OXIDATION OF NUCLEIC ACIDS:
CHEMISTRY OF PURINE QUINONES AND DEVELOPMENT OF NOVEL
PHOTOOXIDIZING AGENTS

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ABSTRACT

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Chemical behavior of the oxopurines 8-oxoadenosine and 8-oxoinosine has been investigated. Purine quinones, pivotal intermediates in oxidation of oxopurines were successfully trapped and the resulting products were characterized. Formation of purine quinones was demonstrated with both chemical and photochemical oxidizing agents. Adducts formed as a result of reaction of purine quinones with nucleophiles were isolated and characterized.

Pyrene dihydrodioxins have been synthesized and shown to be effective agents for release of pyrene-4,5-dione. Pyrene-4,5-dione is an effective DNA photo-oxidation agent. Pyrene dihydrodioxin incorporating a pyridinium salt as a possible internal electron trap has been shown to release pyrenequinone rapidly. Dihydrodioxins containing two pyridine and pyridinium salt moieties have been synthesized and characterized by variety of methods including \(^1\)H and \(^{13}\)C NMR spectroscopy. Dihydrodioxin containing two pyridine moieties was separated into D and L enantiomers via liquid chromatography on a chiral sorbent. Affinities of D and L enantiomers of these dipyridinium pyrene dihydrodioxins for nucleic acids were compared.
This dissertation is dedicated to my loving parents, Yuriy G. Komarov and Larisa P. Komarova.
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INTRODUCTION

One of the most important sources of mutagenesis in cells is oxidative damage to nucleobases.\textsuperscript{1a-e} In living cells, nucleic acids can be subjected to various sources of oxidative stress. One such source is reactive oxygen species such as \textsuperscript{1}O\textsubscript{2}, O\textsubscript{2}\textsuperscript{−}, HO\textsuperscript{−} and H\textsubscript{2}O\textsubscript{2} which are continuously formed during normal metabolic processes in organisms, and their formation can be augmented by inflammation and exposure to certain agents.\textsuperscript{2a,b} Nucleic acids can also be oxidized upon exposure to ionizing radiation, high-intensity UV-radiation,\textsuperscript{3} and direct oxidation by various chemical and photochemical agents. DNA is sensitive to oxidative stress and \textit{in vivo} oxidative damage can result in strand breaks, base modifications, and DNA-protein cross-links.\textsuperscript{2a,4} Unrepaired oxidative damage to nucleic acids is often mutagenic and seems to be implicated in aging, carcinogenesis, and neurological disorders.\textsuperscript{2b,5a,b}

Purines are more susceptible to oxidation than pyrimidines (redox potentials of unmodified bases are G (1.3 V), A (1.4 V), C (1.6 V), and T (1.7 V)).\textsuperscript{6a-d} Oxidation of purine bases often leads to modification at the C8 position and can occur both within existing DNA and RNA strands\textsuperscript{7a-c} causing miscoding in the DNA replication process,\textsuperscript{8a,b} as well as in purines in the nucleotide pool,\textsuperscript{9a-c} which can result in mispairing as they incorporate into new DNA strands. Oxopurines such as 8-oxoguanosine, 8-oxoinosine, and 8-oxoadenosine (Fig. 1) frequently occur in mammalian cells.\textsuperscript{10a,b}

The work presented in this dissertation is divided into two chapters. The first chapter investigates chemical behavior of oxopurines under oxidative stress. The second chapter is devoted to development of dipyridinium dihydrodioxin, a chiral photooxidizing agent designed to effectively oxidize nucleobases.
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CHAPTER I
CHEMISTRY OF PURINE QUINONES

Abstract

Chemical behavior of the oxopurines 8-oxoadenosine and 8-oxoinosine has been investigated. Purine quinones, pivotal intermediates in oxidation of oxopurines were successfully trapped and the resulting products were characterized. Formation of purine quinones was demonstrated with both chemical and photochemical oxidizing agents. Adducts formed as a result of reaction of purine quinones with nucleophiles were isolated and characterized.
1. INTRODUCTION

1.1. Background

A considerable fraction of cellular mutations is derived from oxidative damage to nucleic acids. Oxidation of purine bases at the 8-position leads to formation of 8-oxopurines, such as 8-oxoguanosine (1), 8-oxoadenosine (2) and 8-oxoinosine (3) (Fig. 1). This can result in conformational changes (flip from anti to syn), and may lead to miscoding in the process of DNA replication. Oxopurines 1 and 2 frequently occur in mammalian cells.\textsuperscript{1a-b} Relatively high concentrations of 2 were found in tumors, which suggests that 8-oxoadenosine might be involved in carcinogenesis.\textsuperscript{1c}

A number of pathways have been found in living organisms that protect cells from oxidative damage, in particular enzymes that recognize and repair 8-oxoG and 8-oxoA sites in double stranded DNA,\textsuperscript{1d-i} and enzymes that cleave the phosphates of 8-oxoATP and 8-oxoGTP.\textsuperscript{2a-c} This topic is of great interest due to the apparent connection of DNA oxidative damage to the origins of cancer.\textsuperscript{3a-e,4a-d}

Among the oxidized lesions in DNA, 8-oxoguanosine (1) is the one that has received the most attention by the scientific community. Since 8-oxoguanosine is mutagenic, and is relatively often formed in cells under oxidative stress, it is used as a biomarker.\textsuperscript{5a-c}
It is well established that 1 can be recognized by enzymes and removed by the base excision repair (BER) pathway, involving glycosidic bond cleavage of the damaged base followed by excision of the abasic site.

The possible biological significance of 1 has led to extensive investigation of its effects on DNA structure and its interactions with polymerases and repair enzymes.
1.2. Anti to Syn flip

One of the important nuances of 8-oxopurine biochemistry is the flip from the anti conformation (the normal conformation for purines in DNA duplexes) to syn. This change in conformation affects the hydrogen binding patterns in DNA duplexes. One of the most extensively studied oxopurines 1, which in the syn conformation often binds to adenine rather than cytosine (its complementary Watson-Creek base), often causes miscoding of 1 for adenine rather than cytosine in DNA replication (Fig. 2). Thus, 1 gives rise to G→T transversions (Scheme 1A) by misdirecting polymerases to incorporate dA opposite it.

Scheme 1. A. G→T transversion. B. A→C transversion

The anti-syn flip also causes alteration in conformation of 2, which has an amino group at C6 and normally pairs with T in an anti-anti conformation. When in syn conformation, 8-oxoadenosine (2) becomes complementary to guanosine (Fig. 2), leading to A→C transversions (see Scheme 1B). The 8-oxoA·dC mismatch was also found in the syn-anti conformation and appears to distort the DNA duplex.
Base pairing selectivity of 3 was reported\textsuperscript{11d} to be similar to that of 8-oxoguanosine.

Transition melting temperatures studies have shown that 3 strongly favors the \textit{syn} conformation and in DNA duplexes is preferably bound to dA. The \textit{anti-syn} conformation flip and subsequent change in binding patterns is believed to be the primary source of mutagenesis caused by oxopurines.

\textbf{Anti}

\begin{align*}
8\text{-oxoG:C} & \quad & 8\text{-oxoG:A} \\
8\text{-oxoI:C} & \quad & 8\text{-oxoI:A} \\
8\text{-oxoA:T} & \quad & 8\text{-oxoA:G}
\end{align*}

\textbf{Syn}

\textbf{Figure 2.} Mutagenic base-pairing structures involving \textit{syn}-oxopurines with comparison to natural \textit{anti} base pairs.

The tendency of 8-substituted purines to adopt \textit{syn}-conformation was explained by steric repulsion between C8-substituent and sugar atoms in the \textit{anti}-isomer\textsuperscript{15}. This conformation
change was found to be a reason for translesion incorporation of dA opposite 8-oxodG in DNA replication processes.\textsuperscript{16} Syn-8-oxodG is thought to form a Hoogsteen base pair with dA (Fig. 2). This idea originated in structural studies of duplex DNA containing 8-oxodG:dA pairs.\textsuperscript{17a-b} It was suggested that there could be a weak interaction between the phosphate backbone of DNA duplex and the 2-amino group of 8-oxodG in syn-conformation and this might facilitate misincorporation of 2’-deoxyadenosine,\textsuperscript{11c} but in recent study,\textsuperscript{11d} this was shown to be unlikely.

