Synthesis of N^2-Aryl-2′-Deoxyguanosine Modified Phosphoramidites and Oligonucleotides

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The N^2-position of 2′-deoxyguanosine (N^2-position in dG) is well known for forming carcinogenic minor groove DNA adducts, which originate from environmental pollutants, chemicals, and tobacco smoke. The N^2-dG DNA adducts have strong implications on biological processes such as DNA replication and repair and may, therefore, result in genomic instability by generating mutations or even cell death. It is crucial to know the role of DNA polymerases when they encounter the N^2-dG damaged site in DNA. To get detailed insights on the in vitro DNA damage tolerance or bypass mechanism, there is a need to synthetically access N^2-dG damaged DNAs. This article describes a detailed protocol of the synthesis of N^2-aryl-dG modified nucleotides using the Buchwald-Hartwig reaction as a main step and incorporation of the modified nucleotides into DNA. In Basic Protocol 1, we focused on the synthesis of five different N^2-dG modified phosphoramidites with varying bulkiness (benzyl to pyrenyl). Basic Protocol 2 describes the details of synthesizing N^2-dG modified oligonucleotides employing the standard solid phase synthesis protocol. This strategy provides robust synthetic access to various modifications at the N^2-position of dG; the modified dGs serve as good substrates to study translesion synthesis and repair pathways. Overall data presented in this article are based on earlier published reports. © 2019 by John Wiley & Sons, Inc.

Keywords: 2′-deoxyguanosine • Buchwald-Hartwig coupling • nucleosides • oligonucleotides • phosphoramidite • solid phase synthesis

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INTRODUCTION

DNA plays a very important role in cell maintenance via faithful transformation of hereditary information. Preservation of the integrity of DNA is crucial for genomic stability. When DNA comes into contact with exogenous and endogenous genotoxic agents, alterations and/or modifications in their structures result, called DNA lesions or adducts (Dipple, 1995). Major DNA adducts are formed due to exposure to polycyclic aromatic hydrocarbons (PAHs), α,β-unsaturated aldehydes, and heterocyclic aromatic amines (HAAs; Geacintov & Broyde, 2010). The presence of DNA damage can result in replication fork stalling by replicative DNA polymerases. Under such circumstances, the cell utilizes the low fidelity polymerases called translesion DNA polymerases to tolerate

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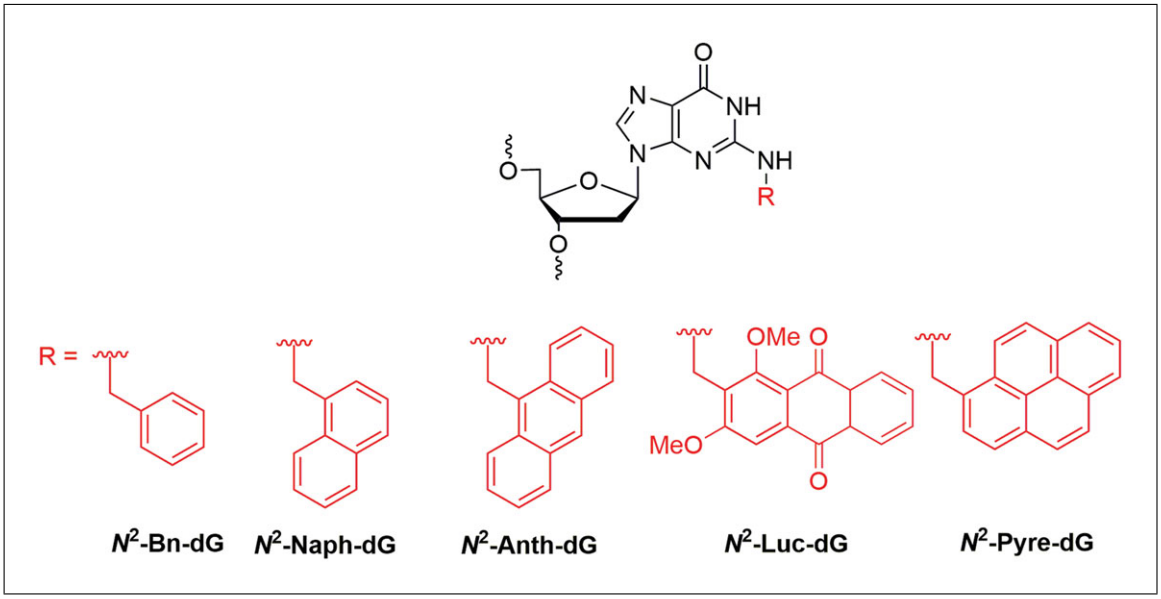

Figure 1 Site-specific N^2-dG DNA modifications.

the damaged sites in DNA and rescue the stalled replication fork (Sale, Lehmann, & Woodgate, 2012).

The N^2-position of dG (Fig. 1) is susceptible to react with various genotoxic agents and leads to the formation of minor groove DNA adducts. These adducts can be repaired either by the nucleotide excision repair pathway (NER; Mu, Geacintov, Min, Zhang, & Broyde, 2017) or they can be tolerated by various translesion DNA polymerases utilizing translesion synthesis pathway (TLS; Choi, Angel, & Guengerich, 2006; Choi & Guengerich, 2004; Choi & Guengerich, 2005; Choi & Guengerich, 2006; Choi & Guengerich, 2008; Jarosz, Godoy, Delaney, Essigmann, & Walker, 2006; Zhang et al., 2009). Among various damaging agents, *N*-nitroso-*N*-benzylurea (BnNU), *N*-nitrosobenzylmethylamine (NBnMA), and benzyl halides are responsible for the benzylation at the N^2-position (N^2-Bn-dG, Fig. 1) of dG (Moon & Moschel, 1998; Moschel, Hudgins, & Dipple, 1980; Peterson, 1997). PAHs such as halomethyl- or sulfoxymethyl-anthracenes as well as 1-methylpyrene contribute to N^2-(9-anthracenylmethyl)-dG (N^2-Anth-dG) and N^2-(1-pyrenylmethyl)-dG (N^2-Pyre-dG) adduct formation (Bendadani et al., 2014; Bendadani, Meinl, Monien, Dobbernack, & Glatt, 2014; Casale & McLaughlin, 1990; Wohak, Monien, Phillips, & Arlt, 2019). Lucidin, a metabolite from madder root, is responsible for forming N^2-Luc-dG DNA adducts (Ishii et al., 2012; Ishii et al., 2009).

To understand the biological implications of various N^2-dG adducts, it is necessary to have a robust synthetic method to obtain site-specific N^2-dG damaged DNAs as substrates for further biological studies. In this article, we focus on the synthesis of five different N^2-dG DNA modifications (benzyl, naphthyl, anthracenyl, a structural analogue of lucidin, and pyrenyl (Fig. 1).

CAUTION: All steps need to be performed in a well-ventilated fume hood. Wear lab coats, safety glasses, and protective gloves.

