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Postsynthetic guanidinylation of primary amino groups in the minor and major grooves of oligonucleotides

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Abstract—Two different postsynthetic strategies were employed to introduce guanidinium moieties into oligonucleotides. A 2′-*O*-(6-aminohexyl) tether, which positions the modification in the minor groove, was selectively deprotected and guanidinylated on the solid support. In the second system, the aminoethoxy spacer of the tricyclic cytosine analog G-clamp, which protrudes into the major groove of the duplex, was guanidinylated in aqueous solution using the fully deprotected oligonucleotide. Both methods were successfully applied to synthesize and characterize various guanidinylated oligonucleotides.

In the past several years, numerous modifications have been designed and synthesized to improve the intrinsic properties of oligonucleotides (ONs) used for diagnostic or therapeutic applications.[1–4] For both types of applications, the binding affinity of the modified oligonucleotide to the target, the specificity and a high on-rate of hybridization are critical parameters. Cationic oligonucleotides[5] (oligonucleotides with positively charged backbones) and zwitterionic oligonucleotides[6–19] (oligonucleotides with positively charged tethers) bind tightly to RNA and to single-stranded as well as to double-stranded DNA (triplex formation). High affinity binding in these modifications is due to charge neutralization and fast on-rates of hybridization. In addition, these oligonucleotides are expected to have good cellular permeation properties.[20]

The 2′-position is attractive for nucleic acid derivatization as it offers the dual advantages of enhanced nuclease resistance and increased binding affinity to a complementary target strand.[21,22] It was shown previously that the introduction of cationic residues at the 2′-position greatly enhances the nuclease resistance of an ON.[18,19,23] The superior nuclease resistance of 2′-*O*-(3-aminopropyl) modified oligonucleotides was attributed to a 'charge effect' of the cationic side chain. Presumably, the side chain interacts competitively with the metal ions involved in the phosphodiesterase activity of the nucleolytic enzymes.[18,19] The 2′-*O*-aminoalkyl group has also served as a site for conjugation of additional groups.[7,9,24]

Recently, several tricyclic cytosine analogs have been developed. These include a phenoxazine analog and G-clamp, which carries an additional aminoethoxy moiety attached to the rigid phenoxazine scaffold.[25,26] Incorporated into ONs, these base modifications were shown to hybridize with guanine and to enhance duplex stability through extended stacking interactions. Binding studies demonstrated that a single incorporation of the G-clamp enhanced the binding affinity of an ON to its complementary target DNA or RNA with a ΔT_m of up to 18° relative to 5-methyl cytosine ($dC5^{Me}$). This is the highest reported affinity enhancement for a single modification. It was suggested that the tethered amino group formed an additional hydrogen bond with O6 in the Hoogsteen face of a complementary guanine (Fig. 1A). This means that the increased affinity of G-clamp is mediated by the combination of extended base stacking and one additional specific hydrogen bond.

The guanidinium group plays an important role in many fundamental organic and biological processes such as catalysis,[27–29] synthetic molecular receptors,[30] protein–nucleic acid interactions,[31,32] including RNA/DNA recognition by arginine rich proteins such as transcriptional elongation factors,[33,34] and regulatory processes including HIV TAR–tat interactions and RNA–guanidiniumglycoside interactions.[35]

The guanidinium group is highly basic ($pK_a \approx 12.5$) and planar and demonstrates directionality in H-bonding

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interactions. Since antisense technology relies on molecular recognition between synthetic oligonucleotides and *m*RNA, introduction of the guanidinium group into ONs at strategically selected positions should improve their pharmacodynamic and pharmacokinetic properties. The significantly higher pK_a value of the guanidinium compared to the amino functionality introduces a positive charge that is maintained over a wide pH range. In addition, such a modified nucleic acid can serve as a probe for understanding specific nucleic acid–nucleic acid interactions as well as specific nucleic acid–protein interactions.

The syntheses of certain guanidinium derivatives of nucleic acids have been described in the literature. Cationic ONs containing a guanidinium backbone have been synthesized by Bruice and his group.[36] Doronina and Behr have synthesized 4-guanidiniumpyrimidine ONs for triple helix formation.[37] Recently, ONs containing 4-guanidinium-2-pyrimidinone nucleobases have been prepared. These nucleobase analogues were designed to mimic the double hydrogen bonding pattern of protonated cytosines in parallel triple helices.[38] However, no convenient method has been reported for site-specifically converting an amino group present in a modified nucleic acid into a guanidinium group.

