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Submitted date: 19/06/2020 • Posted date: 22/06/2020

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Citation information: Bhoge, Bapurao; Saraogi, Ishu (2020): Selective Functionalization at N2-Position of Guanine in Oligonucleotides via Reductive Amination. ChemRxiv. Preprint.

<https://doi.org/10.26434/chemrxiv.12511661.v1>

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Selective functionalization at N²-position of guanine in oligonucleotides via reductive amination

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ABSTRACT: Chemo- and site-specific modifications in oligonucleotides have wide applicability as mechanistic probes in chemical biology. Here we have employed a classical reaction in organic chemistry, reductive amination, to selectively functionalize the N²-amine of guanine/2'-deoxyguanine monophosphate. This method specifically modifies guanine in several tested DNA oligonucleotides, while leaving the other bases unaffected. Using this approach, we have successfully incorporated desired handles chemoselectively into DNA oligomers.

Methods to synthesize modified oligonucleotides are of prime importance due to their role in a variety of applications. For example, modified oligonucleotides are useful to understand the effect of specific chemical modifications on nucleic acid stability, ability of DNA to replicate, and to study nucleic acid-protein interactions.^{1–4} In addition, modified oligonucleotides also have diagnostic or therapeutic applications; for example in nucleic acid sequencing and identification, or in small interfering RNA technology for enhanced stability and target affinity *in vivo*.⁵ However, selectively modified oligonucleotides are difficult to obtain, and are most commonly synthesized on a solid-phase using the corresponding phosphoramidite derivative. The synthesis of the desired phosphoramidite, in turn, involves several steps including nucleotide protection and deprotection, and purification processes such as ion exchange and HPLC. This makes the overall process challenging, time-consuming, and expensive.

One approach to circumvent this problem is to introduce the desired modifications after DNA (or RNA) synthesis. However, this would require methods that can chemoselectively target one out of the four nucleobases. Although a number of reagents are known to form adducts with DNA^{6,7}, very few chemical methods exist for selective targeting of one nucleobase over others. One such example was reported by Okamoto *et al.*, in which they showed that 5-methylcytosine, an important epigenetic modification, can be sequence selectively oxidized using osmium based reagents for the selective cleavage of a DNA strand.⁸ In another example, Geigle *et al.* have performed selective modification at the O⁶ position of guanine in DNA using metal carbenes.⁹

The selective modification of guanine in DNA is biologically relevant. In particular, modifications at the N²-position of guanine frequently lead to mutagenic DNA adducts, and the availability of such substrates is essential to elucidate the biological ramifications of adduct formation. Recently, N²-modified deoxyguanine oligonucleotides were used as substrates to study the activity of the enzyme Top1, which is highly susceptible to modifications in the DNA bases.¹⁰

Guanine nucleotide and its derivatives are also interesting as they self-assemble to form higher order structures in the presence of some metal ions. These have a variety of applications as anticancer hydrogels¹¹, inducers of effective cell growth¹², and selective absorption of anionic dyes from water¹³. These properties can be tuned by appropriate chemical modifications. Moreover, substituted guanine containing deoxy-oligonucleotides have been shown to efficiently fold into G-quadruplexes, biologically relevant structures comprised of four G-rich DNA strands. The modified G-quadruplexes have several desirable characteristics like enhanced thermal stability,¹⁴ and anti-thrombin and anti-HIV properties.^{15,16} Antisense oligonucleotides containing guanine, substituted at its N²-position with cationic groups, show enhanced DNA (and RNA) binding, while preserving RNase H activity required for gene silencing.^{17,18} In summary, the utility of N²-modified guanine necessitates development of methods for selective post-synthetic modifications at the N²-position of guanine.

Despite its importance, very few reagents are available for the post-synthetic functionalization of the exocyclic amine of guanine nucleotide selectively over other nucleotides. Although previous studies have reported reductive alkylation of the exocyclic amine of guanine derivatives,^{19,20,21} a systematic study of the reactivity and selectivity of the exocyclic amine of guanine in a complex chemical environment such as DNA is lacking. The availability of chemical methods for guanine N²-modification are particularly important as the N²-alkyl deoxyguanine nucleotides are not substrates for DNA polymerases (except for polymerase κ^{22}), precluding the use of enzymatic methods for modified nucleotide incorporation into DNA. Here, we have systematically studied reductive amination of guanine with various aliphatic aldehydes to selectively make guanine N²-substituted DNA oligonucleotides. We show that this simple yet powerful reaction can selectively modify the N²-position of guanine, leaving the other aromatic exocyclic amine bases (viz. cytosine and adenine) unaffected under the reaction conditions.