SYNTHESIS OF N^2-dG MODIFIED PHOSPHORAMIDITES

BASIC PROTOCOL 1

This protocol describes the synthesis of various N^2-aryl-dG modified nucleotides employing the Buchwald-Hartwig reaction (Fig. 2). Synthesis is achieved utilizing common precursor-protected 2-bromo-2′-deoxyinosine (2-Br-dI) **2** which was synthesized using reported O^6-*p*-nitrophenylethyl (NPE)-protected nucleoside **1** (Current Protocols

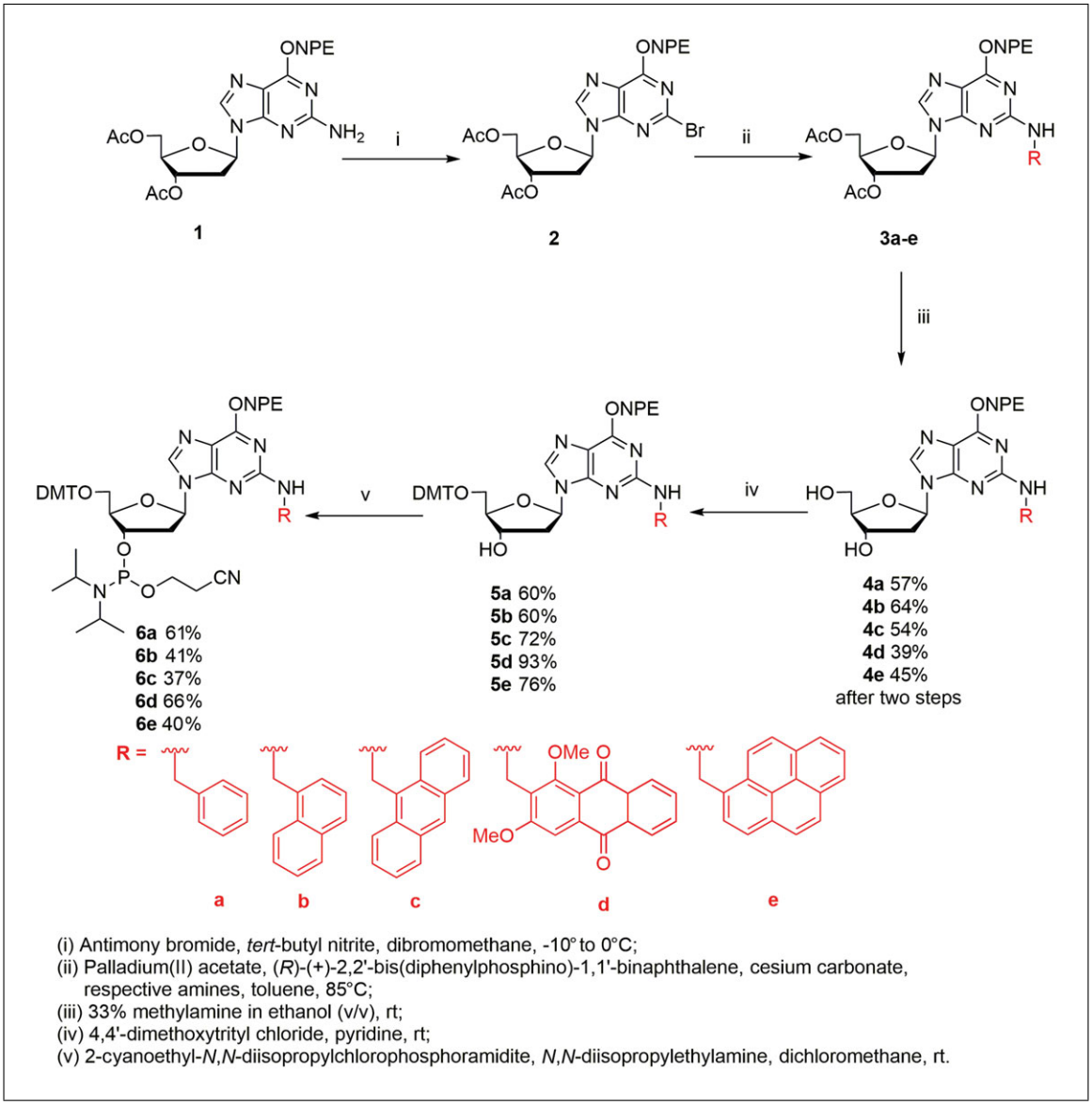

Figure 2 Synthetic strategy for N^2-dG modified nucleotides. rt, room temperature.

article: Harris & Harris, 2001; Harwood, Sigurdsson, Edfeldt, Reid, & Hopkins, 1999). Buchwald-Hartwig coupling occurred between nucleoside **2** and the respective amines to afford N^2-dG modified nucleosides (**3a** to **3e**; Champeil, Pradhan, & Lakshman, 2007). The commercially available amines were used for the synthesis of N^2-Bn and Naph-dG modifications. For N^2-Anth, Luc, and Pyre-dG, the respective amines were synthesized in house using published procedures (Abiraj & Gowda, 2003; Ghodke, Harikrishna, & Pradeepkumar, 2015; Lu, Lei, Tian, Wang, & Zhang, 2012). Further, deacetylation yielded the modified diols (**4a** to **4e**). Subsequent tritylation of the 5′-OH produced tritylated modified nucleosides (**5a** to **5e**). Phosphitylation of tritylated nucleosides furnished the modified phosphoramidites (**6a** to **6e**; Ghodke et al., 2015; Ghodke, Bommisetti, Nair, & Pradeepkumar, 2019).

Materials

Nitrogen (N_2) gas
2′-Deoxyguanosine monohydrate (dG, Innovassynth Technologies)
Triethylamine, anhydrous (Merck Scientific)
Ethyl acetate (EtOAc, Memba Chemicals)

Petroleum ether (Memba Chemicals)
Dichloromethane (DCM, Memba Chemicals)
4 Å molecular sieves
Antimony bromide ($SbBr_3$, MilliporeSigma)
tert-Butyl nitrite (TBN, MilliporeSigma)
Dibromomethane (CH_2Br_2, TCI)
Palladium(II) acetate ($Pd(OAc)_2$, MilliporeSigma)
(*R*)-(+)-2,2′-Bis(diphenylphosphino)-1,1′-binaphthalene ((*R)*-BINAP, MilliporeSigma)
Toluene, anhydrous (Merck Scientific)
Benzylamine (MilliporeSigma)
1-Naphthylmethylamine (MilliporeSigma)
Cesium carbonate (Cs_2CO_3, MilliporeSigma)
33% methylamine in ethanol (v/v, MilliporeSigma)
N, *N*-Diisopropylethylamine, anhydrous (DIPEA, MilliporeSigma)
Pyridine, anhydrous (Merck Scientific)
4,4′-Dimethoxytrityl chloride (DMT-Cl, Alfa Aesar)
2-Cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (CEP-Cl, MilliporeSigma)
1,4-Diazabicyclo[2.2.2]octane (DABCO, Kemphasol)
Sodium bicarbonate ($NaHCO_3$, Merck Scientific)
Sulfuric acid (H_2SO_4, Merck Scientific)
Absolute ethanol (EtOH)
Methanol (MeOH, Finar Scientific)
Celite (Merck Scientific)
Sodium sulfate (Thermo Fisher Scientific)

Sealed tube
Aluminum TLC plates (silica gel coated 60 F_{254}, Merck Scientific)
Reflux condensers (J-SIL Scientific Industries)
Heating mantle (Abhishek Scientific)
Filter paper (Whatman)
Glass funnel (J-SIL Scientific Industries)
Silica gel (60 Å, 40 to 100 µm, Finar Chemicals)
5-, 10-, 25-, 50-, 100-, 250-, 500-ml round bottom flasks (J-SIL Scientific Industries)
Magnetic stir plate and stir bars (Heildolph Instruments)
Teflon tape
UV lamp (254 nm)
1-, 5-, and 10-ml plastic syringes (BD Biosciences)
Rubber septa (MilliporeSigma)
125-, 500-ml separatory funnels (J-SIL Scientific Industries)
Test tubes (J-SIL Scientific Industries)
100- and 250-ml conical flasks (J-SIL Scientific Industries)
Rotary evaporator (Buchi)
Silicone oil bath
High-vacuum oil pump (ED15, Hind High Vacuum)
Chromatography columns (J-SIL Scientific Industries)

Prepare 2-bromo-2′-deoxyinosine (2-Br-dI) 2

NOTE: TLC was visualized by UV light or by dipping into a solution of 5% (v/v) concentrated H_2SO_4 in ethanol and heating. For preparing NMR samples, DABCO can be used.