Since oxopurines might exhibit base pairing selectivity different from their unmodified analogues, it is of considerable interest to determine how the anti-syn flip would affect their behavior in the DNA replication process.

Activities of 1 with polymerases have been extensively studied by Grollman, Greenberg and others.\textsuperscript{11a-d} The reported ratios of specificity constants for nucleotide incorporation opposite 1 show that when using the Klenow fragment of DNA Pol I, the insertion of dC is approximately 4 times more probable than that of dA.\textsuperscript{11d,18}

To our knowledge only one study of nucleotide incorporation opposite 3 has been published.\textsuperscript{11d} Surprisingly, 3 exhibits strong promutagenic behavior. According to the reported data, dA incorporation opposite 3 is approximately 75 times more efficient than opposite 1.

Therefore, due to their mutagenic behavior, oxopurines present significant interest to the scientific community. We, however, are more interested in the investigation of chemical behavior of oxopurines under oxidative stress.

\textbf{1.3. Oxopurines as potential cross-linking agents}

Oxopurines also present interest as potential cross-linking or photoaffinity labeling (PAL) agents. Protein-nucleic acid cross-links formed via guanosine oxidation have been well
documented.\textsuperscript{5-14} The initial oxidation of purine bases themselves probably proceeds through the widely proposed radical cation intermediates. However, oxidations of their 8-oxo derivatives seem to proceed through the quinone-like intermediates 4, 5, and 6 (Scheme 2). The oxidation of guanosine has been widely studied, and yields products that are thermally unstable.\textsuperscript{6,10,11,13} Therefore, 2 and 3 seem to show more promise as cross-linking agents. For that purpose, 2 would seem to be much better suited for the following reasons: 1) 8-oxoA is nearly as easily oxidized as 8-oxoG (0.9 V vs. 0.7 V, NHE, respectively), and both are more easily oxidized than G\textsubscript{n} steps,\textsuperscript{19-21} 2) cross-linking should lead to quite stable, unrearranged products as indicated by the reaction of 2 in Scheme 1, 3) 8-oxoA can easily be incorporated into oligonucleotides via phosphoramidite procedures or by enzymatic synthesis (\textit{in vitro} transcription from DNA templates, PCR),\textsuperscript{22,23} and 4) Adenosine is the universal base. Not only it is a major component of DNA and RNA, but it is also present in many of the major coenzymes such as NAD/NADH, FAD/FADH\textsubscript{2}, CoASH, and \textit{S}-adenosylmethionine. Furthermore, 3 would also seem to be another suitable cross-linking agent for many of the same reasons listed above, and the fact that inosine is incorporated into many ncRNA (non-coding RNA) molecules in post-transcriptional editing processes\textsuperscript{24} making it a particularly interesting base for study in this work. Furthermore, numerous RNA editing enzymes exist which convert adenosine to inosine and are unique to primates. Apparently, this plays a role in their higher brain function.\textsuperscript{25}

In addition, a wide range of work\textsuperscript{5-14} indicates that each oxidation step can be followed by the attack of a nucleophile and that the regiochemistry of these attacks on the purine ring generally will be: initial attack at the 8-position, followed by the 2-position, and finally, the 5-position in that order as outlined in Scheme 3.
Scheme 2. Regiochemistry of nucleophile attack on purine quinones. Arrow indicates sites susceptible to nucleophile attack.

Scheme 3. Stepwise oxidation and nucleophile addition to purine ring

Therefore, the chemical behavior of 2 and 3 under oxidative stress seems to have considerable biological significance. Our goal is to investigate this behavior and to determine
whether oxopurines can potentially be utilized to generate thermally stable cross-links and might be useful PAL agents.

1.4. Purine quinones

Purine quinone intermediates were demonstrated to play pivotal role in chemistry of 8-azidoadenosine.26 When methanolic solution of 8-azidoadenosine is irradiated, a purine diimino quinone intermediate is formed. This purine quinone intermediate can either be reduced to form 8-aminoadenosine or undergo nucleophilic addition to yield 8-amino-2-methoxyadenosine as shown in the Scheme 4.

Scheme 4. Trapping of purine quinone intermediate obtained via irradiation of 8-azidoadenosine.26
Formation of this intermediate was observed both by Ultra-fast laser spectroscopy and by UV-Vis spectroscopy at low temperatures.\textsuperscript{26} The same purine quinone is thought to be involved in oxidation of 8-aminoadenosine in methanolic solutions, since this oxidation leads to formation of 8-amino-2-methoxyadenosine as demonstrated on Scheme 5.

\textbf{Scheme 5.} Oxidation of 8-aminoadenosine.\textsuperscript{26}
2. METHODS

2.1. General methods

All chemicals were from Aldrich or Acros and were used without further purification unless specified otherwise.

Removal of solvents under reduced pressure was performed using a Büchi rotary evaporator connected to Vacuubrand PC 101NT diaphragm pump.

Analytical TLC was performed using E.Merck aluminum-backed sheets of silica gel 60 F254. For preparative TLC Analtech 20×20 cm glass-backed 2 mm thick silica gel 60 F254 plates were used.

HPLC analyses and separations were conducted on Rainin HPLC system coupled with Agilent 8453 UV-Vis spectrometer. For analytical purposes a Waters C18 Spherisorb ODS1 column (4.6×250 mm) was used, whereas separations were performed on a Dynamax C18 preparative column (60 Å, 21.2 mm×250 mm).

One-dimensional $^1$H and $^{13}$C were acquired on a Bruker AM300 spectrometer. All chemical shifts are reported in ppm downfield ($\delta$). $^1$H and $^{13}$C spectra are referenced to internal TMS. $^{13}$C spectra were acquired with broadband $^1$H decoupling. Abbreviations for splitting patterns are as follows: br, broad; s, singlet; d, doublet, t, triplet; m, multiplet; etc. UV absorbance spectra were recorded in water on an Agilent 8453 UV-Vis spectrometer. ESI Q-TOF HRMS spectra were obtained by Larry Sallans at the mass spectrometry facility at the University of Cincinatti.
3. EXPERIMENTAL

3.1. Oxidation of 8-Oxoadenosine (2) with NBS in the Presence of Methanol

A solution of 8-oxoadenosine (2) (203 mg, 0.7224 mmol) in methanol (175 mL) was prepared and stirred at dry ice temperature, then a solution of NBS (161 mg, 0.9045 mmol) in methanol (30 mL) was added. Sodium methoxide (0.2 mL, 1M solution in methanol) was added to catalyze the reaction. A spatula full of potassium carbonate was added to neutralize the HBr formed during the course of reaction. The solution was gradually warmed to room temperature over the course of ~3 h, then left in the freezer overnight. Methanol was evaporated under reduced pressure to yield an oily residue. To the residue, 5 mL of DCM and 1.5 mL of acetic anhydride were added and this mixture was left to stir overnight. The resulting mixture was extracted 2 times with water, then 2 times with saturated sodium bicarbonate solution and with water again. The organic layer was dried over magnesium sulfate, and filtered through celite. The filtrate was reduced to ~1.5 mL and its components were separated on a preparative TLC plate (DCM, 0-4% MeOH). Two major components of the mixture were \( N \)-acetyl-8-oxoadenosine triacetate and \( N \)-acetyl-2-methoxy-8-oxoadenosine 2’,3’,5’-triacetate (8) (21.4 mg, 6% yield).

8, 300 MHz \(^1\)H NMR in CDCl\(_3\), \( \delta \), 6.06 (m, 2H), 5.78 (dd, \( J_1=5.7 \text{ Hz}, J_2=5.85 \text{ Hz}, 1\text{H} \)), 4.49 (dd, \( J_1=3 \text{ Hz}, J_2=11.7 \text{ Hz}, 1\text{H} \)), 4.31 (m, 1H), 4.23 (m, 1H), 4.05 (br.s., 1H), 3.97 (s, 3H),
3.65 (m, 1H) (Presence of many solvent signals in the aliphatic region obstructs identification of signals from acetate methyl groups).

