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Regioselective Syntheses of 7-Nitro-7-deazapurine Nucleosides and Nucleotides for Efficient PCR Incorporation

Tomohiko Kawate,[†,‡] Charles R. Allerson,[†,§] and Jia Liu Wolfe*[,†,‡]

Variagenics, Inc., Cambridge, Massachusetts 02139

wolfe@molbio.mgh.harvard.edu

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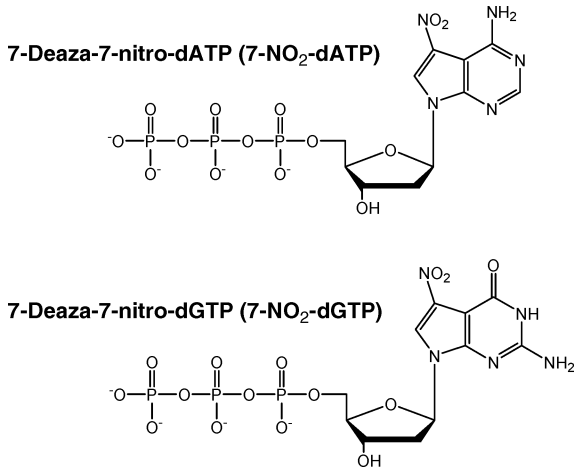
ABSTRACT



Substitution at the C[7] position of purine nucleotides by a potent electron-withdrawing nitro group facilitates the cleavage of glycosidic bonds under alkaline conditions. This property is useful for sequence-specific cleavage of DNA containing these analogues. Here we describe the preparation of 7-deaza-7-NO_2-dA and 7-deaza-7-NO_2-dG using two different approaches, starting from 2′-deoxy-adenosine and 6-chloro-7-deaza-guanine, respectively. These modified nucleosides were converted to nucleotide triphosphates, each of which can replace the corresponding, naturally occurring triphosphate to support PCR amplification.

Modified purines and purine nucleosides have demonstrated broad utility as pharmaceuticals as well as biochemical tools. Compounds containing the pyrrolo[2,3-*d*]pyrimidine system, which often is referred to as 7-deazapurine, have shown useful pharmaceutical properties, either as naturally occurring antibiotics or synthetic derivatives of 7-deazapurine.[1] In addition, oligonucleotides containing 7-deazapurine derivatives often exhibit interesting properties, such as increased duplex stability,[2] diminished nuclease susceptibility,[3] and altered chemical reactivity,[4,5] that are useful for biological applications.

Recently, 7-nitro-7-deazapurines have been utilized in a DNA genotyping method (Incorporation and Complete

[†] Variagenics, Inc.

[‡] Current affiliation: Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114.

[§] Current affiliation: Isis Pharmaceuticals, Inc., Carlsbad, CA 92008.

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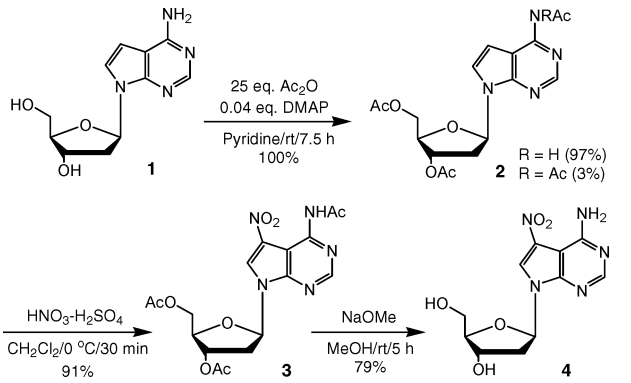
Chemical Cleavage, ICCC)[6] and a footprinting technique (Template Directed Interferance, TDI) that maps DNA−protein interactions.[5] Both technologies are based on incorporation of a chemically labile nucleotide by PCR followed by specific chemical cleavage of the resulting amplicon at modified bases. Whereas TDI uses each modified nucleotide to partially substitute a particular base, ICCC requires the complete displacement of a selected base by its cleavable analogue for subsequent, complete cleavage at the sites of modification. Modeled after N^7-methyl-dGTP, which is labile under alkaline conditions as a result of its electron-withdrawing CH_3-N^{7+} moiety,[7] 7-deaza-7-nitro-dATP (7-NO_2-dATP) and 7-deaza-7-nitro-dGTP (7-NO_2-dGTP) were designed to provide similar sensitivity to alkaline treatment while gaining thermal stability to survive PCR cycles.[5,6]