1. Prepare NPE-protected deoxyguanosine **1** using the reported procedure (Current Protocols article: Harris & Harris, 2001; Harwood et al., 1999).

   Vacuum and heat drying of 2′-dG monohydrate is recommended for this step.

2. Dry 108 mg NPE-protected dG **1** (0.21 mmol) by co-evaporation on a rotary evaporator using anhydrous toluene (12 ml; to remove residual water) for 30 min.

3. Add 111 mg $SbBr_3$ to the round bottom flask containing dry NPE-protected dG **1** and purge reaction mixture with N_2.

4. Place bottle containing anhydrous CH_2Br_2 in an ice bath, maintain at 0°C then add 2.5 ml (12 ml/mmol) CH_2Br_2 to the flask in step 3 with continuous stirring.

5. Cool reaction mixture to –10°C (using an ice and salt bath) and add 0.10 ml TBN dropwise over 5 min.

6. Stir the resulting reaction mixture at –10°C for 2 hr then at 0°C for 2 hr.

7. After 4 hr, monitor by TLC using 70:30 EtOAc/petroleum ether (v/v, $R_f = 0.55$ of product).

8. After completion of reaction, extract reaction mixture using 3 × 30 ml DCM in a 250-ml separatory funnel.

9. Dry organic layers over Na_2SO_4, filter, and concentrate using a rotary evaporator.

10. Purify crude product 2-Br-dI **2** by column chromatography (4 to 6 g silica gel, 1.5-cm diameter column) using 55:45 EtOAc/petroleum ether (v/v) solvent system.

11. Evaporate product-containing fractions and dry under vacuum.

12. Yield of compound **2**: 98 mg, 82% as a pale yellow solid.

13. Store 2-Br-dI **2** at 4°C; the compound is stable at 4°C for 6 months.

14. Characterize product by ^1H, ^{13}C NMR, HRMS, and melting point measurements.

    *The characterization data for 2-Br-dI **2** is available in the published report (Ghodke et al., 2015).*

Perform Buchwald-Hartwig coupling and deacetylation reaction

15. Weigh 0.1 equivalents $Pd(OAc)_2$ and 0.3 equivalents (*R*)-BINAP into a screw-capped tube, flush with N_2 gas then add anhydrous toluene (9.9 ml/mmol).

    a. For **3a**: 5.9 mg $Pd(OAc)_2$, 50 mg (*R*)-BINAP, 3 ml toluene.
    b. For **3b**: 11 mg $Pd(OAc)_2$, 88 mg (*R*)-BINAP, 7 ml toluene.
    c. For **3c**: 3.9 mg $Pd(OAc)_2$, 33 mg (*R*)-BINAP, 2 ml toluene.
    d. For **3d**: 18 mg $Pd(OAc)_2$, 142 mg (*R*)-BINAP, 8 ml toluene.
    e. For **3e**: 16 mg $Pd(OAc)_2$, 132 mg (*R*)-BINAP, 7 ml toluene.

16. Stir the above mixture 5 min at room temperature.

17. Add 1.4 equivalents Cs_2CO_3, 1.1 equivalents of respective amines, and 1 equivalent 2-Br-dI **2**, consecutively.

    a. For **3a**: 131 mg Cs_2CO_3, 0.03 ml benzylamine, 150 mg 2-Br-dI **2**.
    b. For **3b**: 214 mg Cs_2CO_3, 0.07 ml 1-naphthylmethylamine, 269 mg 2-Br-dI **2**.
    c. For **3c**: 87 mg Cs_2CO_3, 41 mg 9-(aminomethyl)anthracene, 100 mg 2-Br-dI **2**.
    d. For **3d**: 375 mg Cs_2CO_3, 250 mg 2-(aminomethyl)-1,3-dimethoxy anthraquinone, 430 mg 2-Br-dI **2**.
    e. For **3e**: 323 mg Cs_2CO_3, 182 mg 1-(aminomethyl)pyrene, 403 mg 2-Br-dI **2**.

18. Purge tube with N_2 gas, seal cap of tube with Teflon tape, and heat tube in an oil bath.

    a. For **3a**: 85°C, 18 hr.
    b. For **3b**: 85°C, 21 hr.
    c. For **3c**: 85°C, 8 hr.
    d. For **3d**: 85°C, 10 hr.
    e. For **3e**: 85°C, 21 hr.

19. Monitor by TLC using 75:25 EtOAc/petroleum ether (v/v).

20. After consumption of starting material, stop reaction by passing through a Celite pad. Wash Celite pad with EtOAc (100 to 250 ml).

21. Concentrate and dry filtrate to obtain crude products **3a** to **3e**.

    *Due to difficulty in removing by-products and excess reagents, the compounds **3a** to **3e** are further subjected to deacetylation.*

22. Perform deacetylation of crude Buchwald-Hartwig coupled products (**3a** to **3e**) using 33% methylamine in ethanol (v/v).

    a. For **4a**: 156 mg **3a**, 5 ml $MeNH_2$ in EtOH.
    b. For **4b**: 260 mg **3b**, 9 ml $MeNH_2$ in EtOH.
    c. For **4c**: 127 mg **3c**, 3.7 ml $MeNH_2$ in EtOH.
    d. For **4d**: 404 mg **3d**, 11 ml $MeNH_2$ in EtOH.
    e. For **4e**: 547 mg **3e**, 15 ml $MeNH_2$ in EtOH.

23. Stir reaction mixture for the respective time at room temperature.

    a. For **4a**: 3 hr.
    b. For **4b**: 2.5 hr.
    c. For **4c**: 3 hr.
    d. For **4d**: 2 hr.
    e. For **4e**: 1 hr 15 min.

24. Monitor reaction by TLC using 10:90 MeOH/DCM (v/v).

    a. For **4a**: $R_f = 0.47$.
    b. For **4b**: $R_f = 0.49$.
    c. For **4c**: $R_f = 0.50$.
    d. For **4d**: $R_f = 0.49$.
    e. For **4e**: $R_f = 0.49$.

25. After completion, concentrate reaction mixture under reduced pressure.

26. Purify crude compounds by column chromatography (6 to 10 g silica gel, 2-cm diameter column) using appropriate solvent system to obtain deprotected nucleosides (**4a** to **4e**).

    a. For **4a**: 4:96 MeOH/DCM (v/v).
    b. For **4b**: 4:96 MeOH/DCM (v/v).
    c. For **4c**: 3:97 MeOH/DCM (v/v).
    d. For **4d**: 3:97 MeOH/DCM (v/v).
    e. For **4e**: 2.5:97.5 MeOH/DCM (v/v).

27. Evaporate product-containing fractions and dry under vacuum.

28. Yield after two steps (**4a** to **4e**):

a. For **4a**: 76 mg, 57% (slightly brownish solid).
b. For **4b**: 171 mg, 64% (slightly yellow solid).
c. For **4c**: 59 mg, 54% (slightly greenish solid).
d. For **4d**: 190 mg, 39% (brownish solid).
e. For **4e**: 202 mg, 45% (slightly yellow solid).