300 MHz $^{13}$C in CDCl$_3$, $\delta$, 170.89, 170.59, 169.57, 169.49, 162.42, 151.39, 150.37, 150.20, 98.01, 84.69, 78.90, 71.64, 70.10, 62.93 (Presence of many solvent signals in the aliphatic region obstructs identification of signals from acetate methyl groups).

HRMS $C_{19}H_{24}O_{10}N_{5}$ $m/z$ calculated 482.1523, found 482.1524.

3.2. Oxidation of 8-Oxoadenosine (2) with NBS in the Presence of Imidazole

Imidazole (400 mg, 5.8823 mmol) and 8-oxoadenosine (2) (225 mg, 0.8007 mmol) were dissolved in methanol (~200 mL). NBS (285 mg, 1.6011 mmol) was added, and the resulting solution was stirred at room temperature for 16 h. More NBS was added (575 mg, 3.2303 mmol), and the reaction mixture was left at room temperature overnight. The methanol was evaporated under reduced pressure, and a sample was sent for analysis by HRMS. The residue was dissolved in dry DMF (4 mL), 1500 mg of TBDMSCl (~9.9522 mmol) were added, the solution stirred at 60°C under argon overnight. The resulting solution was dissolved in DCM (50 mL) and extracted...
with water, saturated sodium bicarbonate solution, water again, and dried over magnesium sulfate. The resulting solution was concentrated under reduced pressure to ~2 mL and separated via flash column chromatography (~40g Merck 60 silica gel, 1-10% methanol/DCM). The product was isolated as a white solid (106 mg, 19.1% yield).

10, 300 MHz $^1$H NMR in CDCl$_3$, $\delta$, 8.48 (s, 1H), 7.74 (s, 1H), 7.13 (s, 1H), 6.0 (br. s., 2H), 5.92 (d, J=6.6 Hz, 1H), 5.28 (dd, J$_1$=4.53 Hz, J$_2$=6.48 Hz, 1H), 4.39 (dd, J$_1$=2.25 Hz, J$_2$=4.41 Hz, 1H), 3.9 (m, 5H), 3.50 (s, 1H) (Presence of many signals in the aliphatic region obstructs identification of signals from TBDMS groups).

10, 300 MHz $^{13}$C in CDCl$_3$, $\delta$, 153.51, 148.83, 148.14, 147.89, 135.83, 129.70, 116.66, 102.131, 86.08, 85.44, 72.80, 71.08, 63.61 (Presence of many signals in the aliphatic region obstructs identification of signals from TBDMS groups).

9, HRMS C$_{13}$H$_{16}$N$_7$O$_5$ m/z calculated 350.12129, found 350.12085

3.3. Oxidation of 8-Oxoadenosine (2) with Sodium Hypochlorite Solution in the Presence of Imidazole

![Chemical Structure](image)

Imidazole (102 mg, 1.5 mmol) and 8-oxoadenosine (2) (26 mg, 0.0925 mmol) were dissolved in ~15 mL of deionized H$_2$O. A solution of sodium hypochlorite solution (150 µL, 0.8M, 0.12 mmol) was added to the reaction mixture. The solution was left stirring overnight. The components of the reaction mixture were separated on an analytical HPLC C-18 column (1 mL/min, 10% ACN/ 90% 20mM NH$_4$OAc in H$_2$O), and collected fractions were lyophilized. A sample of the solid was sent to obtain HRMS. HPLC analysis indicated presence of 2-(N-imidazolyl)-8-oxoadenosine (9) (23% HPLC yield).
3.4. Oxidation of 8-Oxoinosine (3) with Sodium Hypochlorite Solution in the Presence of Imidazole

Imidazole (255 mg, 3.75 mmol) and 8-oxoinosine (106 mg, 0.3746 mmol) were dissolved in 15 mL of deionized water, and a solution of sodium hypochlorite (400 µL, 0.8M, 0.32 mmol) was added to the reaction mixture. The resulting solution was stirred overnight. HPLC (C-18, 7%ACN – 93% 20mM NH4OAc, 1 mL/min) analysis showed presence of two compounds – starting material and product. Mixture was separated on a preparative HPLC C-18 column, and collected fractions were lyophilized to afford 11 (52.5 mg, 40.5% yield).

11, NMR 300 MHz $^{13}$C in DMSO, δ, 160.99, 152.77, 149.28, 145.49, 135.59, 129.00, 117.05, 106.42, 86.04, 85.05, 71.40, 70.74, 62.91

300 MHz $^1$H in DMSO, δ, 10.4 (br. s., 1H), 8.27 (s, 1H), 7.70 (s, 1H), 6.95 (s, 1H), 5.64 (d, J=5.7 Hz, 1H), 5.16 (d, J=6.3 Hz, 1H), 5.00 (d, J=4.2 Hz, 1H), 4.90 (m, 2H), 4.20 (s, 1H), 3.81 (dd, J$_1$=4.2 Hz, J$_2$=7.8 Hz, 1H), 3.63 (m, 1H), 3.45 (m, 1H)

HRMS C$_{13}$H$_{13}$N$_{6}$O$_{6}$ m/z calculated 349.09021, found 349.09021.
3.5. Photosensitized Peroxodisulfate Photooxidation of 8-Oxoinosine (3) in the Presence of Imidazole

Imidazole (160 mg, 2.3529 mmol), 8-oxoinosine (3) (54 mg, 0.1908 mmol), $K_2S_2O_8$ (45 mg, 0.1667 mmol) and riboflavin (6 mg, 0.01594 mmol) were dissolved in 20 mL of deionized water. The resulting solution was irradiated with a sodium lamp (General Electric, 400 lumen) for 3 h. HPLC analysis (0.8 mL/min 20% MeOH/80% H$_2$O) and QTOF-HRMS indicated formation of 2-(N-imidazolyl)-8-oxoinosine (11) (11% HPLC yield).

11, HRMS C$_{13}$H$_{13}$N$_6$O$_6$ m/z calculated 349.0902106, found 349.09026

3.6. Peroxodisulfate Photooxidation of 8-Oxoinosine (3) in the Presence of Imidazole

Imidazole (200 mg, 2.9412 mmol), potassium peroxodisulfate (120 mg, 0.4444 mmol), and 8-oxoinosine (3) (28 mg, 0.0989 mmol) were dissolved in 25 mL of deionized water. The
solution was irradiated in Rayonet Photochemical reactor at 254 nm for 5 h. The resulting mixture was resolved on preparative RP-HPLC column (Dynamax C18 60A 250mm X 21 mm, 25% methanol/ 75% water, 8 mL/min flow rate). Fractions were collected, evaporated under reduced pressure, and lyophilized. The product was identified as 2-(N-imidazol)-8-oxoinosine (11) (12 mg, 34.8% yield).

11, HRMS C_{13}H_{13}N_{6}O_{6} \ m/z \ \text{calculated} \ 349.0902106, \ \text{found} \ 349.09008.

3.7. Oxidation of 8-Oxoinosine (3) with Sodium Hexachloroiridate Solution in the Presence of Imidazole

![Chemical Structures]

Imidazole (56 mg, 0.8235 mmol), 8-oxoinosine (3) (27 mg, 0.0954 mmol) and Na_{2}IrCl_{6}*6H_{2}O (80 mg, 0.1431 mmol) were dissolved in 10 mL of deionized water. The color changed from dark brown to light brown. HPLC indicated formation of 2-(N-imidazolyl)-8-oxoinosine (11). Imidazole adduct was isolated via preparative RP-HPLC column chromatography (Dynamax C18 60A 250mm X 21.2 mm, 15% methanol/ 85% water, 4 mL/min flow rate). Fractions were collected, evaporated under reduced pressure and lyophilized. ^{1}H NMR corresponds to that of 2-(N-imidazolyl)-8-oxoinosine (11) (23 mg, 69% yield).
3.8. Oxidation of 8-Adenosine with Sodium Hexachloroiridate Solution in the Presence of Imidazole

Imidazole (254 mg, 3.7353 mmol) and 8-oxoadenosine (102 mg, 0.3630 mmol) were dissolved in 50 mL of deionized water. Solution of Na$_2$IrCl$_6$•6H$_2$O (110 mg in 2 mL of deionized water) was added dropwise. Imidazole adduct 9 was isolated via preparative RP-HPLC column chromatography (Dynamax C18 60A 250mm X 21.2 mm, 15% methanol/85% water, 4 mL/min flow rate). UV-spectrum and retention time were characteristic of 9 (*vide supra*, p. 19) (~21% HPLC yield).
4. RESULTS AND DISCUSSION