Although nonsubstituted 7-deaza-dATP and 7-deaza-dGTP have been successfully incorporated by PCR, only the latter could completely replace the corresponding native nucleotide dGTP.[3] In our initial PCR attempts, it was observed that even though both 7-NO_2-dATP and 7-NO_2-dGTP can partially replace dATP and dGTP, respectively, it was challenging to incorporate 7-NO_2-dGTP without the dGTP supplement. Since it is well-known that the quality of nucleotide triphosphates is important for the success of PCR, we set out to improve the synthesis and purification process for 7-NO_2-dATP and 7-NO_2-dGTP. Described in this report are our optimized synthetic procedures that led to their successful application in ICCC.[6]

7-NO_2-dA (**4**) was prepared according to a procedure described by the Verdine group[5a] with some changes that are beneficial to isolation and purification of the intermediates. As summarized in Scheme 1, **4** was conveniently

Scheme 1. Synthesis of 7-NO_2-7-deaza-dA (**4**)



prepared in three steps from now commercially available 7-deaza-dA (**1**, also known as 2′-deoxytubercidin), in substantially better yields (72% overall) than what was previously reported (33% overall). One key feature of this improved procedure was the use of a large molar excess of acetic anhydride to effect acetylation at the exocyclic amino group of **1** in addition to the two hydroxyl groups. This change resulted in quantitative conversion of **1** to a 97:3 mixture of tri-acetylated and tetra-acetylated 7-deaza-dA (**2**). Nitration of **2** with 1:1 fuming nitric acid and concentrated sulfuric acid yielded 91% of **3** after purification by silica column chromatography. Removal of acetyl protecting groups generated **4** in a purified yield of 79%. It should be noted here that Seela et al. have also described a similarly improved procedure for the preparation of **4** and definitively assigned its structure using X-ray crystallography.[8]

The much improved synthesis due to additional acetylation of exocyclic amino group may be attributable to reduced electrophilic attack at C^8. As illustrated in Figure 1,

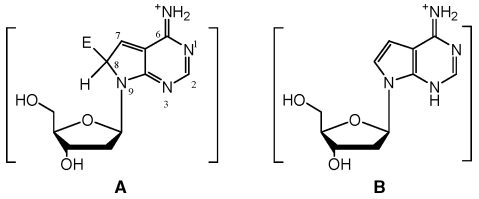


Figure 1. Mesomeric stabilization of electrophilic attack at C^8 (**A**) and protonation of N^3 (**B**) of 7-deaza-dA (**1**).

unprotected NH_2 may direct electrophilic attack at C^8 and/or increase the likelihood of N^3 protonation and subsequent depurination, resulting from mesomeric stabilization of σ-complexes **A** and **B**, respectively. Acetylation of NH_2 reduces the electron density on the nitrogen and thus favors the nitration at 7-position.

The synthesis of 7-NO_2-dG (**12**) has been reported by Storek et al., starting from 6-Cl-7-deazaguanine and involving glycosylation, 6-methoxylation, and exocyclic amine protection, followed by nitration. Unfortunately a mixture of 7- and 8-NO_2 substituted 7-deaza-dG analogues were obtained that were difficult to separate.[5b] To improve the regioselectivity of the nitration reaction, we attempted to predict what synthetic intermediates could promote C^7-nitration based on a stabilized transition state. Shown in Figure 2 are a few postulated σ-complexes that would be involved in the nitration of a particular synthetic intermediate. Structure **A** illustrates that electrophilic attack at the 8-position is promoted by 2-NH_2 in 7-deaza-dG, which was first recognized by the Seela group.[9] Structure **B** shows that the 6-OMe group could direct electrophilic attack to the 8-position of 6-OMe-7-deaza-dG, even though 2-NH_2 is protected. Structure **C** shows how N^9 could help direct an attack by an electrophile (E) to the desired 7-position, as previously proposed by Seela et al.[9] Both σ-complexes **B** and **C** could be involved in the aforementioned nitration procedure[5b] that