In the present paper, we report the postsynthetic modification of a primary amino group to a guanidinium moiety on either the 2′-position of a nucleotide or the aminoethoxy tether of G-clamp. In the double helical structure of nucleic acids, the 2′-*O*-(6-aminohexyl) tether faces the minor groove of the duplex, while the aminoethoxy spacer of G-clamp protrudes into the major groove (Fig. 1A). We rationalized that the extension of the aminoethoxy spacer of the G-clamp to a guanidinium derivative would provide another Hoogsteen bond between the imino or amino nitrogens of the tethered guanidinium and N7 of a complementary guanine base (Fig. 1B). In fact, crystallographic X-ray studies, of a decamer duplex containing the guanidinium-G-clamp nucleotides revealed this additional hydrogen bond.[39] This is the first direct observation of a single base pair with five hydrogen bonds including both Watson–Crick and Hoogsteen interactions.

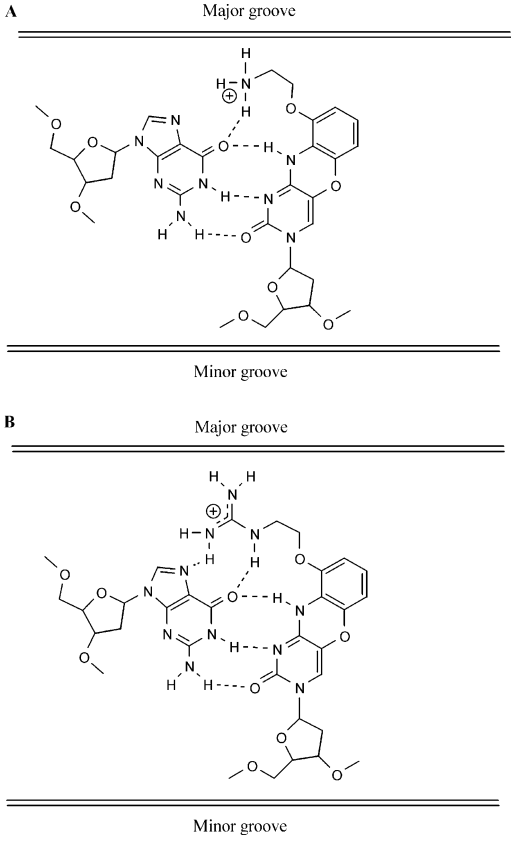
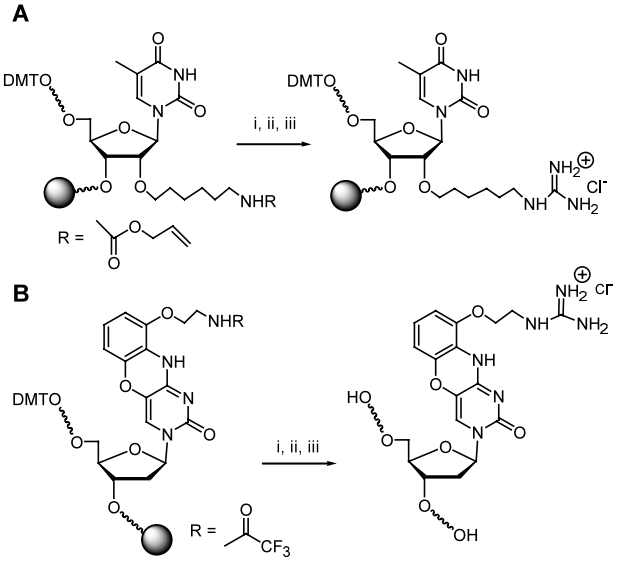


Figure 1. (A) Proposed base pairing between G and G-clamp; (B) base pairing between G and guanidinium-G-clamp.

As outlined in Scheme 1, two different strategies have been employed to convert an existing amino function into a guanidinium group: (A) selective deprotection of the primary amino group followed by guanidinylation on the solid support and (B) guanidinylation of a completely deprotected ON in solution. 2′-*O*-Aminoalkyl modifications can be introduced using simple alkylation procedures reported.[7,9,18,40] The amino group has been protected with phthalimido,[7,9,18] trifluoroacetyl,[18] cyanoethyloxycarbonyl[41] and allyloxycarbonyl (Alloc) groups.[40] In the present work, Alloc was chosen as a protection group for the primary amino function of 2′-*O*-(6-aminohexyl) as it can be



Scheme 1. Guandinylation of primary amino groups. (A) *Reaction conditions* (1 μmol scale): (i) 10 mg $Pd_2[(Ph\text{-}CH{=}CH)_2CO]_3{\cdot}CHCl_3$, 26 mg $P(Ph)_3$ in 0.5 ml of 1.2 M *n*$BuNH_2$/HCOOH in THF, 50°C, 1.5 h, 100%, (ii) washing with DCM, acetone, sodium *N*,*N*-diethyldithiocarbamate (ddtc Na^+), H_2O, acetone, DCM, diethyl ether, (iii) 1.0 M of 1*H*-pyrazole-1-carboxamidine hydrochloride and DIEA in DMF, rt, 5 h, 90%. (B) (i) 40% aq. CH_3-NH_2/conc. aq. NH_3 (1:1), 55°C, 1 h, (ii) HPLC purification and DMT-deprotection, (iii) 1.0 M solution of 1*H*-pyrazole-1-carboxamidine hydrochloride in 1.0 M aq. Na_2CO_3, 3 h at rt, for ON-3, ON-4 and 12 h at 55°C, for ON-5, ON-6, isolated yield after 2nd HPLC purification: 50–60%.

