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Synthesis of Site-specific Crown Ether Adducts to DNA Abasic Sites, 8-Oxo-7,8-dihydro-2'-deoxyguanosine and 2'-Deoxycytidine

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Abstract

Formation of adducts to DNA is of great benefit to DNA sequencing and damage detection technology and to enzymology. Here we describe the synthesis and characterization procedures of 18-crown-6 adducts formed to abasic (AP) sites, 8-oxo-7,8-dihydro-2'-deoxyguanosine (OG), and 2'-deoxycytidine (C) residues in DNA oligodeoxynucleotides. These crown ether adducts were used as site-specific modifications to facilitate nanopore technology. The methods described can be readily expanded to attach other suitable primary amines of interest.

Keywords

18-crown-6; Site-specific chemistry; DNA adducts; Abasic sites; 2'-deoxycytidine; 8-Oxo-7; 8-dihydro-2'-deoxyguanosine; DNA sequencing

1. Introduction

Post-synthetic site-specific modification of oligodeoxynucleotides (ODNs) has been well investigated due to its important role in studying the mechanism of DNA repair enzymes (1–5), the study of various carcinogens (6–8), the development of DNA sequencing and modification detection technologies (9–12), and furthermore a number of ODN modifications are useful in surface and analytical chemistry (13,14). Herein, we describe step-by-step synthetic protocols for forming 18-crown-6 (18c6) adducts to DNA abasic (AP) sites, 8-oxo-7,8-dihydro-2'-deoxyguanosine (OG) and 2'-deoxycytidine (C) via site-specific chemistry (Fig. 1). We used the commercially available primary amine derivative of 18c6, 2-aminomethyl-18-crown-6, to demonstrate these reactions; however, these protocols can also be readily used to attach other suitable primary amines of interest with similar yields. Thus, these methods provide high potential to use these three nucleotides as 'convertible nucleosides' in DNA chemistry (15).

These adducts were designed to better understand the interactions between DNA and the ion-channel forming bacterial protein α -hemolysin (α -HL) as an approach to advance nanopore technology in our laboratory and others (16–18). Single-stranded DNA (ssDNA) can be electrically drawn through α -HL, generating blockages to the open channel current

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that are characteristic for the identity of the nucleotides (19). To investigate closely the electrical signature of a specific nucleotide, streptavidin can be used to complex biotinylated ssDNA, which is too large in dimension to enter the ion channel (19). As a result, the ssDNA chain is immobilized inside α -HL, positioning the modified site at the sensing zone (Fig. 2a) (20,21). The blockage current was recorded for 2 s (Fig. 2b), and then the voltage was briefly reversed to release the DNA allowing another strand to be captured. The blockage current so obtained was then compared to that of an unmodified control strand (C₄₀, Fig. 2c). By labeling AP sites and OG with these adducts, we were able to generate modulated electrical signatures (Fig. 2c) (11,22,23). The 18c6 moiety was unique in its ability to interact with the electrolyte, providing clear changes in electrical current when the crown ether was present in the ion channel (Fig. 2c). A variety of primary amines were studied, from simple alkyl amines, polyamines, amino acids, short peptides, and aminoglycosides (23–25). While these amines all readily formed adducts to DNA under the reaction conditions described here, only the crown ethers were able to slow the entry of the nucleotide into the constriction zone of the wild-type α -HL ion channel, yielding significant current modulations and providing readout of the presence of the modified nucleotide. This report therefore focuses on adduct formation with the 18c6 pendant amine.

Three methods to introduce the 18c6 adduct are illustrated: (I) Reductive amination, which is appropriate for covalent attachment of a primary amine to an aldehyde group, such as the AP sites generated by the treatment of a 2'-deoxyuridine (U) containing ODN with uracil-DNA glycosylase (UDG) (Fig. 1a). (II) Oxidative addition of a primary amine to the electrophilic intermediate created from base oxidation of OG. This is best demonstrated by the addition of amines to the OG oxidation intermediate (26); in the absence of a better nucleophile, H₂O adds at C5, generating a spirocyclic product (27). In the presence of low concentrations of a primary amine, the amine adduct is produced (Fig. 1b). (III) In the case of 5-bromo-2'-deoxycytidine, ammonia can add to C5 of 2'-deoxycytidine to generate 5-amino-cytidine (28). In the present example, we find 5-iodo-2'-deoxycytidine to be more conveniently converted to a 5-alkylamino-2'-deoxycytidine adduct (Fig. 1c).