29. Store compounds at 4°C.

*Compounds **4a** to **4e** are stable at 4°C for up to 1 year.*

30. Characterize product by ^{1}H, ^{13}C NMR, HRMS, and melting point.

*The characterization data for **4a** to **4e** is available in the published reports (Ghodke et al., 2015, 2019).*

Perform tritylation

31. Co-evaporate N^2-dG-modified nucleosides **4a** to **4e** (1 equivalent) using anhydrous pyridine (5 ml) then dissolve in pyridine (10 ml/mmol).

a. For **5a**: 251 mg **4a**, 5 ml dry pyridine.
b. For **5b**: 171 mg **4b**, 3 ml dry pyridine.
c. For **5c**: 162 mg **4c**, 3 ml dry pyridine.
d. For **5d**: 140 mg **4d**, 2 ml dry pyridine.
e. For **5e**: 151 mg **4e**, 3 ml dry pyridine.

32. Add 1.22 equivalents DMT-Cl and stir at room temperature for the respective time under N_2 atmosphere.

a. For **5a**: 332 mg DMT-Cl, 24 hr.
b. For **5b**: 126 mg DMT-Cl, 10 hr.
c. For **5c**: 180 mg DMT-Cl, 10 hr.
d. For **5d**: 135 mg DMT-Cl, 10 hr.
e. For **5e**: 102 mg DMT-Cl, 12 hr.

33. Monitor reaction by TLC in 2:98 MeOH/DCM plus 2% Et_3N.

a. For **5a**: $R_f = 0.55$.
b. For **5b**: $R_f = 0.50$.
c. For **5c**: $R_f = 0.50$.
d. For **5d**: $R_f = 0.55$.
e. For **5e**: $R_f = 0.55$.

34. Extract reaction mixture using DCM (100 ml) and wash with saturated $NaHCO_3$ and water using a 500-ml separatory funnel.

35. Dry DCM layer over Na_2SO_4, filter, and evaporate on rotary evaporator.

36. Purify crude DMT-protected compounds by column chromatography using appropriate solvent system.

a. For **5a**: 90:10 DCM/petroleum ether plus 2% Et_3N (v/v).
b. For **5b**: 1:99 MeOH/DCM plus 2% Et_3N (v/v).
c. For **5c**: 1:99 MeOH/DCM plus 2% Et_3N (v/v).
d. For **5d**: 1:99 MeOH/DCM plus 2% Et_3N (v/v).
e. For **5e**: 1:99 MeOH/DCM plus 2% Et_3N (v/v).

37. Evaporate product-containing fractions and dry under vacuum.

38. Yield of compounds:

    a. For **5a**: 237 mg, 60% (pale yellow solid).
    b. For **5b**: 154 mg, 60% (yellow solid).
    c. For **5c**: 177 mg, 72% (slightly greenish solid).
    d. For **5d**: 187 mg, 93% (dark brown solid).
    e. For **5e**: 178 mg, 76% (pale yellow solid).

39. Store compounds **5a** to **5e** at 4°C.

    *Compounds **5a** to **5e** are stable at 4°C for up to 6 months.*

40. Characterize products by ^{1}H, ^{13}C NMR, HRMS, and melting point.

    *The characterization data for **5a** to **5e** is available in the published reports (Ghodke et al., 2015, 2019).*

Perform phosphitylation

41. Dissolve DMT-protected nucleosides **5a** to **5e** (1 equivalent) in DCM (10 ml/mmol). Add 8 equivalents DIPEA followed by 2 equivalents CEP-Cl.

    a. For **6a**: 150 mg tritylated nucleoside **5a**, 0.25 ml DIPEA, and 127 mg CEP-Cl.
    b. For **6b**: 150 mg tritylated nucleoside **5b**, 0.25 ml DIPEA, and 83 mg CEP-Cl.
    c. For **6c**: 170 mg tritylated nucleoside **5c**, 0.26 ml DIPEA, and 88 mg CEP-Cl.
    d. For **6d**: 150 mg tritylated nucleoside **5d**, 0.2 ml DIPEA, and 71 mg CEP-Cl.
    e. For **6e**: 200 mg tritylated nucleoside **5e**, 0.3 ml DIPEA, and 100 mg CEP-Cl.

42. Stir reaction mixture under N_2 atmosphere at room temperature for the respective times.

    a. For **6a**: 2 hr.
    b. For **6b**: 2 hr 15 min.
    c. For **6c**: 1 hr.
    d. For **6d**: 1 hr 30 min.
    e. For **6e**: 1 hr 15 min.

43. Monitor reaction by TLC using appropriate solvent system.

    a. For **6a**: $R_f = 0.65$ (90:10 DCM/petroleum ether plus 2% Et_3N).
    b. For **6b**: $R_f = 0.65$ (75:25 DCM/petroleum ether plus 2% Et_3N).
    c. For **6c**: $R_f = 0.60$ (75:25 DCM/petroleum ether plus 2% Et_3N).
    d. For **6d**: $R_f = 0.56$ (75:25 DCM/petroleum ether plus 2% Et_3N).
    e. For **6e**: $R_f = 0.66$ (75:25 DCM/petroleum ether plus 2% Et_3N).

44. After completion, quench reaction using ~0.1 to 1 ml anhydrous MeOH and stir 10 to 30 min.

45. Dilute reaction mixture with DCM and perform extraction using DCM (100 ml) and aqueous $NaHCO_3$. Dry organic layer over Na_2SO_4, filter, and evaporate.

46. Purify compounds using column chromatography and solvent systems described below to obtain phosphitylated compounds **6a** to **6e**.

    a. For **6a**: 35:65 DCM/petroleum ether plus 2% Et_3N (v/v).
    b. For **6b**: 35:65 DCM/petroleum ether plus 2% Et_3N (v/v).
    c. For **6c**: 40:60 DCM/petroleum ether plus 2% Et_3N (v/v).
    d. For **6d**: 30:70 DCM/petroleum ether plus 2% Et_3N (v/v).
    e. For **6e**: 45:55 DCM/petroleum ether plus 2% Et_3N (v/v).

47. Evaporate product-containing fractions and dry under vacuum.

48. Perform precipitation using petroleum ether (40 ml) to remove triethylamine if necessary.

49. Yield of compounds **6a** to **6e**:

    a. For **6a**: 111 mg, 61% (pale yellow solid).
    b. For **6b**: 74 mg, 41% (pale yellow solid).
    c. For **6c**: 75 mg, 37% (slightly greenish solid).
    d. For **6d**: 118 mg, 65% (brown solid).
    e. For **6e**: 93 mg, 40% (pale yellow solid).

50. Store compounds **6a** to **6e** at −20°C.

    *Compounds **6a** to **6e** are stable for up to 2 years under inert conditions. Drierite can be used for long-term storage of phosphoramidites.*

51. Characterize product by ^{31}P NMR, HRMS, and melting point.

    *The characterization data for **6a** to **6e** is available in the published reports (Ghodke et al., 2015, 2019).*

SYNTHESIS OF N^2-dG MODIFIED OLIGONUCLEOTIDES

BASIC PROTOCOL 2

This section describes the detailed synthesis of various N^2-dG modified oligonucleotides as outlined in Figure 3. Here we describe the details of the incorporation of modified phosphoramidites (see Basic Protocol 1) into oligonucleotides using a MerMade-4 DNA/RNA synthesizer (Bioautomation). All N^2-dG modified oligonucleotides sequences (Table 1) were synthesized using appropriate controlled pore glass (CPG) solid support on a 1 µmol scale. Coupling of phosphoramidites is carried out as per standard synthesis cycles (2 min), except for the modified phosphoramidites (6 to 10 min) due to their bulky nature. Use phosphoramidites dissolved in CH_3CN within 2 days. The deprotection of oligonucleotides is carried out in four different steps, which is discussed in detail in this section. The purification and characterization of modified oligonucleotides were carried out using PAGE and MALDI spectrometry, respectively.