These experiments strongly indicate that purine quinones are in fact pivotal intermediates in the oxidation of oxopurines. The oxidation of 8-oxoadenosine was investigated using several oxidizing agents in the presence of a number of nucleophiles (methanol, isopropanol, imidazole, benzimidazole, ammonia, diethylamine, triethylamine, piperidine, pyridine, DMAP, 2-thioethanol, thiophenol, phenol, 4-nitrophenol, tetramethylguanidine, adenine, adenosine, inosine, urea, lysine, histidine, and glycine). Among the investigated oxidizing agents (NBS, NCS, sodium hypochlorite, hydrogen peroxide, potassium ferricyanide, and sodium periodate; photochemical – riboflavin, rose bengal, tetrachlorobenzoquinone, and potassium peroxodisulfate), NBS in methanol, sodium hypochlorite in water, and aqueous sodium hexachloroiridate were found to be most effective. Thus, treatment of 2 with NBS in cold methanolic solutions leads to the formation of a bright yellow intermediate (Fig. 2). This yellow intermediate bleaches over ca. two hours at room temperature to yield the methanol adduct of 8-oxoA 7 (R = 2′-ribosyl, Nuc = OCH₃), Scheme 6.

Scheme 6. Oxidation of 8-oxoadenosine (2) in methanolic solutions.
Figure 3. Growth of absorption spectrum generated from the oxidation of 8-oxoA in methanolic NBS solution at -78º C and warmed to room temperature.

Similarly, oxidation of 8-oxoinosine (3) with NBS in cold methanolic solutions gives rise to pale yellow intermediate (Fig. 3). This intermediate remains in cold methanolic solutions for hours and is perceived to be inosine quinone 6 (Scheme 7).

Scheme 7. Oxidation of 8-oxoinosine (3) with NBS.
Figure 4. Growth and decay of absorption spectrum generated from the oxidation of 8-oxoinosine (3) in methanolic NBS solution at -78°C and warmed to room temperature.

However, the 8-oxoadenosine (2) oxidation leads to the formation of products that are themselves susceptible to further oxidation as outlined in Scheme 3. The nucleophilic attack at the 2-position in the first oxidation step yields a product that is more easily oxidized than the starting 8-oxopurine. Furthermore, HBr produced in the course of reaction seemed to have detrimental effect on the yield of products. Yields of methoxy adduct 7 were improved by conducting the oxidation in the presence of solid potassium carbonate, added to neutralize the HBr. Another possible reason for increase in the yield is the formation of methoxide anion, which is a much better nucleophile than methanol. Adduct 7, formed as a result of addition of methanol to purine quinone 5, was isolated as N-acetyl-2-methoxy-8-oxoadenosine triacetate to allow purification via chromatography on silica gel.
The transient yellow intermediate 5, that is produced in methanolic solutions of NBS, persists in the cold (-78° C) for hours, since methanol is a poor nucleophile, but the addition of aliphatic amines quench the intermediate rapidly. However, the attempts to isolate the product(s) of aliphatic amine (diethyl amine) addition to 5 were unsuccessful, probably because the resulting products are highly susceptible to further oxidation and are destroyed more rapidly than they are formed. Imidazole is an amine that does not function as an activating substituent. When 8-oxoA (2) is oxidized in the presence of imidazole, the imidazole adduct 9 (Nuc = imidazole) (Scheme 6) is formed. Imidazole appears to be an unusual nucleophile in that the n-lone pair on nitrogen serves as a good nucleophile, but once imidazole is attached to the purine ring, the lone pair on the attacking nitrogen becomes part of an aromatic sextet, and does not activate the purine ring toward further oxidation. UV spectrum of imidazole adduct 9 demonstrates a considerable red shift when compared to 8-oxoadenosine (Fig. 3). This red shift in UV absorption of 9 indicates that imidazol group provides for extended conjugation.
Figure 5. UV spectra of adenosines

Since the imidazole adduct 9 seems to be more stable towards oxidation than 2 itself, we were able to successfully isolate 2-imidazolyl-8-oxoadenosine (9) when oxidizing 8-oxoadenosine (2) with a plethora of oxidizing agents.

The first oxidizing agent we employed to obtain 9 was a methanolic solution of NBS. A large excess of imidazole was used for two reasons – to minimize competition from other nucleophiles, such as methanol, and to neutralize HBr formed in the course of reaction. This strategy proved to be a success and the over-oxidation was largely suppressed. The alcohol groups on the ribose residue of imidazole adduct were treated with TBDMS chloride to allow separation on a chromatography column.
Although a successful strategy for trapping purine quinone 5, oxidation of 8-oxoadenosine (2) in methanol is not the best way to mimic oxidation of oxopurines in living organisms. Therefore, we decided to conduct oxidation of 2 in aqueous environment. Sodium hypochlorite was chosen as oxidant, because of its similarity to NBS. Oxidation of 8-oxoA (2) with sodium hypochlorite in the presence of imidazole successfully yielded 9.

However, formation of 9 in oxidations with either sodium hypochlorite or NBS (Scheme 8) does not necessarily provide proof for formation of purine quinone 5. Alternative mechanisms can be proposed.

Scheme 8. Proposed mechanism for oxidation of 8-oxoadenosine (2) with NBS in the presence of imidazole.
A similar mechanism is proposed for the oxidation of aqueous 8-oxoadenosine in the presence of imidazole with solution of sodium hypochlorite as shown on Scheme 9.

**Scheme 9.** Oxidation of 8-oxoadenosine (2) with solution of sodium hypochlorite

To prove that oxidation of 2 proceeds through formation of purine quinone 5 we used Na₂IrCl₆*6H₂O. Sodium hexachloroiridate is an oxidizing agent different in *modus operandi* from either NBS or sodium hypochlorite. Sodium hexachloroiridate is a one-electron oxidizing agent. Since Na₂IrCl₆*6H₂O is a strong oxidant, it is often used in organic syntheses and kinetic studies. Sodium hexachloroiridate was found to have tumoricidal activity.²⁷

The oxidation of 8-oxoadenosine (2) with sodium hexachloroiridate seems to yield lower quantity of products of over-oxidation than with sodium hypochlorite and NBS.

“Active” oxygen species, such as singlet oxygen and hydroxyl radicals, are some of the most prominent sources of oxidative stress in the living cells. Therefore, we examined
the visible light irradiation of aqueous solutions of 2 in the presence of imidazole and rose bengal or riboflavin in order to determine if singlet oxygen might produce products similar to 9. The results of these experiments have been inconclusive. However, when K$_2$S$_2$O$_8$ was added to the riboflavin reactions, the imidazole adduct 9 is formed, although in low yields. These yields might be improved by increasing the intensity of light source and the time of irradiation.

An alternative approach to inhibiting the further oxidation of the products is to begin with a less electron-rich purine. Therefore, 3 is an ideal candidate for testing this approach. When the potent electron-donating 6-amino group of adenosine is replaced by the electron-withdrawing carbonyl group in inosine, the purine ring becomes considerably more electron-deficient. Consequently, 3 is not as readily oxidized as 2. Thus, treatment of 8-oxoinosine with methanolic solution of NBS does not result in reaction. However, treating 3 with aqueous solution of sodium hypochlorite, which is a more potent oxidizing agent, in the presence of imidazole yields 11, the corresponding 8-oxoinosine adduct in the 2-position (Scheme 10).

![Scheme 10. Oxidation of 8-oxoinosine (3) in the presence of imidazole](image-url)
Oxidation of 3 with sodium hypochlorite, conducted in the presence of imidazole, produced adduct 11 (40.5%) with yields considerably higher than in corresponding oxidations of 2 (23%). Noticeably, the amount of products of over-oxidation was considerably reduced. In most instances 11 was the only product detected via HPLC.

**Figure 6.** UV spectra of inosines

Adduct 11 can be successfully obtained by employing photo-oxidizing agents. When aqueous solutions of 3 are irradiated with 254 nm in the presence of imidazole and potassium peroxodisulfate, imidazole adduct 11 is formed (34.8%).