2. Materials

2.1 Reagents used for 18-crown-6 DNA adduct synthesis

1. ddH₂O (18 M Ω)
2. Sodium phosphate buffer solution (PBS)
3. Hydrochloric acid (HCl)
4. Phosphoric acid (H₃PO₄)
5. Sodium hydroxide (NaOH)
6. Ethanol
7. Tris(hydroxymethyl)aminomethane (Tris)
8. Ethylenediaminetetraacetic acid disodium salt (Na₂EDTA)
9. Sodium acetate (NaOAc)

10. Ammonium acetate (NH₄OAc)
11. Dithiothreitol (DTT)
12. Acetonitrile (CH₃CN)
13. Ammonium hydroxide (NH₄OH)
14. Sodium hexachloroiridate (Na₂IrCl₆)
15. Sodium cyanoborohydride (NaBH₃CN)
16. 2-aminomethyl-18-crown-6 (18c6)
17. Sodium chloride (NaCl)
18. Potassium iodide (KI)
19. 3-(*N*-Morpholino)propanesulfonic acid (MOPS)
20. Potassium peroxymonosulfate as Oxone (KHSO₅)
21. Uracil-DNA glycosylase (UDG) supplied by New England Biolabs (NEB)

2.2. DNA preparation and purification procedures

2.2.1. DNA synthesis: The 3'-biotinylated oligodeoxynucleotides (ODN) used here were synthesized from commercially available phosphoramidites by the DNA-Peptide Core Facility at the University of Utah. Their sequences are 5'-CCCCC CCCCC CCCCC CCCCC CCCCC CXCCC CCCCC CCCCC-Btn, where X = U, OG, or 5-iodo-2'-deoxycytidine (5-I-C) and Btn = biotin-triethyleneglycol (TEG) linker. After synthesis, each ODN was cleaved from the synthetic column and deprotected according to the manufacturer's protocol.

2.2.2. DNA purification: The ODNs were purified with a semi-preparative anion-exchange HPLC column (A = 10% CH₃CN/90% ddH₂O, B = 20 mM PBS (pH 7.0), 1 M NaCl in 10% CH₃CN/90% ddH₂O, flow rate = 3 mL/min). A linear gradient of 25% to 100% B over 30 min was used while monitoring the absorbance at 260 nm. Desalination was performed with a dialysis cassette possessing 7000 molecular weight cut-off (MWCO) membrane against ddH₂O at 4 °C for 2 d and changing the ddH₂O three times daily, followed by vacuum concentration to reduce the volume.

2.2.3. DNA characterization: The concentrations of the ODNs were measured in ddH₂O by UV-vis spectroscopy at 260 nm under room temperature (24 °C). The corresponding extinction coefficients for the strands were determined from their primary sequence with the assumption that the modified bases had the same extinction coefficients as their parent base (i.e., OG and G have the same $\epsilon_{260\text{nm}}$). The identities of the ODNs were determined by negative ion electron spray mass spectrometry (ESI⁻-MS) on a Micromass Quattro II mass spectrometer equipped with Zspray API source in the mass spectrometry laboratory at the Department of Chemistry, University of Utah. All ODNs were stored at -20 °C.

2.3. Preparation of reactive reagents and buffers

Three reactions described in this article require different buffer conditions and reagents elaborated below:

2.3.1. 18c6 adduct to DNA AP sites: UDG buffer: 20 mM PBS (pH 8.0), 1 mM DTT, and 1 mM EDTA.

Reductive amination reaction buffer: 150 mM MOPS (pH 6.5).

Stock solutions of reagents: 2 M NaBH₃CN solution and 2 M NaOH,

2.3.2. 18c6 adduct to OG: Reaction buffer: 75 mM PBS (pH 8.0).

Stock solutions of reagents: 200 mM Na₂IrCl₆ and 50 mM Na₂EDTA (pH 8.0).

2.3.3. 18c6 adduct to 5-I-C: Reagent: 1 M 2-aminomethyl-18-crown-6.

3. Methods

3.1. Synthesis of AP-18c6 adduct

3.1. step 1. Thermally equilibrate a 100-μL solution of UDG buffer with 10 μM U-containing ODN and 1 unit UDG at 37 °C for 30 min to generate AP sites and then dialyze the strands against ddH₂O at 4 °C for 12 h (see Notes 5.1 and 5.2).