All oligonucleotides were characterized using MALDI/ESI-MS. The characterization data is available in the published reports (Ghodke et al., 2015, 2019; Yockey et al., 2017).

Materials

Argon gas (Medigas)
Phosphorous pentoxide (P_2O_5)
Molecular sieves (4 Å, Alfa Aesar)
Acetonitrile (CH_3CN, anhydrous)
Reagents for DNA synthesis:
  Deblock: Trichloroacetic acid/dichloromethane (3:100, v/v, MilliporeSigma)
  Cap A: 2,6-lutidine/acetic anhydride/tetrahydrofuran (8:1:1, v/v/v, MilliporeSigma)
  Cap B: *N*-methylimidazole/tetrahydrofuran/pyridine (8:1:1, v/v/v, MilliporeSigma)
  Oxidizer: tetrahydrofuran/water/pyridine/iodine (90.54:9.05:0.41:0.43, v/v/v/w, MilliporeSigma)
  Activator: acetonitrile/5-(ethylthio)-1*H*-tetrazole (ETT; 100:3, v/w; MilliporeSigma)
N^4-Acetyl-dC phosphoramidite (Innovassynth Technologies)
N^6-Benzoyl-dA phosphoramidite (Innovassynth Technologies)
N^2-Isobutyryl-dG phosphoramidite (Innovassynth Technologies)

dT phosphoramidite (Innovassynth Technologies)
N^4-Acetyl or benzoyl-dC derivatized CPG (500 Å; MilliporeSigma)
N^6-Benzoyl-dA derivatized CPG (500 Å; MilliporeSigma)
N^2-Isobutyryl-dG derivatized CPG (1000 Å; MilliporeSigma)
dT derivatized CPG (500 Å; MilliporeSigma)
Nuclease-free water (Merck Millipore)
10% diethylamine (MilliporeSigma) in anhydrous CH_3CN (v/v)
1 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, MilliporeSigma) in anhydrous CH_3CN (stock solution)
Ammonium hydroxide solution (30% to 33%, w/v, MilliporeSigma)
3 M sodium chloride (NaCl; Merck Scientific) solution (dissolve 87.66 g NaCl in 500 ml nuclease-free water)
Fast-moving DNA dye (see recipe)
40% acrylamide/bisacrylamide (see recipe, MP Biomedicals)
20% PAGE solution (see recipe)
TEN buffer (see recipe)
1× TBE running buffer (see recipe)
10% ammonium persulfate (APS, Merck; 1 g in 10 ml water; prepare fresh)
N,*N*,*N*′,*N*′-Tetramethylethane-1,2-diamine (TEMED, MilliporeSigma)
Ammonium acetate (SD Fine Chemicals)
Absolute ethanol (MP Biomedicals)
Ammonium citrate dibasic (MilliporeSigma) solution (2.5 mg in 50 µl water)
3-Hydroxypicolinic acid (3-HPA; MilliporeSigma) solution (2.5 mg in 50 µl 50% aqueous CH_3CN)

MerMade-4 Nucleic Acid Synthesizer (Bioautomation)
Empty CPG column (Biosearch Technologies)
Frits (big, 3 mm and small, 1.5 mm; Biosearch Technologies)
Screw cap vial (Corning)
Vortex mixer (Vortex Genie-2)
Benchtop centrifuge (Eppendorf)
Shaker (Grant-bio)
1.5-ml microcentrifuge tubes (Tarson)
Heating block set to 55°C (Grant-bio)
Centrifugal vacuum evaporator (Eppendorf)
0.5- and 1-mm spacers (Biotech R&D Laboratories)
0.5- and 1-mm combs (Biotech R&D Laboratories)
Gel system (Biotech R&D Laboratories)
Glass plates for PAGE (20 × 30 cm; Biotech R&D Laboratories)
Electrophoretic power supply (EPS 3501 XL, GE Healthcare)
Micro pipets (Eppendorf)
Pipet tips (10, 200, and 1000 µl, Tarson)
5-ml syringe
15- and 50-ml centrifuge tubes (Tarson)
PVDF filter (0.45 µm, Whatman)
C_{18} Sep-Pak column (Waters)
UV instrument (Lambda Bio+, PerkinElmer)
1-cm quartz glass cuvettes (Hellma Analytical)
−30°C freezer (SANYO biomedical freezer)
Centrifuge (4°C, Eppendorf)
ESI-MS or MALDI-TOF-MS system (UltrafleXtreme or AutofleXtreme, Bruker)

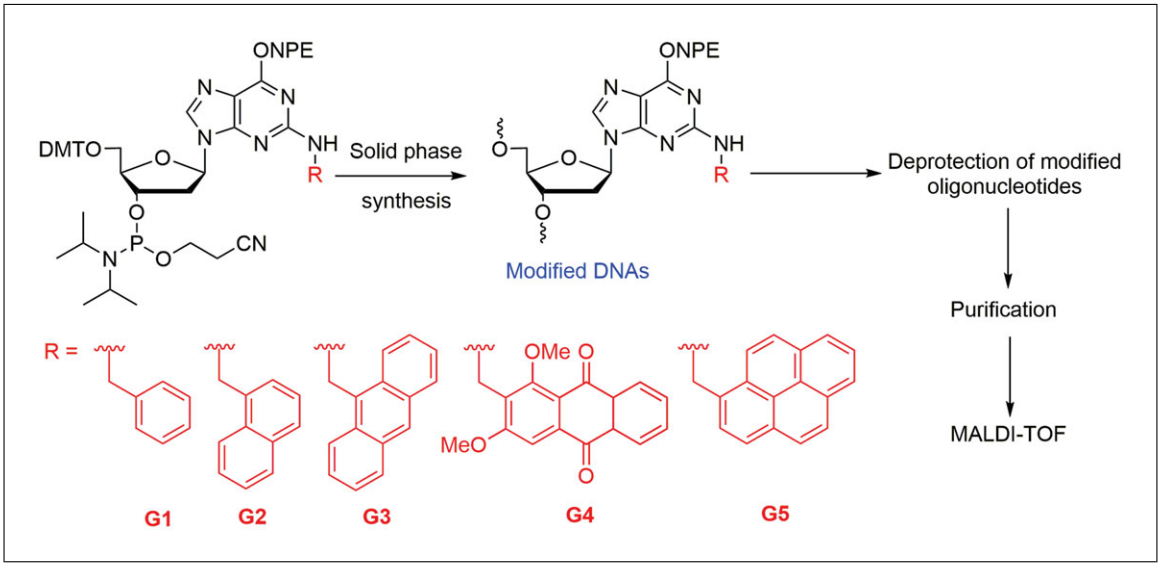

Figure 3 Flow chart for synthesis of N^2-dG modified DNAs.

Synthesize modified oligonucleotides

NOTE: These settings are applied for MerMade-4 only. Make sure humidity of the room is <40%. Also, make sure there is no leakage of argon gas.