Potassium peroxodisulfate is a one-electron photooxidizing agent. Peroxodisulfate anion forms radicals upon irradiation with short UV-light as shown on Scheme 11.
Thus formed, sulfate radicals oxidize 8-oxoinosine (3) to form purine quinone 6, which reacts with imidazole to yield adduct 11. Attempts of oxidizing 3 with rose bengal and riboflavin were not successful, possibly due to the higher oxidation potential of 8-oxoinosine (3). The oxidation potential of 3 is not recorded in the literature and has yet to be determined.

Imidazole adduct 11 can be successfully obtained with high yields (up to ~70%) when 3 is oxidized with sodium hexachloroiridate in the presence of imidazole. Thus, adduct 11 seems to be more stable towards over-oxidation than 9. Therefore, we expect that 8-oxoinosine will form more stable cross-links upon oxidation than 8-oxoadenosine.

Overall, both 5 and 6 are more attractive targets for cross-linking than 4, since both form stable adducts after nucleophilic attack at C2. However, 8-oxoadenosine occurs more frequently than 8-oxoinosine in nucleic acids, and therefore is more likely to occur in P/NA cross-linking or PAL.

While the observations discussed above provide good circumstantial evidence for existence of purine quinone intermediates 5 and 6, alternative mechanistic explanations of these reactions are conceivable. Furthermore, all oxidative methods for formation of these intermediates suffer from the fact that many of products formed are themselves more susceptible
to further oxidation than the starting 8-oxopurines. Therefore, an alternative strategy for the formation of purine quinone 5 has been devised. In a related project Denis Nilov attempts to observe the same purine quinone intermediate by investigating photochemistry of 6-azido-8-oxopurine. In preliminary experiments, when 6-azido-8-oxopurine triacetate is irradiated with UV light in the presence of imidazole, corresponding imidazole adduct is formed. These experiments support the idea of purine quinones as pivotal intermediates in the chemistry of oxopurines.
5. REFERENCES


(d) Nakabeppu, Y.; Sakumi, K.; Sakamoto, K.; Tsuchimoto, D.; Tsuzuki, T.; Nakatsu, Y. 


CHAPTER II
DEVELOPMENT OF PYRIDINIUM DIHYDRODIOXINS AS NUCLEIC ACID PHOTO-OXIDIZING AGENTS

Abstract

Pyrene dihydrodioxins have been synthesized and shown\textsuperscript{1} to be effective agents for release of pyrene-4,5-dione. Pyrene-4,5-dione is an effective DNA photo-oxidation agent. Pyrene dihydrodioxin incorporating a pyridinium salt\textsuperscript{1} as a possible internal electron trap has been shown to release pyrenequinone rapidly. Dihydrodioxins containing two pyridine and pyridinium salt moieties have been synthesized and characterized by variety of methods including \textsuperscript{1}H and \textsuperscript{13}C NMR spectroscopy. Dihydrodioxin containing two pyridine moieties was separated into D and L enantiomers via liquid chromatography on a chiral sorbent. Affinities of D and L enantiomers of these dipyridinium pyrene dihydrodioxins for nucleic acids were compared.
1. INTRODUCTION

1.1. Background

It was generally believed that primary function of DNA is to encode the production of messenger RNA, which in turn is used for the production of protein, but recent studies have shown that only ca. 2% of the human genome code is encoding protein synthesis. The largest part of genome information remains unassigned, but it was suggested that this remaining code is largely devoted to the production of noncoding RNAs (≤ 100kb) that are believed to play pivotal roles in the regulation of many biochemical processes. The specific mechanisms by which these complex regulatory biochemical systems function are of great interest, and protein-nucleic acid (PNA) cross-linking may provide a powerful tool to investigate them.

It is now believed that oxidative damage to DNA may be the source of various destructive biological effects including aging and mutagenesis, and diseases, including cancers, neurodegenerative disorders, and chronic inflammatory diseases. Oxidation of DNA can lead to base modification, DNA strand breaks, and DNA-protein crosslinks. DNA-protein crosslinks can be produced by a variety of endogenous and environmental agents such as UV light, ionizing radiation, chemical and photochemical oxidants. Although a variety of methods have been shown to induce DNA-protein cross-linking, the molecular structure of these cross-links and mechanisms of their formation are largely unknown.

Studies of oxidative damage to DNA over the last decade was mostly devoted to oxidation of the guanosine residue, since it is the most easily oxidized nucleic acid base. Guanosine is initially oxidized to 8-oxo-7,8-dihydroguanosine (8oxoG), which in turn is even...
more easily oxidized than guanosine itself (0.7 V vs 1.29 V, NHE, respectively).\textsuperscript{16-17} Processes that may be involved in oxidation of guanosine are shown in Scheme 1.

**Scheme 1. Oxidation of guanosine**

Oxidation of DNA is characterized by most of workers as electron transfer from nucleic acid bases to oxidizing agents thus creating a “hole” (radical cation) in the DNA stack. The hole created can lose a proton and be further oxidized or it can migrate through the stack until it encounters a “hole sink”, a site at which the radical cation is particularly stable. Experimental results reported from several workers show that a hole introduced into duplex DNA in solution
will migrate relatively long distances before being trapped by chemical reaction.\textsuperscript{7,19-21} The mechanism for hole transport has been intensively investigated using both experimental observations and theoretical calculations.

It was suggested that a radical cation moves through the DNA stack by a series of short hops, moving from one low-energy sight (usually guanine) to another until it reaches a “hole sink” or will be trapped by reaction with a base or nucleophile.\textsuperscript{21}

To date, several “hole sinks” present in the DNA stack have been reported. Among these are GG, GGG and other polyG sequences\textsuperscript{21-23}, and 8oxoG. Most interesting is the observation that 8-oxo-7,8-dihydroguanosine (8-oxoG) seems to be the most effective “hole sink” (oxidation potential of 0.7 V, NHE), and radical cations initially created at random sites by oxidation of DNA will often migrate to 8oxoG locations.\textsuperscript{24}

It was reported by several laboratories that oxidation of DNA can lead not only to strand breaks, but also to reaction with various nucleophiles, e.g. proteins. Intermediates formed in guanosine oxidation are particularly susceptible to nucleophile attack.\textsuperscript{25-27} Protein-DNA crosslinks formed via guanosine oxidation by various agents have been well documented.\textsuperscript{10,12,28}

Lysine and argenine residues constitute the best nucleophiles in proteins and are most probably involved in cross-linking with DNA. It was shown that in oxidative guanosine cross-linking initially occurs at the 8-position via nucleophilic attack by the amino group of lysine.\textsuperscript{29} Further oxidation leads to nucleophile attack at the 5-position. It was reported, however, that cross-links at the 5-position form reversibly and are not particularly stable.\textsuperscript{30}

While the P/NA cross-linking aspects of guanosine oxidation have been widely studied, guanosine is not the ideal system for development as a protein-nucleic acid (P/NA) cross-linking system due to low thermal stability of its cross-links, which are prone to undergo loss of the
nucleophile, and a variety of rearrangements and fragmentations of the purine ring system.\textsuperscript{30} For that purpose, 8-oxo-7,8-dihydroadenosine (8oxoA) would seem to be much better suited for the following reasons: 1) 8oxoA is nearly as easily oxidized as 8oxoG (0.9 V vs. 0.7 V, NHE, respectively) and also more easily oxidized than G\textsubscript{n} steps,\textsuperscript{18} 2) it should lead to more stable, unrearranged cross-linked products.\textsuperscript{30}

In order to generate PNA cross-links by oxidation of 8-oxoA one needs a suitable oxidizing agent. It was shown that dihydrodioxins (DHDs) are effective tools for oxidation of DNA.\textsuperscript{1,33}

1.2. DHDs as a Tool for Controlled Oxidation of Nucleic Acids

Oxidation of nucleobases can be utilized for both cleavage of nucleic acid strands and generation of P/NA cross-links. A number of oxidizing agents are already been used for manipulation of nucleic acids. To name a few, 1,10-phenantroline-copper complexes are employed in studies of protein-DNA interactions\textsuperscript{34} and hydroxyl radicals are used for footprinting.\textsuperscript{35}

