conditions compared to the ones reported here. Eq 10 exploits UV-Vis measurement of the purified product to assess the extent of labeling, which provides the relative percentages of labeled T and of labeled C.

This study showed the feasibility of labeling T in native DNA effectively, predictably, and selectively with Osbipy. In addition to determining optimal labeling conditions this report discloses: (i) How to analyze the reactions, (ii) how to evaluate the labeling selectivity, and

(iii) a quick and effective sample purification. The important findings from this work are that the osmylation reaction has good selectivity for T over C with no competing reaction or coordination with the purine bases. Additionally it was found that the products have good chemical stability, and that the reaction is insensitive to both the length and sequence of the target DNA. This has important implications in avoiding systematic errors in sequence determination. Labeling conditions are mild and produce stable products with potential utility for EM sequencing. Moreover the approach followed for evaluation of Osbipy is anticipated to be transferable to other labels for T or for any of the other nucleobases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Oligos	oligodeoxynucleotides
Osbipy	osmium tetroxide 2,2'-bipyridine
T	thymidine
EM	electron microscopy

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Figure 1. ^1H NMR spectra from the reaction of 10 mM dTMP with 20 mM OsO_4 in the presence of 10 mM bipyridine.

Figure 2a:

Figure 2b:

Figure 2.

Figure 2a. CE profiles from a reaction of 2.2 mM Osbipy (migration time, *mt*, at 3 min, not shown) with 0.16 mM dTTP at 25 °C monitored at 260 nm; consecutive analyses of the same reaction mixture show that the dTTP peak decreases and a product peak, Osbipy-dTTP, appearing as a doublet, increases with time (see discussion in text).

Figure 2b. Overlapping UV-Vis spectra obtained with the Diode Array Detector of the CE instrument. Left: blue, Osbipy; red, Osbipy-dTTP. Right: blue, dTTP; red, Osbipy-dTTP. The spectra clearly suggest that Osbipy-dTTP has substantial absorbance at 320nm, in contrast to dTTP, OsO₄, or bipyridine (not shown).

Figure 3.

CE profiles from the consecutive analyses of a sample with 1.3 mM Osbipy and 0.08 mM oligoT1 (AAA ATA AAA) in water at 25°C. Bottom profile shows the excellent stability of the Osbipy-labeled-oligo in the presence of excess Osbipy at 25°C.

Figure 4. CE profiles from the consecutive analyses of a sample with 1.3 mM Osbipy and 0.08 mM oligoT2 (AAA TTA AA) at 25°C.

Figure 5. Comparison of the CE profiles from the analyses of two samples incubated for 19 hours at 25°C: Top, 1.3 mM Osbipy with 0.08 mM oligoT2; bottom, 1.3 mM Osbipy with 0.08 mM oligoC2. Impurity present in the oligoC2 reaction mixture remains unchanged during incubation. After 19 hours no oligoT2 can be detected, whereas oligoC2 shows very little reactivity. The appearance of multiple peaks for P1 and P2 is discussed in the text.

Figure 6a:

Figure 6b:

Figure 6.

Figure 6a. CE profiles from the consecutive analysis of a reaction mixture with 1.3 mM Osbipy and 0.04 mM oligoT3 (AAT AAT AAT AA) at 25°C (see text for discussion). Impurity present in the reaction mixture remains unchanged as shown. Reaction is practically completed overnight. Final product exhibits excellent stability over 3 days at 25°C in the presence of excess label.

Figure 6b. Tri-Osbipy labeled oligoT3 (AAT AAT AAT AA) analyzed by CE equipped with a long capillary exhibits at least 6 partially resolved peaks. The 8 predicted isomers are: AAT^a AAT^a AAT^a AA, (in analogy 7 more: aab, aba, baa, abb, bab, bba, and bbb)

Figure 7. Rates of oligoTn (n=1, 2 or 3, see Table 1) disappearance in the reaction with 1.3 mM Osbipy at 25°C as a function of the number of Ts in oligoTn.

Figure 8.

Data from the reaction of three different concentrations of oligoT8, at 0.025, 0.050 and 0.075 mM, with 3 mM Osbipy at 25°C. Ratio 320/260 stands for the ratio of the CE peak areas at 320 over 260nm; this ratio varies with time. Average value at the plateau is 1.51 ± 0.2 , and $k_{\text{obsd}} = 0.041$ per min from all the data plotted together. For rate determinations see Experimental Section.

Figure 9a:

Figure 9b:

Figure 9.

Figure 9a. Consecutive 15 min CE analyses of reaction mixture composed of L1 (see Table 1), an 80 base long oligo, and 2.6 mM Osbipy in water at 44°C in the presence of 0.042 mM dCTP as internal standard. Analysis #1 is obtained after 3 min. Osbipy migrates at 2.3 min (not shown) and it is a large peak compared to the others. Peak migration time (mt) of dCTP internal standard does not exhibit a shift. L1 peak mt shifts exponentially with time towards the peak mt attributed to L1 where all Ts are Osbipy-labeled. In the absence of Osbipy L1 peak mt=6.4 min, approximately 0.4 min later compared to peak mt #1, clearly showing that even after 3 min there is substantial labeling.

Figure 9b. Migration time (mt) of main peak as a function of incubation time at 44°C for the reaction of long oligo L1 with 2.6 mM Osbipy (see figure 9a). The point at 0 time corresponds to the mt of an L1 standard in water analyzed under the above conditions.

Figure 10a:

Figure 10b:

Figure 10.

Figure 10a. Data obtained from the reaction of long oligo L1 with 3 mM Osbipy in water at 25°C. Two kinetic processes are seen: the fast one is attributed to T-labeling and the slow one to C-labeling. For determination of k_{obsd} see Discussion.