We first treated an aqueous solution of each of the four nucleotide monophosphates (NMPs) individually with propanal in the presence of sodium cyanoborohydride (Table 1). Near quantitative conversion was obtained for guanine (both dGMP and GMP) to give the monoalkylated product after six hours. The product identity and site of the reaction was confirmed by NMR methods. The product assignment was further supported by the observation that inosine triphosphate (ITP), lacking the N²-amine, did not react under these conditions.

Table 1: Screening of nucleotide monophosphates for reductive functionalization with propanal.

Nucleotide ^a	Time (h)	Conversion ^b (%)
dGMP	6	≥ 96
GMP	6	≥ 98
AMP	24	0
dAMP	24	0
dCMP	24	0
CMP	24	0
dTMP	24	0
UMP	24	0
ITP	24	0

^aReaction was done at 150 mM NMP concentration; propanal = 50 equiv. w.r.t NMP; ^b% conversion was calculated from HPLC based on area under the peak ($\lambda = 253\text{nm}$) with respect to the starting material.

Under the same reaction conditions, no conversion was observed for adenine and cytosine nucleotides, both of which contain an exocyclic amine, even after 24 hours, indicating that the reaction was selective for guanine (Table 1). Similarly, uracil and thymine derivatives did not react under these conditions. We also treated a mixture of all the four deoxy NMPs (dNMPs) under the given reaction conditions. After 6 hours, the crude reaction mixture was analysed by HPLC, which indicated that even in the mixture, only dGMP successfully reacted with propanal to form N²-propyl substituted dGMP, while others remained unchanged (Fig 1). Not surprisingly, screening several aliphatic aldehydes in this reaction revealed that the reaction became more sluggish as the hydrophobicity of the aldehyde increased. This was likely due to the reduced solubility of the aldehyde in aque-

ous medium, and correlated well with the calculated logP values for the aldehydes. In agreement with this, we also found no reaction with aromatic aldehydes under the same reaction conditions.

Although the exact reason for selectivity towards guanine needs to be investigated, it is likely that the N²-amine of guanine is significantly more nucleophilic, as it is part of a guanidinium system. In comparison, the amidines of cytosine and adenine are less nucleophilic.^{23,24} We further note that aliphatic ketones showed negligible reactivity with guanosine under the given reaction conditions, indicating that in addition to the reactivity of guanine, electronic and steric properties of the carbonyl substrate also play an important role in differentiating the exocyclic amine of guanine.

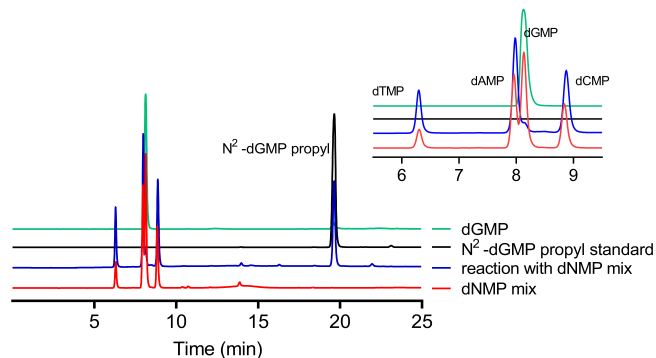


Fig 1: HPLC analysis of a mixture of all four dNMPs under our reaction conditions. Only the dGMP starting material was consumed (inset: compare red and green), and one new peak corresponding to the N²-modified dGMP was observed.

The selectivity observed for guanine was remarkable because of the ability of a simple reaction to distinguish between GMP/dGMP and other nucleotide bases, indicating that this reaction could potentially be applied to post synthetic modification of oligonucleotides, allowing guanine base to be selectively targeted. To test this idea, we started with a four nucleobase DNA sequence containing all four nucleotides, i.e., d(GTCA). When this oligonucleotide was treated with propanal and NaCNBH₃, a new peak appeared in HPLC, but complete consumption of the starting material was not observed. So, we performed systematic optimization of the reaction conditions, including temperature and reagent concentrations. We found that although an increase in temperature up to 45°C increased yield to 70%, any further increase led to significantly lower yields likely due to decomposition of the reducing agent. In contrast, increasing aldehyde concentration pushed the reaction towards completion, with almost complete disappearance of starting material within 3 hours, and appearance of a new peak in the HPLC profile (See Fig. 2, and Table 2). A mass spectrometric analysis of this peak showed a single propyl modification in the oligonucleotide, and was consistent with a modification on guanine. We note that even at 1500-fold excess of aldehyde, other nucleobases remained unreacted.