Photochemical oxidizing agents offer many advantages over regular DNA oxidizing agents, the most important of which is the ability to control the oxidation both spatially and temporally. Among photochemical activated agents used for oxidation of nucleic acids are metal complexes and organic chemicals such as riboflavins, enedyines, pterins, naphthalimide derivatives and quinones.\textsuperscript{36,37}

The type of damage caused by photooxidizing agents to nucleic acids is largely dependent on the way these agents interact with DNA. Small molecules can bind to DNA in several ways, such as intercalation between the base pairs, formation of covalent bond with
DNA, non-covalent binding into minor or major grooves, and electrostatic binding. The structure of the molecule defines its mode of interaction with nucleic acids. Binding into the grooves is often stabilized by formation of hydrogen bonds or by electrostatic interactions, whereas intercalation is generally based on hydrophobic interactions between nucleobases and flat aromatic molecules. Therefore, intercalators mostly damage nucleic acids by oxidizing nucleobases via electron transfer, while groove binders usually damage the backbone.38

Several decades ago Schönberg and Mustafa demonstrated that upon irradiation with visible light ortho-quinones undergo photocycloaddition with olefins resulting in dihydrodioxins (DHDs) as demonstrated on Scheme 2.39

![Scheme 2. Schönberg-Mustafa reaction](image_url)

**Ortho-quinone**

**DHD**

DHDs serve as masking agents from which ortho-quinones can be released via irradiation with UV light. The mechanism of release of pyrene-4,5-dione (12) from pyrene dihydrodioxin 13 is outlined in Scheme 3.1,40
The most interesting observation regarding release of 12 from dihydrodioxin 13 (Scheme 3) is that it is autocatalytic. Following initial release of quinone by irradiation with UV, further quinone release can be achieved by excitation of pyrene quinone (12) with visible light, which will lead to photo-oxidation of dihydrodioxin and release of additional quinone.

Quinone, released from dihydrodioxins will oxidize purines upon excitation with visible light. Two possible mechanisms for photooxidation of the purine base 2 with 12 are outlined in Scheme 4.
Scheme 4. Possible mechanisms for photooxidation of 2 with pyrene-4,5-dione (12)

A major problem encountered in the developing of DHDs as nucleic acids oxidizing agents was that while release of 12 occurred quite effectively in the absence of DNA, it was effectively quenched by DNA (Fig. 1).\(^1\) This seems to be due to back-electron transfer from the DNA stack to the initially formed DHD radical cation as indicated in Scheme 3.

In order to avoid the problem of reverse-electron transfer, it was suggested that a new type of dihydrodioxins incorporating powerful electron sinks such as pyridinium moieties might be effective. This approach have been shown not only to successfully eliminate back electron
transfer (BET) to DHD radical cation (Scheme 3), but also to promote the effective release of pyrenequinone (Scheme 5).

\[ \text{Scheme 5. Quinone release facilitated by intramolecular electron transfer. Mack, E.T.; Wilson, R.M, unpublished results.} \]
It was shown\textsuperscript{40} that quinone release from compound 14 is not suppressed in presence of DNA (see Figure 2).

\textbf{Figure 1.} DNA quenching of quinone release from dihydrodioxin 13.\textsuperscript{40}
It was found that in the initial quinone release process, the pivotal intermediates, the pyrene radical cations of the types shown in Schemes 3 and 5, have a very intense absorption band that is easily detected even in transient spectra of complex mixtures. A striking difference was found in the lifetimes of their respective pyrene radical cation bands ($\lambda_{\text{max}} = 453$ nm) for [10,11-dihydro-10,10-bis-(4-methoxyphenyl)-9,12-dioxabenz[e]pyrene] (13) (6.3±0.1 μs, Figure 3) and 14 (1.14 ps, Scheme 5 and Fig. 4). These observations are consistent with the electron-transfer mechanisms proposed in Schemes 3 and 5.
Figure 3. Long-lived pyrene radical cation, R=Me ($\tau = 6.3\pm0.1$ ms). Mack, E. T.; Carle, A. B.; Liang, J. T.-M.; Coyle, W.; Wilson, R. M. *J. Am. Chem. Soc.*, **2004**, *126*, 15324-15325.

Figure 4. Short-lived pyrene radical cation ($\tau = 1.14$ ps). Mack, E.T.; Wilson, R.M, unpublished results.
Introduction of positively charged $N$-methyl pyridinium groups into DHDs not only promotes faster release of pyrene quinone 12, but also increases solubility in water and facilitates binding to nucleic acids. In this chapter we discuss synthesis and possible applications of pyridinium dihydrodioxins.
2. METHODS

2.1. General methods

See page 14.

Irradiations were conducted using Southern New England Rayonet system using 419 nm lamps for Schönberg-Mustafa reactions and 350 nm lamps for photolysis of dihydrodioxins.

Circular dichroism (CD) spectra were obtained on an Aviv 202DS spectrometer (Aviv Biomedical Inc., Lakewood, NJ) equipped with a Peltier temperature control unit. Measurements were conducted using a 1 mm path-length cell at room temperature.

In oligonucleotides UV-melting experiments, melting curves were obtained using a 10 mM sodium cacodylate buffer (10mM sodium cacodylate, 1 mM Na₂EDTA, 100 mM NaCl, pH=7.1). The concentrations employed for UV melting experiments of DNA duplexes as well as DNA dimers were generally between 6.5 and 10 μM in strands. Samples were placed in 1 cm path length quartz cuvettes (V_{min}=60 μL), and a Cary 100 Bio UV-Visible spectrophotometer (Varian, Inc.) was used to measure absorbance as a function of temperature at 260 nm with a heating rate of 1°C/min. Absorbance readings were taken every 0.2 °C over a range of temperature from 20 to 90 °C. Three melting transitions were obtained for each sample to make the data more precise.

2.2 DNA Strand Scission as a Quantitative Measure of DHD Activity

The DNA strand scission can be utilized to quantitatively measure the ability of photooxidizing agents to damage nucleic acids. Supercoiled plasmid relaxation essay is the one of the most sensitive tests for DNA strand cleavage. φX174 is a small icosahedral E. coli bacteriophage that has considerable historical significance. The φX174 plasmid was the first
DNA that was completely sequenced. In 1977, F. Sanger et al sequenced φX174 and showed it to have overlapping genes. The bacteriophage φX174 is a circular single stranded DNA 5386 bases long. The supercoiled circular duplex form of φX174 is formed only during replication phase and is referred to as a replicative form (RF I). When a cleavage of a single strand occurs the supercoiled φX174 becomes relaxed, which results in the formation of an open circular double-stranded plasmid form (RF II). If the another strand of relaxed form of φX174 is cut in the proximity of an existent single-stranded cleavage, the formation of linear form (RF III) results. The three forms of φX174 described above can be separated by agarose gel electrophoresis. When the bands corresponding to different forms of the plasmid are quantified, the relative activities of DNA photooxidizing agents can be compared.
3. EXPERIMENTAL

3.1. Preparation of [4-(10,11-dihydro-9,12-dioxabenz[e]pyren-10-yl)-pyridine] (15)

Dihydridioxin 15 was prepared according to procedure taken from dissertation of E.T. Mack. Pyrene-4,5-dione (12) and 4-vinylpyridine were purified by filtration through silicagel mesh 60 prior to synthesis. A solution of pyrene-4,5-dione (29 mg, 0.125 mmol in 25 mL of benzene) was added to 4-vinylpyridine (504 mg, 4.8 mmol). This mixture formed a gel that was removed by filtration through the pad of silica gel. The solution was degassed and saturated with argon for 10 min, then irradiated for 2 h using the 419 nm lamps in a Rayonet photoreactor at ~70°C. Solvent was removed under reduced pressure to form an oily residue that was kept for 1 h at high vacuum to eliminate the excess of olefin. The crude product was purified by preparative TLC (two elutions with 1:1 ethyl acetate: hexanes) and then recrystallized from dichloromethane/methanol mixture to yield white crystals (31 mg, 76% yield). The spectroscopic data was consistent with that of authentic material.43
3.2. Preparation of [4(10,11-dihydro-9,12-dioxa-benzo[e]pyren-10-yl)-N-methyl] Pyridinium Tetrafluoroborate Salt (14)