1. Vacuum dry phosphoramidites at room temperature, 24 hr over P_2O_5.
2. Attach all reagent bottles for cap A, cap B, deblock, activator, and oxidizer reagents to the DNA synthesizer.
3. Use 5-(ethylthio)-1*H*-tetrazole (ETT) as an activator.
4. Dissolve the appropriate modified phosphoramidites (**6a** to **6e**) and unmodified phosphoramidites in anhydrous CH_3CN to maintain concentrations of 0.1 M and 0.067 M, respectively.
5. Prepare all the above amidite solutions and add to their respective DNA synthesizer reagent bottles under a N_2 atmosphere.
6. Store amidite solutions over molecular sieves (4 Å) 10 hr at 4°C prior to use.
7. Attach bottles containing phosphoramidite solutions (step 6) to the MerMade-4.
8. Attach solid support containing columns to the instrument. Pack CPG column as mentioned below:
   a. Take a clean column, rinse with anhydrous CH_3CN, and flush with N_2.
   b. Insert small frit to the end of the column slowly.
   c. Weigh CPG on the top of small frit as per requirement.
   d. Carefully insert big frit on the top of CPG support.
   e. Flush column with N_2 to remove excess CPG outside the frits.
9. Input desired oligonucleotide sequence containing N^2-dG modification (at particular position, see Table 1) and coupling details using the vendor software installed in the computer of DNA synthesizer.
10. Set coupling time to 2 min for unmodified phosphoramidites and to 6 to 10 min for modified phosphoramidites.
11. Start solid-phase synthesis to obtain modified DNAs.

Table 1 N^2-dG Modified DNAs

Code	Oligonucleotide sequences (5′-3′)[a]	Molecular weight (calculated)	Molecular weight (found)
D1	TCTAG1GGTCCTAGGACCC	5566.7	5566.0
D2	TCTG1GGGTCCTAGGACCC	5581.7	5581.9
D3	TCTAGGG1TCCTAGGACCC	5566.7	5567.3
D4	TCCTACCGTGCCTACCTGAACAGCTG GTCACACTG1ATGCCTACGAGTACG	15358	15354.8
D5	TCTAG2GGTCCTAGGACCC	5616.8	5616.8
D6	TCTG2GGGTCCTAGGACCC	5632.8	5635.1
D7	TCTAGGG2TCCTAGGACCC	5616.8	5617.4
D8	TCCTACCGTGCCTACCTGAACAGCT GGTCACACTG2ATGCCTACGAGTACG	15408.1	15409.1
D9	TCTAG3GGTCCTAGGACCC	5666.9	5667.6
D10	TCTG3GGGTCCTAGGACCC	5682.9	5682.0
D11	TCTAGGG3TCCTAGGACCC	5666.9	5666.9
D12	TCCTACCGTGCCTACCTGAACAGCTG GTCACACTG3ATGCCTACGAGTACG	15458.2	15453.8
D13	TCTAG4GGTCCTAGGACCC	5757	5756
D14	TCTG4GGGTCCTAGGACCC	5773	5773
D15	TCTAGGG4TCCTAGGACCC	5757	5757
D16	TCCTACCGTGCCTACCTGAACAGCTG GTCACACTG4ATGCCTACGAGTACG	15547	15547
D17	AGCTGGTCACACTG4AAGCGTTAGCCATTA	9183	9183
D18	GCCGG4AATAGCGCA	4579	4579
D19	TGCG4CTATTCCGGC	4512	4513
D20	ATGG4CTGATCCGCGCGGATCAG	7058	7057
D21	CTATG4TCGATCCGCGGATCGAC	6993	6994
D22	TCTAG5GGTCCTAGGACCC	5690.9	5690.2
D23	TCTG5GGGTCCTAGGACCC	5706.9	5705.8
D24	TCTAGGG5TCCTAGGACCC	5690.9	5689.9
D25	TCCTACCGTGCCTACCTGAACAGCTG GTCACACTG5ATGCCTACGAGTACG	15482.2	15480.9

[a]Structures of N^2-dG modifications (see Fig. 1): G1, N^2-Bn-dG; G2, N^2-Naph-dG; G3, N^2-Anth-dG; G4, N^2-Luc-dG; G5, N^2-Pyre-dG.

12. Extend DNA sequence (Table 1) using the respective N^2-dG modified and unmodified phosphoramidites in DMT-off mode.
13. After completion, remove CPG column from the instrument, flush with N_2, and store in −30°C freezer.

Remove cyanoethyl protecting group

14. Carefully transfer CPG support from the column to the 1.5-ml screw cap vial.
15. Add 800 µl 10% diethylamine in anhydrous CH_3CN (v/v) to the CPG support.
16. Shake 5 min at room temperature.

17. Discard supernatant layer and wash CPG support with CH_3CN (2 × 400 µl).

18. Air dry CPG support ~1 hr.

Remove O^6-NPE protecting group

19. Perform NPE deprotection using 1 M DBU in anhydrous CH_3CN solution as per Glen Research Protocol (*https://www.glenresearch.com/media/productattach/import/tbn/TB_2-F-dI.pdf*, see Critical Parameters section for more details).

    Shaking at 350 rpm is recommended.

20. After 1 hr of treatment, discard solution and wash solid support with anhydrous MeOH and anhydrous CH_3CN.

21. Air dry CPG support ~1 hr.

Remove solid support

22. Treat CPG support with 30% to 33% aqueous ammonium hydroxide (v/v, 1 ml) at room temperature, 3 hr; shaking at 350 rpm is recommended.

23. After 3 hr, collect supernatant in a new fresh 1.5-ml microcentrifuge tube.

24. Wash CPG support with water (2 × 400 µl) and collect supernatant in the above tube (step 23).

25. Evaporate supernatant solution under vacuum using a centrifugal evaporator to obtain the dry oligonucleotide pellet.

Remove base protecting groups

26. Resuspend dry oligonucleotide pellet in 30% to 33% aqueous ammonium hydroxide (v/v, 1 ml) and heat 16 hr at 55°C.

27. Evaporate solution under vacuum using centrifugal evaporator. Dissolve crude oligonucleotide in nuclease-free water.

28. Check concentration of crude oligonucleotide.

Purify modified oligonucleotides: Perform analytical gel

For long oligonucleotides (50 nt) use 12% PAGE solution. Performing steps 29 to 51 qualitatively confirms the correct length of oligonucleotides.

29. Keep a 0.05-cm gel thickness and gel dimension of 20 cm × 30 cm.

30. Prepare 50 ml 20% PAGE solution.

31. Gently stir PAGE solution.

32. Clean both glass plates using 70% aqueous ethanol (v/v) and wipe with paper towels.

33. Assemble glass plates by placing a 0.05-cm spacer between them in a horizontal manner.

34. Add freshly prepared 10% (150 µl, w/v) ammonium persulfate to the gel solution.

35. Add TEMED (35 µl) to the above gel solution.

36. Carefully pour gel solution immediately between the two glass plates; avoid air bubble formation.

37. Immediately place comb (sixteen teeth) between the glass plates.

38. Polymerization of the acrylamide and bisacrylamide will take ~30 to 45 min.

39. Monitor gel polymerization using the leftover gel solution.
40. After 45 min, remove comb carefully and wash wells with water using a syringe.
41. Connect assembly to the gel system.
42. Fill gel system using 1× TBE running buffer.
43. Wash all wells with 1× TBE using a 5-ml disposable syringe and add fast-moving dye in one of the wells.
44. Turn on the power supply and start pre-run and run 30 min at 30 W.
45. After the pre-run, load crude oligonucleotide samples carefully (1 nmol each mixed with equal volume of stop solution).
46. Perform electrophoresis at 30 W for ~2.5 hr and monitor fast-moving DNA dye (bromophenol blue).
47. Turn off power supply and dismantle gel system.
48. Set gel plates in a horizontal manner.
49. Carefully separate gel plates. Remove one glass plate and cover gel with plastic film. Flip gel and remove another glass plate carefully.
50. Make sure that gel is covered with plastic film on both sides.
51. Visualize the desired oligo bands using UV at 260 nm (by keeping the gel on TLC plate).