3.1. step 2. Lyophilize the sample to dryness and resuspend the resulting AP-containing ODN in 100-μL MOPS buffer; then introduce by pipette 20 μL of 1 M 2-aminomethyl-18-crown-6 into the reaction, immediately followed by the addition of 5 μL of 2 M stock NaBH₃CN solution (see Note 5.3). Keep the reaction at 37 °C for 24 h.

3.1. step 3. Add 10 μL of 2 M NaOH solution to cleave the unreacted ODNs to generate strand breaks. Keep the mixture at 90 °C for 10 min.

3.1. step 4. Dialyze the reaction mixture against ddH₂O in a 7000 MWCO membrane dialysis cassette at 4 °C for 12 h before HPLC analysis (see Notes 5.4–5.6).

^{5.1.} During the synthesis of AP-18c6 adduct, the pH (6.5) we chose to use is based on a series of pH-dependent studies. DNA AP sites are vulnerable to elimination under basic conditions, generating strand breaks. On the other hand, higher pH facilitates the amination process. Therefore, a pH ranging from 5.5–6.5 produced the highest product yield.

^{5.2.} It is highly recommended to use PBS instead of Tris buffer in the UDG treatment step. Being a primary amine, Tris can aminate AP sites, thus generating an undesirable side product. Similarly, we also recommend not using Tris buffer for OG-containing ODNs as well.

^{5.3.} In order to guarantee a good yield in the reductive amination reaction, we recommend that one could add a second aliquot of NaBH₃CN (100 mM, 10 μmole) 12 h after the first addition.

^{5.4.} Similar reactions have been conducted with heterosequence ODN strands previously, and we observed similar yields with ODNs up to 50 nucleotides in length.

^{5.6.} Similar product yields were achieved when the 2-aminomethyl-18-crown-6 in the AP and OG reactions was substituted with *N*^α-OAc-*O*-methyllysine, spermine, spermidine, *O*-methyl-arginine, glucosamine, Gly-Pro-Arg-Pro carboxamide, and 2-aminomethyl-15-crown-5.

3.2. Synthesis of Sp-18c6 adduct

3.2. step 1. Incubate a 100- μ L solution of 75 mM PBS (pH 8) containing 10 μ M OG-containing ODN and 2 mM aminomethyl-18-crown-6 at 45 °C for 30 min to achieve thermoequilibrium (see Note 5.7).

3.2. step 2. Add 200-mM stock Na_2IrCl_6 solution in three 7.5- μ L aliquots into the reaction mixture, and then incubate at 45 °C for another 30 min (see Note 5.8).

3.2. step 3. Add stock Na_2EDTA to a final concentration of 1 mM to quench the reaction.

3.2. step 4. Dialyze the reaction mixture against ddH₂O at 4 °C for 12 h before HPLC analysis (see Notes 5.4, 5.5, and 5.9).

3.3 Synthesis of 5-I-C and conversion to an amine adduct in a nucleoside model

The iodination of 2'-deoxycytidine was achieved by following published protocols with the following modification (29,30).

3.3. step 1. Briefly, a reaction with 1 mM 2'-deoxycytidine nucleoside, 8 mM KI, and 8 mM KHSO_5 was incubated in PBS (pH 6.0) at 45 °C for 24 h.

The identity and site of iodination on the C heterocycle was established by ESI⁺-MS and ¹H-NMR on the nucleoside model reaction. ESI⁺-MS [M + H⁺] calcd mass = 354.1, found = 354.0. ¹H NMR (D₂O) δ 7.22 (s, 1H), 6.20 (dd, 1H), 4.45 (ddd, 1H), 4.08 (ddd, 1H), 3.84 (td, 2H), 2.45 (td, 1H), 2.33 (td, 1H). UV-vis: λ_{max} = 294.0 nm.