Dihydridoxin 14 was prepared according to procedure taken from dissertation of E.T. Mack. To a solution of 4-(10,11-dihydro-9,12-dioxa-benzo[e]pyren-10-yl)-pyridine (15) (40 mg, 0.119 mmol in 12 mL of CH₂Cl₂ (freshly distilled from P₂O₅)) was added trimethyloxonium tetrafluoroborate (23 mg, 0.155 mmol). The reaction mixture was stirred in a closed vessel for 40 min. Yellow crystals precipitated from the solution. The volume of the reaction mixture was reduced to 3 mL under reduced pressure, precipitated solid was removed by filtration and recrystallized from boiling methanol then dried under high vacuum for 1 h (50 mg, yield 96%). The spectroscopic data was consistent with that of authentic material.⁴³
3.3. Preparation of 4,4′-dipyridine-pyrenedihydrodioxin (16)

![Chemical Structure]

Pyrene-4,5-dione (46 mg, 0.198 mmol) and (E)-bis-1,2(4-pyridyl) ethylene (61 mg, 0.335 mmol) were dissolved in 25 mL of benzene, then the solution was degassed and irradiated with 419 nm in a Rayonet photoreactor at ~7°C for 18 h. The benzene was removed from the reaction mixture under reduced pressure to afford a brown oil mixed with pale crystals. This residue was recrystallized from methanol to yield tan crystals. The tan powder was washed with methanol and dried under high vacuum to yield 16 (35.5 mg, 43% yield).

C_{28}H_{18}N_{2}O_{2} 414.14 g/mole; HRMS (ESI Q-TOFMS) calculated for C_{28}H_{19}N_{2}O_{2} 415.1447, found 415.1431; ^1H NMR (300 MHz, CDCl$_3$) δ 8.62 (d, J=6.9 Hz, 4H), δ 8.45 (d, J=7.8 Hz, 2H), δ 8.169 (d, J=7.8 Hz, 2H), δ 8.090 (s, 2H), δ 8.021 (t, J=7.8 Hz, 2H), δ 7.148 (d, J=6.9 Hz, 4H), δ 5.173 (s, 2H); ^13C NMR (300 MHz, CDCl$_3$) δ 150.13, 144.32, 134.17, 130.99, 128.33, 127.45, 126.11, 124.91, 124.36, 122.38, 121.26, 118.13.

Fluorescence spectrum is on p. 92.
3.4. Preparation of Bis-N-methylpyridinium Pyredenedihydriodioxin (17)

To a solution of 4,4'-dipyridine-pyredenedihydriodioxin (16) (23 mg, 0.055 mmol) in 100 mL of CH₂Cl₂ (freshly distilled from P₂O₅) was added trimethyloxonium tetrafluoroborate (28 mg, 0.189 mmol). The solution was stirred in a closed vessel for 22 h. The reaction mixture changed color from yellow to pale yellow and some precipitation occurred. Dichloromethane was removed under reduced pressure. The residue was dissolved in acetonitrile and filtered through cotton. Acetonitrile was evaporated to ~0.5 mL and 2 mL of methanol where gradually added which initiated the crystallization. Pale yellow crystals were removed by filtration and dried under high vacuum (22 mg, 64%).

C₃₀H₂₄N₂O₂(BF₄)₂ 614.14 g/mole; HRMS (ESI Q-TOFMS) calculated for C₃₀H₂₄N₂O₂BF₄, m/z=531.1873, found 531.1850; ¹H NMR ( 300 MHz, CD₃CN) δ 8.56 ( d, J=6.9 Hz, 4H), δ 8.472 (d, J=7.8 Hz, 2H), δ 8.287 ( d, J=7.8 Hz, 2H), δ 8.183 ( s, 2H), δ 8.133 ( t, J=7.8 Hz, 2H), δ 8.089 ( d, J=6.9 Hz, 4H), δ 6.322 ( s, 2H), δ 4.24 ( s, 6H); ¹³C NMR (500
MHz, CD$_3$CN) δ 154.37, 145.86, 142.20, 132.98, 131.05, 127.58, 126.75, 126.15, 125.16, 124.25, 121.17, 118.07, 75.13.

3.5. Chiral separation of (±) 4,4’-dipyridine-pyrenedihydrodioxin (16)

Solution of (±) 4,4’-dipyridinepyrenedihydrodioxin camphor sulfonic acid salt (obtained from Alexei Shamaev) in methanol (~1mg/mL) was resolved via reverse phase HPLC on chiral sorbent. HPLC analyses and separations were conducted on Rainin HPLC system coupled with Agilent 8453 UV-Vis spectrometer and Chiralcel OJ-R column (4.6x150mm). Separation was performed using isocratic flow (90% methanol, 10% H$_2$O, flow rate 0.8 mL/min). Fractions were collected and concentrated under reduced pressure. Resulting aqueous solutions were lyophilized to yield separated enantiomers of 4,4’-dipyridinepyrenedihydrodioxin (16). Circular dichroism spectra of corresponding methanolic solutions (obtained with help of Daniil Zaitsev) showed complete separation of (+) and (-) enantiomers (Fig. 5).
Figure 5. Circular dichroism spectra of (+) and (-) 4,4’-dipyridinepyenedihydrodioxins (15) (this spectrum was obtained with help of Daniil Zaitsev).
4. RESULTS AND DISCUSSION

Dihydrodioxins containing pyridine and pyridinium moieties were successfully synthesized and characterized by a variety of methods including HR-MS and NMR spectroscopy.

We have found that introduction of positively charged pyridinium groups into dihydrodioxins facilitates binding to nucleic acids and increases their solubility in water and alcohols. Furthermore, pyridinium groups will act as electron traps and will suppress back electron transfer (BET) from nucleic acid strands and thus will promote the release of pyrene quinone from dihydrodioxins.

In the syntheses of dihydrodioxins, the initial step of photocycloaddition is the most challenging. The temperature of solution has to be kept slightly above the freezing point of benzene in order to decrease formation of side products. It is possible that at higher temperatures olefins used for photocycloaddition may polymerize. Another type of side reaction that frequently occurs in the course of Schönberg-Mustafa reaction is shown on Scheme 6.

![Scheme 6](image)

_Scheme 6._ Side reaction that occurs in the course of Schönberg-Mustafa syntheses of DHDs.40
The second step involved methylation of pyridine moieties. To convert 15 into corresponding pyridinium dihydrodioxin 14, we used trimethylxonium tetrafluoroborate. This reaction produced 14 with high yields (96%). When we used the same approach to obtain 17 from dipyridine dihydrodioxin 16, yields were considerably lower. One of the reasons for decrease in the yield of 17 is a competing reaction of dihydrodioxin 16 with tetrafluoroboric acid (Scheme 7), which can occur if the trimethylxonium tetrafluoroborate has been exposed to water prior to its use in methylating the pyridine moieties.

Scheme 7. Protonation of dihydrodioxin 16

To neutralize the tetrafluoroboric acid formed during the course of reaction and in hydrolysis of trimethylxonium tetrafluoroborate, we tried to add solid potassium carbonate to reaction mixture. This caused a decomposition of dihydrodioxins present in reaction mixture back to pyrene quinone and other compounds.

Another possible reason for lower yields of 17 is the precipitation of mono-methylated dihydrodioxin 18 (Scheme 8). To eliminate this problem we used considerably more diluted
solutions of 16 to conduct the reaction. This allowed us to improve the yield of 17 up to *circa* 65%.

![Scheme 8. Formation of mono-methylated pyrene dihydrodioxin 18](image)

The dipyridinium salt 17 is of particular interest. Employing molecular modeling in the gas phase it was found that 17, which has the trans geometry, exists in the surprising “diaxial” conformation. The diaxial geometry appears to be energetically favored in respect to the diequatorial geometry (6.55 kcal/mol, DFT B3LYP 6-31G* in the gas phase). The prevalence of the diaxial conformation can be explained by considerable steric and electronic repulsions between the two large pyridinium groups (Fig. 6). However, in recent calculations we have found that diequatorial conformation may be more favorable in aqueous phase. Therefore, this equilibrium is very sensitive to the environment of the dipyridinium salt 17.
Compound 17 is synthesized as a racemic mixture of RR and SS enantiomers. The diaxial geometry is probably imparts high degree of chirality to dihydrodioxin 17, which should affect binding of enantiomers to double-stranded DNA as it shown on Figure 7. For DHD 16 we determined specific rotation of enantiomers at the sodium D-line (589 nm) to be ~2.2 deg (this measurement was performed with help of Daniil Zaitsev).
The next step was to resolve dihydrodioxin 16 into its RR and SS enantiomers using liquid chromatography on chiral sorbent. As shown on Figure 7, both enantiomers might bind into the major groove of DNA, but the RR isomer would be expected to bind much more effectively.