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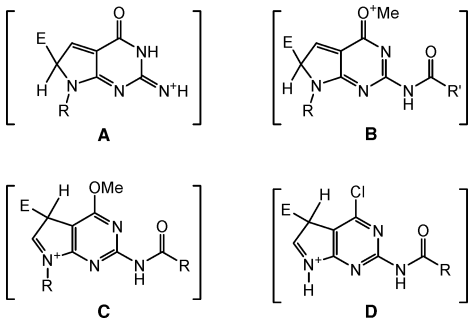


Figure 2. Mesomeric stabilization of electrophilic attack at C^8 of 7-deaza-dG (**A**), C^8 of 2-NH_2-protected 6-OMe-7-deaza-dG (**B**), C^7 of 2-NH_2-protected 6-OMe-7-deaza-dG (**C**), and C^7 of 2-NH_2-protected 6-OMe-7-deazaguanine (**D**).

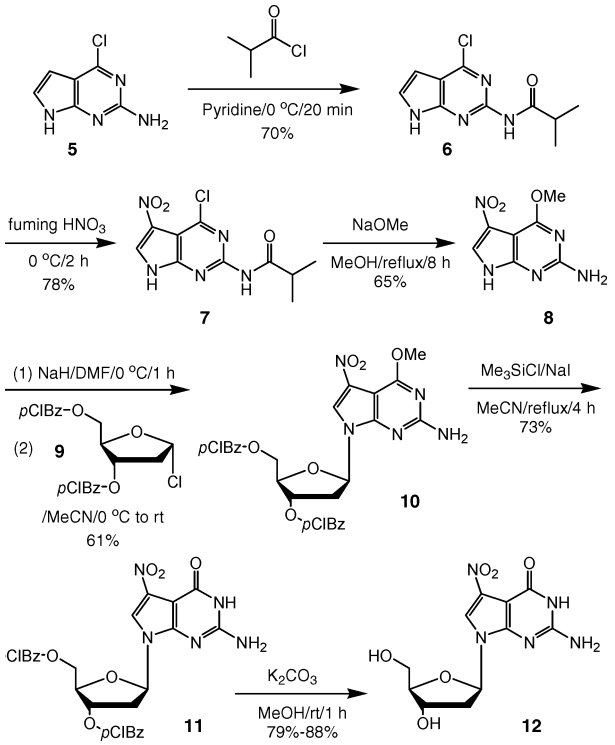
resulted in both 7- and 8-substitution. On the basis of these considerations and the commercial availability of 6-Cl-7-deaza-guanine, it was used as the starting material for nitration. In addition, we reasoned that although the free electron pair on N^9 in structure **C** seems to be helpful for 7-substitution, an unglycosylated guanine might be an even better choice (structure **D**), because the unsubstituted N^9 could direct electrophilic attack to the 7-position without increasing the risk of deglycosylation.

Scheme 2 depicts the six-step synthetic procedure for the preparation of 7-NO_2-dG (**12**). First the exocyclic amine of 6-Cl-7-deaza-guanine (**5**) was protected in 70% yield to afford **6**. Treatment with a 1:1 mixture of fuming nitric acid and concentrated sulfuric acid generated the single regioisomer 6-Cl-7-nitro-7-deaza-guanine (**7**), which readily precipitated from the reaction mixture and was isolated in 78% yield. This excellent regioselectivity made chromatographic purification unnecessary and was likely the result of a favorable conjugation shown in Figure 2**D**. Treating **7** with sodium methoxide displaced the 6-chloro substituent with a methoxy group while concurrently removed the isobutyryl protection group of the exocylclic amine to afford compound **8**.