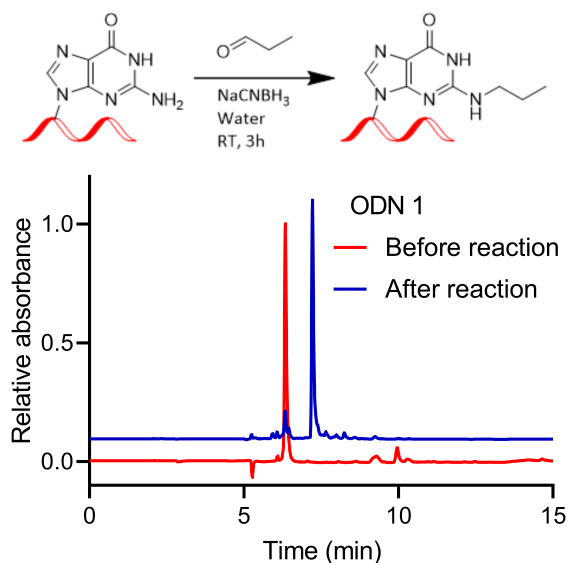


Fig 2: Scheme showing reductive alkylation of DNA oligonucleotide d(GTCA) (labeled as ODN1) with propanal (1500 equiv.). The blue line corresponds to the HPLC profile of the crude reaction after 3 hours.

Table 2: Reaction optimization with DNA oligonucleotide d(GATC)

Aldehyde (eq.)	Temperature (°C)	% conversion (from HPLC) ^a	
		3 h	24 h
300	25	14	35
300	37	43	44 ^b
300	45	70	71
300	60	44	39
1500	25	90	95

DNA concentration was 5 mM in water, ^a % conversion was calculated based on area under the peak with respect to starting material; ^b reaction was stopped after 8 hours since there was no significant change in % conversion after 3 hours.

We then tested a series of DNA oligonucleotides using the above reaction protocol, and found that method works efficiently with all tested DNA sequences irrespective of the position of guanine, and the number of cytosine and adenosine bases (Table 3). In all G-containing oligonucleotides, a new peak appeared in the RP-HPLC profile indicating likely formation of one new product in the reaction mixture. This new peak was attributed to reaction at the single guanine site, as oligonucleotides lacking guanine did not show any change in HPLC profile after six hours (Table 3, Entry ODN5, ODN7, ODN10). To further confirm this result, ODN11 was treated with phosphodiesterase I from *Crotalus adamanteus* venom followed by calf intestine phosphodiesterase (CIP) digestion. This treatment hydrolyzes the DNA into individual nucleosides and allows product identification by HPLC. Analysis of the hydrolyzed products showed the presence of N²-propyl deoxyguanosine nucleoside, along with other unreacted deoxynucleosides (dC, dT and dA). Taken together, the data presented above strongly support that the reaction takes place regio- and chemo-selectively at the N²-position of guanine in DNA, leaving all other nucleobases unaffected.

Table 3: Reductive alkylation of propanal with DNA oligonucleotides

Entry	DNA sequence ^a	Time (hours)	% Conversion ^b (from HPLC)
ODN2	d(GATC)	3	88
ODN3	d(ATGC)	3	95
ODN4	d(ATCG)	3	95
ODN5	d(ATATC)	6	0
ODN6	d(AAAGAAA)	3	87
ODN7	d(AAACAAA)	6	0
ODN8	d(CCCGCCC)	3	95
ODN9	d(TTTGTTT)	3	93
ODN10	d(TTTCTTT)	6	0
ODN11	d(AACTGACTCA)	3	90

^a DNA concentration was 5mM in water; aldehyde = 1500 equiv. and NaCNBH₃ = 150 equiv. w.r.t DNA; ^b conversions are based on area under the peak (λ = 253nm).