conveniently and selectively removed on the solid support by a Pd(0) complex, while leaving all other protection groups of the oligonucleotide unaffected.[40] In both strategies, the primary amino groups were guanidinylated by using 1*H*-pyrazole-1-carboxamidine hydrochloride.[42] Although this reagent has been widely used for guanidinylation of peptides and small organic molecules, it had not been tested for nucleic acids, yet. This may have been due to the concern about the potential reactivity of this reagent towards exocyclic amines present in the nucleobases.

The guanidinium-modified ONs synthesized during this study are summarized in Table 1. Interestingly, in the case of the self-complementary sequences, ON-5 or ON-6, complete guanidinylation of the amino groups could only be achieved by performing the reaction at elevated temperature (55°C) and extended reaction time (12 h). This shows that the primary amino group, which is involved in the base pairing interactions, is not susceptible to the guanidinylation reaction within the double-stranded structure of these palindromic ONs. In all these cases, even under elevated temperatures and extended reaction times, selective and quantitative guanidinylation of only the alkyl amino group was observed. After desalting (Sephadex G25) and purification of the compounds by another reversed phase HPLC step, the guanidinylated compounds were analyzed by electrospray mass spectrometry (ES-MS) and capillary gel electrophoresis (CGE) analysis. There was no evidence for residual starting material bearing the primary amino function or any other side product in the purified samples.

Guanidinylation of the primary amino groups slightly increased the hydrophobicity of the corresponding ONs, as detected by minor changes in RP-HPLC retention time. We also attempted to evaluate the change in binding affinity towards target RNA in converting an amino group into a guanidinium group. As ON-1 has the tendency to form both duplexes and triplexes with its target strand, the thermal melting analysis was carried out only with the G-clamp-containing ON-2, ON-5 and their guanidinylated analogues ON-3 and ON-6. All T_m experiments were carried out in buffer containing 100 mM NaCl, 10 mM phosphate and 0.1 mM EDTA, pH 7.0.

The melting experiments with the self-complementary sequences ON-5 and ON-6 were performed without an additional target strand and the transition temperature of their helix to coil conversion could not be determined (T_m >95°C). However, this observation certainly confirmed the intrinsic high affinity nature of the G-clamp modification and its guanidinium analogue. The corresponding unmodified DNA shows a T_m <50°C. The T_m analysis of guanidinylated ON-3 in comparison with ON-2 did not show the additional increase in affinity expected for the formation of an additional (5th) hydrogen bond. With this particular sequence setup, in which the G-clamp modification is flanked by two Cs and the target strand had a five base overhang at its 5′-end, the guanidinylation resulted in a decrease in T_m for RNA and DNA target strands of 5.9° (64.9 versus 70.8°C) and 5.7° (53.5 versus 59.2°C), respectively. A more detailed study of the hybridization properties of G-clamp oligonucleotides and their guanidinylated counterparts is in progress.

In summary, two methods for postsynthetic modification of oligonucleotides have been developed. Both involve the conversion of primary amino functions into guanidinium groups by using 1*H*-pyrazole-1-carboxamidine hydrochloride. Although this reagent is known to react with aromatic amines under forcing conditions, the exocyclic amines of the nucleobases were not affected under the conditions used in the present work. For conversion on the solid support, the amino groups were protected by Alloc, which can be selectively removed without cleaving the ON from the support, and the guanidinylation was carried out in 10% DIEA in DMF. For postsynthetic guanidinylation in aqueous solution, primary amino groups were protected with Tfa, which can be readily removed under the conditions of oligonucleotide deprotection and cleavage. Using these methods several modified ONs bearing guanidinium moieties, located in either the minor or major groove, have been prepared and analyzed. The detailed binding affinity and other antisense properties of these modifications are being evaluated.

Table 1. Sequence and modification of the oligonucleotides synthesized

ON	Sequence 5′→3′	Modification	MW_{calcd}	MW_{found}
ON-1	TTT TU*T TTT T	All PO[a]; U*: 2′-*O*-hexylguanidinium-U^{5me}	3281.6	3281.7
ON-2	TCT CC*C TCT C	All PO; C* = 2′-deoxy-G-clamp	3039.1	3039.4
ON-3	TCT CC*C TCT C	All PO; C* = 2′-deoxy-guanidinium-G-clamp	3081.1	3080.8
ON-4	CTC GTA CCC* TCC CGG TCC	All PO; C* = 2′-deoxy-guanidinium-G-clamp	5553.7	5552.1
ON-5	GC*G TAU_M ACGC	All PO; U_M = 2′-*O*-MOE[b]-U^{5me}; C* = 2′-deoxy-guaninidium G-clamp	3293.3	3292.8
ON-6	GCG TAU_M AC*GC	All PO; U_M = 2′-*O*-MOE-U^{5me}; C* = 2′-deoxy-guanidinium-G-clamp	3293.3	3293.0

[a] Phosphodiester backbone.
[b] 2′-*O*-Methoxyethyl.