3.3. step 2. The amination of 5-I-C was achieved by incubating the 5-I-C nucleoside with 1 M 2-aminomethyl-18-crown-6 in ddH₂O at 55 °C for 24 h

The identity and site of amination on 5-I-C was determined by ESI⁺-MS and ¹H-NMR from the nucleoside model reaction conducted when 5-I-C was allowed to react with methylamine. ESI⁺-MS [M + H⁺] calcd mass = 257.3, found = 257.1. ¹H NMR (D₂O) δ 7.22 (s, 1H), 6.20 (dd, 1H), 4.45 (ddd, 1H), 4.08 (ddd, 1H), 3.84 (td, 2H), 2.56 (s, 3H), 2.45 (td, 1H), 2.33 (td, 1H). UV-vis: λ_{max} = 305.0 nm.

3.4 Synthesis of 5-I-C and conversion to an amine adduct in an ODN

In order to make site-specific 5-18c6-C adducts, 5-I-C was incorporated into the ODN through solid-phase synthesis; alternatively, site-specific iodination can be conducted by adding an n-1 complement to the strand such that the desired C residue is present as a bulged nucleotide (17). The attachment of 18c6 is described below, which was adapted from published protocols (28):

5.7. Do not use Tris buffer because it is a primary amine and will compete for adduct formation during the oxidation reaction to label OG with 2-aminomethyl-18-crown-6.

5.8. The biotin linker at the 3' terminus of the ODNs is used to immobilize the molecules inside the α -HL ion channel by forming a complex with streptavidin during the nanopore measurements, and it is not relevant to the chemical reactions. However, it needs noting that during the synthesis of Sp-18c6, biotin can be oxidized with Na_2IrCl_6 yielding an M+16 product that does not change its ability to bind streptavidin (31).

5.5. The yield decreases with the length of the ODN, and the ability to purify the adducts by HPLC also diminishes.

5.9. The Sp-18c6 adduct is recommended to be studied immediately, due to its instability (32–35).

3.4. step 1. Incubate a 10- μ M ODN containing 5-I-C and 1 M 2-aminomethyl-18-crown-6 in ddH₂O at 55 °C for 24 h.

3.4. step 2. Dialyze the reaction mixture against ddH₂O in a 7000 MWCO dialysis cassette at 4 °C for 12 h before HPLC analysis.

4. Purification and characterization of adducted ODNs

4.1. HPLC analysis and purification of the adducted oligomers

4.1. step 1. An analytical anion-exchange column can be used to analyze these reactions with the following mobile-phase conditions: A = 10 % CH₃CN/90% ddH₂O and B = 20 mM PBS (pH 8.0), 1 M NaCl in 10% CH₃CN/90% ddH₂O. A linear gradient of 25% to 100% B over 30 min (flow rate = 1 mL/min) can be used while monitoring the absorbance at 260 nm.

4.1. step 2. The chromatograms of these reactions should be compared to those of their corresponding starting materials and new peaks (products) can be identified and collected (Fig. 3).

4.1. step 3. Dialyze the collected product solutions against ddH₂O in a 7000 MWCO dialysis cassette at 4 °C for 24 h before vacuum concentration to reduce the volume.

4.2. Preparation of the ODNs for ESI⁻-MS Analysis

Ethanol precipitation was used to prepare the ODNs for ESI⁻-MS, which is briefly described below:

4.2. step 1. Add 3 M NH₄OAc (1/10 volume of the ODN, pH 5.2) to achieve a final concentration of 0.3 M and mix well.

4.2. step 2. Add 2–3 volumes of 100% ethanol and mix well.

4.2. step 3. Keep the tube at –20 °C for 30 min.

4.2. step 4. Spin in a centrifuge at 14,000 rpm at 4 °C for 20 min and carefully decant supernatant.

4.2. step 5. Add 1 mL 70% ethanol (–20 °C), mix, and then spin briefly. Decant supernatant. Repeat twice.

4.2. step 6. Vacuum dry pellet and resuspend in 3 mM NH₄OAc.

All the reaction products were characterized with ESI⁻-MS.

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Fig. 1. Synthetic schemes for making 18c6 adducts to AP sites (a), OG (b) and 5-I-C (c) via site-specific chemistry.

Fig. 2. Illustrations of the nanopore immobilization experiment. (a) Experimental setup (b) Typical current-time trace of an immobilization event (c) Percentage residual current histograms of 18-crown-6 and 15-crown-5 adducts of AP sites, compared to a control DNA C_{40} ($\% I/I_0$ set to 0).

Fig. 3. HPLC chromatograms of these three reactions: AP-18c6 (a), Sp-18c6 (b), and 5-18c6-C (c), and their comparisons to starting materials.