Purify modified oligonucleotides: Perform preparative gel

52. Keep a 0.1-cm gel thickness and gel dimensions of 20 cm × 30 cm.
53. Make sure to use comb with two teeth and a 0.1-cm spacer (~100 ml of gel solution is required). Load 100 nmol DNA sample into each well; mix with equal volume of stop solution. Carry out steps 31 to 50 from the previous analytical gel section.
54. Perform step 51.
55. Mark and cut desired band; do not expose the gel for a long time (not more than 5 min).
56. Crush gel band into fine particles using a 5-ml syringe (i.e., place gel pieces inside the syringe and crush using the piston) and collect residue in a 15-ml centrifugal tube.
57. Add 10 ml TEN buffer to the centrifugal tube, keep at −80°C 1 hr then heat at 90°C, 5 min.
58. Extract gel particles by shaking the centrifugal tube at 300 rpm at room temperature overnight. Collect supernatant in a fresh 15-ml centrifugal tube.
59. Perform second extraction using 10 ml TEN buffer for 6 hr. Collect supernatant in the same 15-ml centrifugal tube (step 58).
60. Filter supernatant using a 0.45–µm PVDF filter prior to loading into the Sep-Pak column.
61. Desalt oligonucleotide using the Sep-Pak column:
    a. Wash Sep-Pak column with 5 ml of 50% CH_3CN in water (v/v), 5 ml water, and 5 ml TEN buffer.
    b. Slowly load filtered oligonucleotide sample from step 60 onto the column.

c. Slowly wash column with TEN buffer (5 ml) and water (5 ml).
d. Elute oligonucleotide sample using 5 ml of 50% CH_3CN in water (v/v) and collect into the 1.5-ml microcentrifuge tubes.
e. Evaporate sample under vacuum using a centrifugal evaporator.

62. Dissolve oligonucleotide pellet in nuclease-free water; check concentration at 260 nm using spectrophotometer.

63. Determine molar extinction coefficients employing an oligo analyzer from IDT (*https://www.idtdna.com/calc/analyzer*).

Characterize modified oligonucleotides

64. Perform MALDI-TOF analysis of modified oligonucleotide sequences (Table 1, calculate the molecular weights of oligonucleotides using Mongo calculator; *https://mods.rna.albany.edu/masspec/Mongo-Oligo*).

65. Prepare oligonucleotide sample for MALDI analysis as per reported protocol (Shah & Friedman, 2008).

a. Dissolve 1 nmol of each modified oligonucleotide in 30 µl water.
b. Add 15 µl of 7.5 M ammonium acetate to the oligonucleotide sample.
c. Gently vortex sample and allow to stand 1 hr at room temperature.
d. Add 115 µl of 100% cold EtOH to the same sample and keep at −80°C for 7 hr or overnight.
e. Centrifuge sample at 17,400 × *g* (4°C) for 30 min and carefully decant supernatant.
f. Add 100 µl of 70% cold EtOH to the oligonucleotide pellet.
g. Centrifuge 20 min at 17,400 × *g* (4°C) and decant EtOH immediately.
h. Air dry oligonucleotide pellet 15 min.
i. Dissolve oligonucleotide pellet in water (3 µl) and use for the MALDI analysis.
j. Prepare matrix of 3-hydroxypicolinic acid (3-HPA) and ammonium citrate (9:1 ratio).
k. Spot matrix and oligonucleotide sample on the MALDI plate and air dry 15 min.
l. Analyze samples in positive mode; perform calibration before analysis using desired standards.

REAGENTS AND SOLUTIONS

For common stock solutions, see Current Protocols article: Current Protocols (2001).

Acrylamide, 40%/bisacrylamide solution

For 1 L: 387 g of acrylamide, 13.8 g of bisacrylamide. Make up volume to 1 L using 18 mΩ water; protect from light.
Store at 4°C for up to 2 months.

EDTA, 0.5 M, pH 8

For 800 ml: 149 g of $Na_2EDTA{\cdot}2H_2O$ in 800 ml of water, add 14 g of NaOH pellets and stir to dissolve. Adjust pH using NaOH solution to pH 8. Sterilize by autoclaving. Prepare fresh.

Fast-moving DNA dye

For 40 ml: 32 ml formamide (v/v), 4 ml 0.5 M EDTA (pH 8; see recipe), 4 ml 10× TBE (see recipe), 10 mg bromophenol blue sodium salt (MilliporeSigma). Prepare fresh.

PAGE solution, 20%

For 500 ml: Prepare 7 M urea denaturing gel solution by mixing 40% (v/v) acrylamide/bisacrylamide solution (250 ml; see recipe), 10× TBE (50 ml; see recipe), urea (210 g; MP Biomedicals), and make up volume using 18 mΩ water; protect from light.

Store at 4°C for up to 4 weeks.

Stop solution

For 40 ml: 32 ml deionized formamide, 4 ml 0.5 M EDTA (pH 8), 4 ml 10× TBE (see recipe), 25 mg bromophenol blue sodium salt (fast moving; MilliporeSigma), 25 mg xylene cyanol FF (slow moving; MilliporeSigma). Prepare fresh.

TBE buffer, 10×

For 1 L: 40 ml of 0.5 M EDTA (pH 8), 55 g of boric acid (MP Biomedicals), 108 g of Tris base (MP Biomedicals); mix thoroughly and sterilize by autoclaving. Prepare fresh.

TBE running buffer, 1×

For 1 L: 100 ml 10× TBE (see recipe) in 900 ml of water.

TEN (Tris/EDTA/NaCl) buffer

For 1 L: 888 ml of 18 mΩ water, 10 ml of 1 M Tris·HCl (pH 8), 2 ml of 0.5 M EDTA (pH 8), 100 ml of 3 M NaCl; sterilize by autoclaving. Prepare fresh.

Final concentrations: 10 mM Tris, 1 mM EDTA, 300 mM NaCl, pH ~8.0.

COMMENTARY

Background Information

DNA "lesions" are formed due to the exposure of exogenous and endogenous agents (Errol et al., 2006). The N^2-position of dG is susceptible to reaction with various cancer-causing agents such as PAHs, aldehydes, and heterocyclic aromatic amines, which leads to the N^2-dG DNA modifications. Specifically, benzo[*a*]pyrene and its diol epoxide metabolite are well known for the formation of N^2-dG DNA adducts (Stowers & Anderson, 1985). Apart from this, benzyl halides and metabolites of anthracene, pyrene, and lucidin also contribute to the formation of N^2-dG DNA adducts as summarized in Figure 4.