We applied this reductive amination strategy to functionalize a G-quadruplex forming sequence TT(GGGT)₄. A mass spectrometric analysis of the modified sequence showed that eleven out of the twelve guanine bases were modified, indicating the potential of this reaction to densely functionalize DNA. When oligonucleotide ODN11 was treated with more hydrophobic aliphatic aldehydes, the reaction was sluggish, and the % conversion was significantly reduced for aldehydes having logP more than 1.5 (Table 4). We further explored the potential of this reaction to introduce bioorthogonal handles such as azide or alkyne into a DNA oligonucleotide (Table 4). Aldehydes with suitable bioorthogonal handles reacted with ODN11 to give moderate to good yields of the monosubstituted product. The bioorthogonal functionality introduced on oligonucleotides in this manner can be further reacted with a desired probe for example a fluorophore or affinity tag to obtain

labeled oligonucleotides. We have demonstrated this using dibenzylcyclooctyne-PEG₄-5/6-tetramethylrhodamine (DBCO-TAMRA) as a representative example to illustrate the scope of the reaction. We first reacted ODN11 with 5-azido-1-pentanal, and the product was washed with ethyl acetate to remove organic reagents. The bioorthogonal handle containing DNA was further reacted with DBCO-TAMRA under strain mediated copper free click conditions. The formation of the fluorescently labeled DNA was confirmed by HPLC, as well as HRMS (Fig. 3).

Table 4: Screening of aldehydes with ODN11

Aldehyde	logP	Conversion (%)	
		6 h	12 h
	0.44	85	90
	1.24	58	68
	1.63	32	41
	-0.28	60	80
	0.12	55	80
	0.25	72	78

Oligonucleotide used is d(AACTGACTCA). DNA concentration was 5 mM in water; aldehyde = 1500 equiv. and NaCNBH₃ = 150 equiv. w.r.t DNA; conversions are based on area under the peak ($\lambda = 253\text{nm}$).

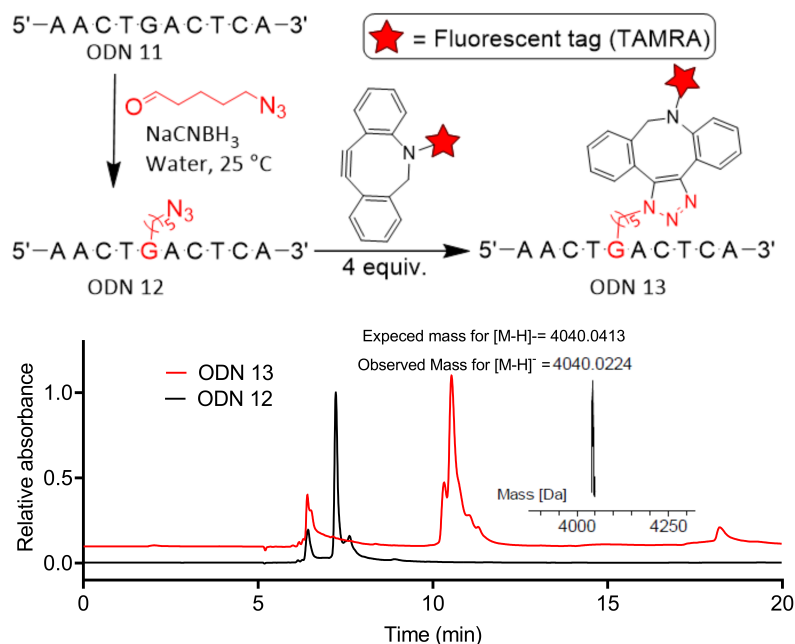


Fig 3: Labeling of a DNA oligonucleotide with a fluorescent tag using the reactive handle installed on DNA by the reductive amination approach.

In summary, we have shown that reductive amination with DNA bases can selectively modify guanine in nucleic acids. This simple yet powerful method can readily produce oligonucleotides modified at the N²-position of guanine. We have also shown an application of this method to access fluorescent DNA labeled specifically at guanine base. The method is simple and readily

accessible even for biologists, and significantly reduces the time and cost involved in using modified phosphoramidites for selective labeling of guanine in nucleic acids.

Acknowledgments

We thank IISER Bhopal and SERB for financial support of this work. B.A.B thanks CSIR for fellowship. I.S is a recipient of the Ramanujan Fellowship from the Science and Engineering Research Board.

Conflicts of interest

There are no conflicts to declare.

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