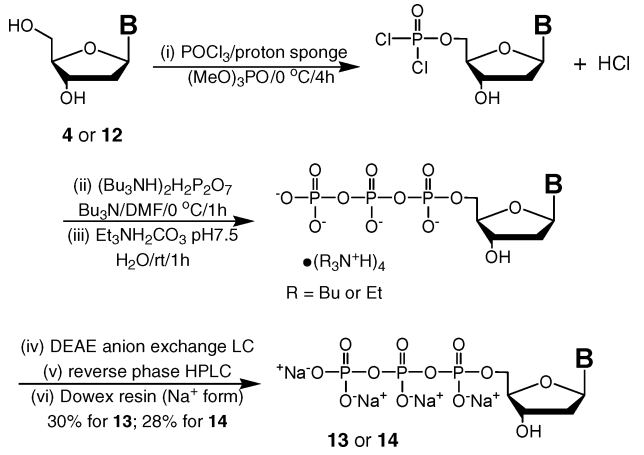
The nucleobase-anion of **8** was generated using NaH and was glycosylated in situ with 2-deoxy-3,5-di-*O*-(*p*-chlorobenzoyl)-α-D-ribofuranosyl chloride (**9**) to produce the desired *β*-isomer **10** in 61% isolated yield. Compound **9** was prepared from 2-deoxy-D-ribose in three steps, following a literature procedure.[10] Demethylation of **10** using iodotrimethylsilane liberated the 6-oxo group in 73% yield after flash column chromatography. Deprotection of hydroxyl groups by methanolysis generated 7-NO_2-dG (**12**) in 79% after column chromatography purification. This synthetic procedure is a significant improvement over the existing method,[5b] which required the separation of 7- and 8-substituted products using preparatory TLC. In comparison, conversion of **6** to **7** was performed at a 3.3-g scale and did not require chromatographical purification. The regiochemistry of the nitro group was confirmed by differential NOE experiments on the N^9-methyl derivative of **7** between the proton at C^8 and the protons of N^9-methyl.

7-NO_2-dATP (**13**) and 7-NO_2-dGTP (**14**) were prepared from 7-NO_2-dA (**4**) and 7-NO_2-dG (**12**), respectively, according to Scheme 3. Initially, we attempted to use a commonly used procedure that involves phosphorylation using $POCl_3$ in $(MeO)_3PO$ followed by in situ reaction with pyrophosphate and subsequent anion exchange column chromatography.[5b,3] This process, however, generated nu-

Scheme 2. Synthesis of 7-NO_2-7-deaza-dG (**12**)



Scheme 3. Preparation of Nucleotide Triphosphates 7-NO_2-dATP (**13**) and 7-NO_2-7-deaza-dGTP (**14**)



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cleotide triphosphates of low purity and in poor yields. Consequently, several modifications to the procedure were implemented. To maintain the neutrality of the phosphorylation reaction, during which HCl is released as a byproduct, 1,8-bis(dimethylamino)naphthalene (proton sponge), a strong base with weak nucleophilic character due to steric effects, was used to balance the pH in place of the more commonly used alkylamines. In addition, we found that anion exchange column chromatography purification alone did not provide sufficiently pure nucleotide triphosphates. Further fractionation by reversed-phase HPLC was necessary to remove impurities that had comigrated with the triphosphates during anion exchange column chromatography. After simple conversion to the Na^+ salt using Dowex-50W×8 resin (Na^+ form), The final yields were 30% and 28% for 7-NO_2-dATP (**13**) and 7-NO_2-dGTP (**14**), respectively. These highly pure purine nucleotide analogues can completely replace dATP and dGTP, respectively, in supporting PCR amplification of DNA samples.[6] These results suggest that (i) 7-NO_2-dATP and 7-NO_2-dGTP can be efficiently incorporated by DNA polymerase, and (ii) DNA products containing 7-NO_2-dA or 7-NO_2-dG can also act as templates for polymerase extension.

In conclusion, we have developed synthetic procedures that can generate highly pure 7-NO_2-dA and 7-NO_2-dG, as well as their nucleotide triphosphates. These reagents are critical components for polymorphism analysis using ICCC,[6] as well as studies of sequence-specific protein−DNA interactions by TDI footprinting.[5] The synthesis and purification principles discussed herein may also be generally applicable for the preparation of other modified purine analogues. For example, to our knowledge, the improved three-step synthesis of 7-NO_2-7-deaza-dA from 7-deaza-dA is the most efficient route that can introduce an electrophilic substitution at the 7-position of 7-deazapurine-2′-deoxynucleoside.[11] In addition, the discovery that 2-NH_2-protected 6-chloro-7-deazaguanine is the most suitable substrate for electrophilic attack at the 7-position may be instructive for the preparation of other 7-substituted 7-deazapurine-2′-deoxynucleosides, such as 7-halogenated and 7-alkylated 7-deazapurine-2′-deoxynucleosides.

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Supporting Information Available: Experimental procedure and spectral data for synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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