The N^2-dG adducts protrude into the minor groove of DNA and have the potential to disturb the normal replication process by high-fidelity DNA polymerases. In this regard, the N^2-dG DNA adducts are well studied with respect to their diversity and effect on biological processes employing various translesion synthesis (TLS) DNA polymerases (Kottur et al., 2015; Yang & Gao, 2018; Yockey et al., 2017). To understand the miscoding potential of TLS polymerases opposite to N^2-dG adducts during replication, the corresponding N^2-dG modified oligonucleotides are required in bulk amount for further studies. Several synthetic strategies have been reported for the N^2-dG modified adducts. Recently, we have summarized the specific applications of Buchwald-Hartwig coupling in the synthesis of various nucleoside modifications (Ghodke & Pradeepkumar, 2018).

Various methods have been utilized to study the TLS of modified oligonucleotides to assess possible misincorporations opposite to the damaged site. To understand the biological implications of DNA damage, biochemical assays such as full-length extension (standing or running start), single nucleotide incorporation, steady-state, pre-steady state, and extension kinetics have been extensively used (Delaney & Essigmann, 2008; Guengerich, 2006). In addition, the demand for damaged oligonucleotides is considerable in order to study their properties by NMR spectroscopy (Lukin & de los Santos, 2006) and X-ray crystallography (Yang, 2014). Liquid chromatography with tandem mass spectrometry (LC-MS/MS) has also been used to provide more insights into TLS processes that are responsible for the frameshift mutations and other mutagenic products (Tretyakova, Villalta, & Kotapati, 2013).

We developed a robust synthesis for N^2-dG damaged oligonucleotides of varying

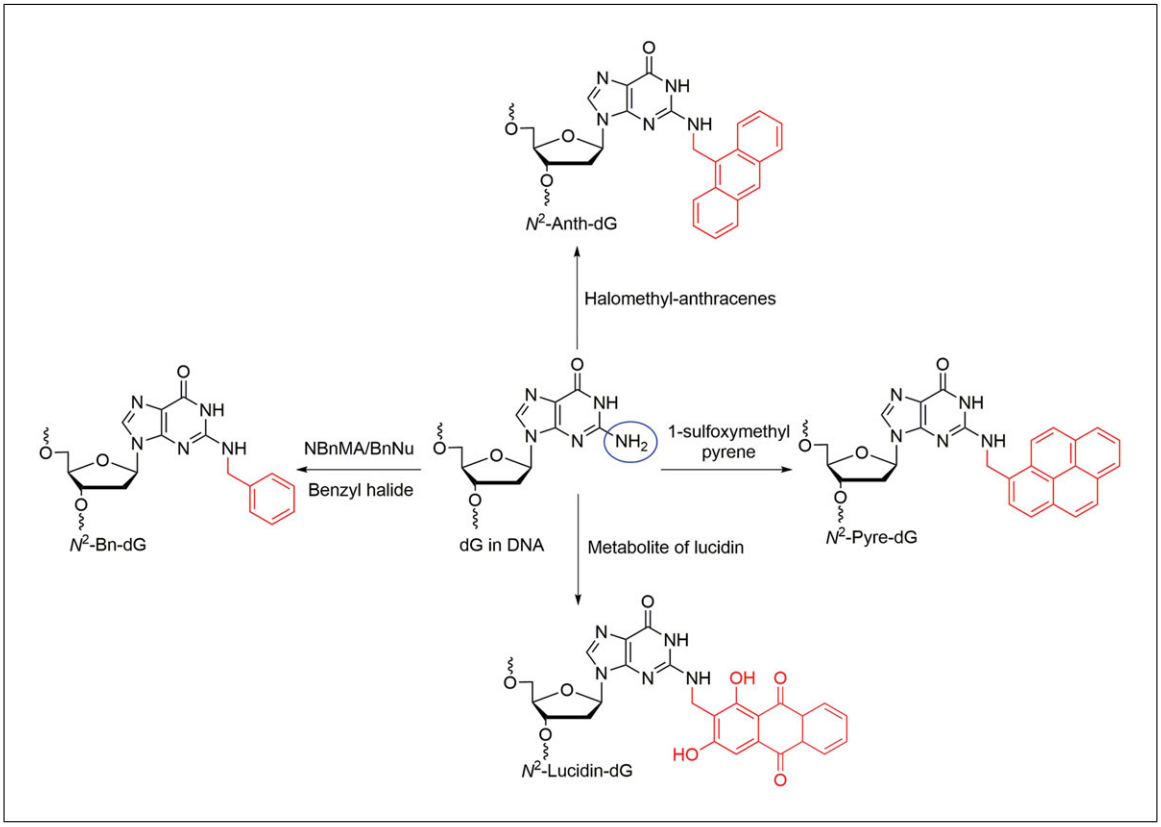

Figure 4 N^2-dG modifications.

bulkiness and nature using a phosphoramidite approach employing the Buchwald-Hartwig reaction. These phosphoramidites were successfully incorporated into DNA (Table 1). The solid phase synthesis was carried out at standard 1 µmol scale and stepwise coupling efficiencies were determined by automated trityl absorbance monitoring. After deprotection, the DNA products were purified by gel electrophoresis and their integrity was confirmed using MALDI-TOF/ESI-MS. We have also synthesized N^2-dG modified oligonucleotides in large amounts for X-ray crystallography (500 µg to 1.3 mg).

Critical Parameters and Troubleshooting

Prior experience with standard organic chemistry laboratory techniques is necessary. Reagents need to be handled carefully and reactions need to be carried out in a well-ventilated fume hood. Acrylamide is neurotoxic and needs to be handled in well-ventilated fume hood. Prior experience in nucleic acid chemistry is necessary to carry out phosphoramidite synthesis. The modified oligonucleotide synthesis and purification requires prior experience using a DNA synthesizer and gel purification techniques. Use of 5-ethylthio 1*H*-tertrazole as activator is recommended. For successful DNA synthesis, phosphoramidites must be vacuum dried over P_2O_5 prior to use. Also, it is important to maintain anhydrous conditions throughout the synthesis. The increase in coupling time for modified amidites is highly recommended to obtain oligonucleotides in good yield. In the case of N^2-Anth and Pyre-dG, the double coupling was preferred (coupling time: 10 min) due to the bulkiness of the modification, whereas for N^2-Bn, Naph, and Luc-dG, the coupling time was 6 min. The deprotection of modified DNAs needs careful optimization and for NPE deprotection, the Glen Research Protocol can be used (*http://www.glenresearch.com/Technical/TB_2-F-dI.pdf*).

Understanding Results

The synthesis of 2-Br-dI **2**, a common intermediate described in the protocol here, gave a good yield (>75%). The Buchwald-Hartwig coupling of **2** with respective amines followed by deacetylation gave moderate yields from 39% to 64% of modified nucleosides. The subsequent tritylation and phosphitylation are anticipated to proceed with moderate yields from 40% to 60%. For N^2-dG modified DNA synthesis, the method described in Basic Protocol 2 results in excellent coupling yields (>90%) as monitored on the basis of

trityl graphs (using MerMade software). The highly pure N^2-dG modified oligonucleotides can be achieved in good yield (mostly >40%) after gel purification.

Time Considerations

In Basic Protocol 1, synthesis of the common intermediate 2-Br-dI **2** can be achieved in 1 week. One modified phosphoramidite can be prepared in 2 weeks. Overall, synthesis and purification of all N^2-dG phosphoramidites can be accomplished in 8 to 10 weeks.

In Basic Protocol 2, synthesis, deprotection, purification, and characterization of one modified oligonucleotide can be done in 2 weeks. Overall, synthesis, deprotection, purification, and characterization of all the oligonucleotides (listed in Table 1) can be completed in 2 months.

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