Figure 10b. Data obtained from the reaction of oligo L1 with 3 mM Osbipy in water at 25°C (see Fig 10a). Evaluation of $k_{\text{obsd}}=0.042$ per min for the fast process, T-labeling.

Figure 11. Correlation between the ratios of CE peak area at 320nm over CE peak area at 260nm, Ratio 320/260 (T) for Osbipy-labeled oligo as a function of the ratio T/N_{total} , i.e. number of Ts over the total number of nucleobases N_{total} . Data obtained from Table 1; see text for discussion. The best fit of the data is linear with slope=1.53, and goes through the origin.

Scheme 1.

Reaction of OsO_4 with bipyridine to form the bipy-OsO₄ reactive complex, which in a second step reacts with dTMP to form the osmylated-dTMP [20–23] (See Results and Discussion).

Table 1

Oligos used in this study, sequences, # of T or C over total nucleobases (N_{total}), k_{obsd} , the rate of product formation with 3mM Osbipy, and values for Infinity Ratio 320/260 for T-labeling or T+C labeling (see text).

ODN	sequence	k_{obsd} , 1/min (T)	T/N _{total} (same in decimal format)	C/N _{total}	Infinity Ratio 320/260 (T)	Infinity Ratio 320/260 (T+C)
dTTP		0.042	1		1.53±0.05	
oligoT1	AAA ATA AAA	0.050	1/9 (0.111)		0.12	
oligoT2	AAA TTA AA	0.037	2/8 (0.250)		0.25	
oligoT3	AAT AAT AAT AA	0.045	3/11 (0.273)		0.32	
oligoT8	TTT TTT TT	0.041	1		1.51±0.03	
oligoA1	TTT TAT TTT	0.041	8/9 (0.889)		1.25	
oligoA2	TTT AAT TT	0.040	6/8 (0.750)		0.97	
oligoA3	TTA TTA TTA TT	0.039	8/11 (0.727)		0.94	
oligoC1	AAA ACA AAA		-	1/9		
oligoC2	AAA CCA AA		-	2/8		
oligoC3	AAC AAC AAC AA		-	3/11		
oligoTC1	TTT TCT TTT		8/9	1/9		1.45
oligoTC2	TTT CCT TT		6/8	2/8		1.43
oligoTC3	TTC TTC TTC TT		8/11	3/11		1.40
L1	80-mer	0.042	24/80 (0.300)	17/80	0.375	0.63
L2	90-mer	0.045	29/90 (0.322)	22/90	0.391	0.70
L3	80-mer	0.039	22/80 (0.275)	17/80	0.372	0.61
L4	89-mer	0.043	19/89 (0.213)	26/89	0.298	0.61

Sequence of L1:

CCTCAGAGTTCCAAGGTGAGTGAGTGGTCCTTCCTTCCTCCGGTGAGTGAGTGGA CGGTAAGCCATT

Sequence of L2:

sequence of L2:

CTCAGAGTTCCAAGTGAGTGAGTGGTCCTTCCTTCCTCCGTGAGTG AGTGAGTGACGGTAAGCCATT

Sequence of L3:

sequence of E3:

CTCAGAGTTCC A GGAGAGAGATT AGTGGTCCTTCCTTCCTTCGGTGAT TGAGAGAGAGGA CGGT AAGCCATT

Sequence of L4:

sequence of L4:
 CTCAGAGTTC AAGCACTC ACGCACTC ACGGACTTC ACGGACTTC ACGGACTTC ACGGACTTC ACGGACTTC ACGGACTTC

Table 2

Selectivity of Osbipy for dTTP compared to dCTP in water at 25°C obtained by competition experiments.

Exp.	CE areas at 260nm			Calculated total areas		T*/Total T	C*/Total C	selectivity
	dTTP	dCTP	T*	Total C	Total T			
1	55	386	2.6	388.6	65.4	0.159	0.0067	24
2	58.5	551	3.6	554.6	71.2	0.178	0.0065	27
3	146	829	4.3	833.3	167.2	0.127	0.0052	24

All three experiments with 3mM Osbipy: exp 1, 0.1 mM dTTP + 0.6 mM dCTP; exp 2, 0.1 mM dTTP + 0.8 mM dCTP; exp 3, 0.2 mM dTTP + 1.0 mM dCTP. C* refers to Osbipy-labeled dCTP and T* refers to Osbipy-labeled dTTP. Observation that at 260 nm a starting material and its Osbipy-labeled product exhibit the same absorbance within experimental error, allows the calculation of Total T and Total C from the sum of T* + dTTP, and C*+ dCTP, respectively.

Table 3

Percent of Osbipy-labeled T and Osbipy-labeled C of an oligo containing all four bases for a reaction conducted in water at 25°C with 3mM Osbipy as a function of incubation time.

Half-lives (T)	Incubation time, min	100x (T*/T)	100x (C*/C)	100x((T*/T) + (C*/C)), calculated	Apparent selectivity T/C, calculated
1	17	50	1.85	51.85	27
2	34	75	3.67	78.67	20
3	51	87.5	5.46	92.96	16
4	68	93.75	7.21	100.96	13
5	85	96.87	8.93	105.80	11
6	102	98.44	10.61	109.05	9

T* stands for Osbipy-labeled-T, C* stands for Osbipy-labeled-C. T and C are the total of number of T and C, respectively, contained in the tested oligo. (T*/T) and (C*/C) is calculated from the corresponding rates of 0.042 and 0.0011 per min for T- and C-labeling, respectively. If T=C, then (T*/T) + (C*/C) = (T*+C*)/T. This table can be easily extended to include other time points besides the ones shown here.