To obtain separated enantiomers of 17 we tried two different approaches. First was to separate enantiomers of 17 directly, and second approach was to separate enantiomers of 16 and then methylate the pyridine groups. Initially, multiple attempts were made to recrystallize 17 from solution of (+) sodium tartrate in order to obtain mixture of diasteriomer tartrates of dipyridinium dihydrodioxin. This series of experiments proved to be unsuccessful. The next approach we used was thin-layer chromatography on reverse-phase silica gel. We used aqueous solution (+) sodium tartrate and acetonitrile as a mobile phase. After multiple runs we observed only one band that contained dihydrodioxin. In another attempt to resolve 17 on reverse-phase silica gel we chose a mobile phase which contained considerable concentrations of β-cyclodextrine. Solubility of β-cyclodextrine in water is very low. In order to reach higher
concentrations of β-cyclodextrine in the mobile phase we used a 20 M aqueous solution of urea. However, this approach proved to be technically difficult and did not succeed in separation of enantiomers.

Since 17 has two positive charges, we attempted separation on chiral ion-exchange sorbent, Sephadex® SP-25. Solutions of NaCl and HCl were used as a mobile phase. No separation of enantiomers of 17 was observed.

In another set of experiments we attempted to resolve 16 on a chiral HPLC column Chiralcel® OJ-R. This chiral sorbent is a reverse-phase silica gel covered with dextrins modified with p-methylbenzoates. Attempts to separate 16 on Chiralcel® OJ-R with acetonitrile or methanol as a mobile phase were unsuccessful. However, sample of (S)-(+) mandelate salt of 16, provided by Alexei Shamaev, was resolved into two bands that contained dihydrdioxin. Integration of the bands showed that ratio of surface under two bands is close two 1:1. Unfortunately, the amount of material in collected fractions was insufficient to obtain CD-spectra.

Once provided with the sample of (-) camphorsulfonic acid salt of 16 by Alexei Shamaev, a method for successful resolution of 16 on a chiral HPLC column Chiralcel® OJ-R was developed. The optimal resolution was obtained when using 90% MeOH/ 10% H2O. Resulting fractions were collected and analyzed. Surprisingly, these fractions contained resolved enantiomers of 16, and not the corresponding (-) camphorsulfonic acid salts. Since separation of methanolic solution of 16 into its enantiomers was unsuccessful, whereas separation of (-) camphorsulphonic acid salt and of (S)-(+) mandelic acid salt yielded enantiomers of 16, it seems that ion-ion interaction with anions and the sorbent may play an important role in this separation. Furthermore, since the resulting fractions contained enantiomers of 16 and not the corresponding
salts, it is possible that deprotonation of dipyridinium form of 16 plays a role in this separation. As the retention time of (-) and (+) enantiomers differs by more than 30 min, it is clear that interaction of the (+) enantiomer with the sorbent is much stronger than that of the (-) enantiomer.

Small oligonucleotide consisting of about five or fewer base pairs do not form stable duplex Watson-Crick assemblies at ambient temperatures at least in significant part due to electrostatic repulsion between their negatively charged phosphate backbones. However, in the presence of suitable intercalative binding agents, the duplex binding of small oligonucleotides is significantly stabilized.

Dihydrodioxin 17 seems to be capable of stabilizing duplex binding particularly well for two reasons. First, duplex stability increases due to intercalation of the pyrene unit between base pairs. Second, positive charges on pyridinium groups counter negative charges on the phosphate backbone of oligonucleotides, and thus promote duplex stability by reducing repulsion between the two strands.

Experiments with dihydrodioxin 17, conducted by Emil Khisamutdinov, demonstrated that addition of 17 stabilizes duplex dodecamer 5’-gtt agt ata tgg-3’/3’-caa tca tat acc-5’ and significantly increases its melting point (Fig. 8). Furthermore, the less strongly binding (-) enantiomer of 17 stabilizes this duplex dodecamer by about 6.31 kcal/mol, and the more strongly binding (+) enantiomer of 17 stabilizes the dodecamer by about 12.49 kcal/mol.
Figure 8. First derivative plots of the melting point profiles of: duplex dodecamer 5’-gtt agt ata tgg-3’/3’-caa tea tat acc-5’, $T_m = 39.4^\circ C$, this dodecamer with the enantiomer (-), $T_m = 61.2^\circ C$, and with the enantiomer (+), $T_m = 68.1^\circ C$. This melting point profiles were obtained by Emil Khisamutdinov.
5. REFERENCES


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CONCLUSIONS

The chemical behavior of the oxopurines 8-oxoadenosine (2) and 8-oxoinosine (3) in the presence of various oxidizing agents and in the presence a number of nucleophiles has been investigated. Purine quinones 5 and 6, which are pivotal intermediates in oxidation of oxopurines 2 and 3 respectively, were successfully trapped and the resulting products 7, 9, 11 were characterized. Formation of purine quinones was demonstrated with both chemical and photochemical oxidizing agents. Over-oxidation seems to be a major challenge in trapping adducts of 5 and 6. Imidazole adducts were demonstrated to be more stable towards further oxidation than adducts of other nucleophiles.

The next logical step for this project will be to investigate oxidation of 8-oxoadenosine when it is incorporated into oligonucleotides. These experiments will bring us closer to our goal of understanding the oxidative processes associated with damage to DNA.

Dihydridoxins containing two pyridine moieties (16) and two pyridinium salt moieties (17) have been synthesized and characterized by variety of methods including $^1$H and $^{13}$C NMR spectroscopy. Dihydridoxin 16, containing two pyridine moieties, was separated into D and L enantiomers via liquid chromatography on a chiral sorbent. Dihydridoxin 17 was demonstrated to stabilize considerably the stability of DNA duplexes. Affinities of D and L enantiomers of 17 to nucleic acids were significally different. Dihydridoxin 17 may have significant applications as a selective template for the assembly of oligonucleotides.
Inosine

Absorbance

Wavelength

A
b
s
o
r
b
a
n
c
\[
\begin{align*}
&\text{H} & & \text{O} \\
&\text{N} & & \text{H} \\
&\text{N} & & \text{O} \\
&\text{O} & & \text{H} \\
&\text{O} & & \text{H} \\
&\text{N} & & \text{H} \\
&\text{O} & & \text{H} \\
\end{align*}
\]
These are Negative Ion Results (Positive Ion results are poor) of ion abundances. FT-ICR better representation on the next slide gives accurate masses and elemental compositions.

Note: First structure that you wrote but not the one on the label (2 H's less)
From hexachloroiridate mediated oxidation

\[
\begin{array}{c}
\text{HO} \\
\text{O} \\
\text{N} \\
\text{O} \\
\text{H} \\
\end{array}
\]
Spectra of Inosines

A b s o r b a n c e

Wavelength

-0.2 0 0.2 0.4 0.6 0.8 1 1.2

200 209 218 227 236 245 254 263 272 281 290 299 308 317 326 335 344 353 362 371 380

Inosine
8-oxoinosine
2-imidazol-8-oxinosine
Elemental Composition Report

Single Mass Analysis
Tolerance = 50.0 mDa  /  DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0  Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
28958 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

Sample ID: mvr-414 (C28 H18 N2 O2) 414 Da (in MeOH/ESIB/766.2817LM)
MARSHALL-WR-061206-MW-414-RUN1.22 (0.424) AM (Con5, 80.00, H1.4000,0.766.28,1.00); Sm (Mn, 2x3.00); Sb (1,40.00); Cm (1:53) 1.82e3

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Maximum: -1.5

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16
Racemic Mixture

H₃C

2BF₄⁻

CH₃

17
Theoretical/Experimental