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References

1. Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1993**, *49*, 6123–6194.
2. Cook, P. D. *Annu. Rep. Med. Chem.* **1998**, *33*, 313–325.
3. Goodchild, J. *Bioconjugate Chem.* **1990**, *1*, 165–187.
4. Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 543–584.
5. Letsinger, R. L.; Singman, C. N.; Histand, G.; Salunkhe, M. *J. Am. Chem. Soc.* **1988**, *110*, 4470–4471.
6. Guinosso, C. J.; Hoke, G. D.; Freier, S. M.; Martin, J. F.; Ecker, D. J.; Mirabelli, C. K.; Crooke, S. T.; Cook, P. D. *Nucleoside Nucleotides* **1991**, *10*, 259–262.
7. Manoharan, M.; Guinosso, C. J.; Cook, P. D. *Tetrahedron Lett.* **1991**, *32*, 7171–7174.
8. Hashimoto, H.; Nelson, M. G.; Switzer, C. *J. Am. Chem. Soc.* **1993**, *115*, 7128–7134.
9. Manoharan, M.; Tivel, K. L.; Andrade, L. K.; Cook, P. D. *Tetrahedron Lett.* **1995**, *36*, 3647–3650.
10. Chaturvedi, S.; Horn, T.; Letsinger, R. L. *Nucleic Acids Res.* **1996**, *24*, 2318–2323.
11. Horn, T.; Chaturvedi, S.; Balasubramaniam, T. N.; Letsinger, R. L. *Tetrahedron Lett.* **1996**, *37*, 743–746.
12. Manoharan, M.; Ramasamy, K. S.; Mohan, V.; Cook, P. D. *Tetrahedron Lett.* **1996**, *37*, 7675–7678.
13. Cuenoud, B.; Casset, F.; Husken, D.; Natt, F.; Wolf, R. M.; Altmann, K.-H.; Martin, P.; Moser, H. E. *Angew. Chem., Int. Ed.* **1998**, *37*, 1288–1291.
14. Potier, P.; Abdennaji, A.; Behr, J.-P. *Chem.-Eur. J.* **2000**, *6*, 4188–4194.
15. Schmid, N.; Behr, J.-P. *Tetrahedron Lett.* **1995**, *36*, 1447–1450.
16. Rana, V. S.; Ganesh, K. N. *Org. Lett.* **1999**, *1*, 631–633.
17. Rana, V. S.; Ganesh, K. N. *Nucleic Acids Res.* **2000**, *28*, 1162–1169.
18. Griffey, R. H.; Monia, B. P.; Cummins, L. L.; Freier, S.; Greig, M. J.; Guinosso, C. J.; Lesnik, E.; Manalili, S. M.; Mohan, V.; Owens, S.; Ross, B. R.; Sasmor, H.; Wancewicz, E.; Weiler, K.; Wheeler, P. D.; Cook, P. D. *J. Med. Chem.* **1996**, *39*, 5100–5109.
19. Teplova, M.; Wallace, S. T.; Tereshko, V.; Minasov, G.; Symons, A. M.; Cook, P. D.; Manoharan, M.; Egli, M. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 14240–14245.
20. Crooke, S. T. *Methods Enzymol.* **2000**, *313*, 3–45.
21. Manoharan, M. *Biochim. Biophys. Acta* **1999**, *1489*, 117–130.
22. Kawasaki, A. M.; Casper, M. D.; Prakash, T. P.; Manalili, S.; Sasmor, H.; Manoharan, M.; Cook, P. D. *Nucleosides Nucleotides* **1999**, *18*, 1419–1420.
23. Prakash, T. P.; Kawasaki, A. M.; Vasquez, G.; Fraser, A. S.; Casper, M. D.; Cook, P. D.; Manoharan, M. *Nucleosides Nucleotides* **1999**, *18*, 1381–1382.
24. Manoharan, M. In *Antisense Research and Applications*; Crooke, S. T.; Lebleu, B., Eds.; CRC Press: Boca Raton, 1993; pp. 303–349.
25. Lin, K.-Y.; Jones, R. J.; Matteucci, M. *J. Am. Chem. Soc.* **1995**, *117*, 3873–3874.
26. Lin, K.-Y.; Matteucci, M. D. *J. Am. Chem. Soc.* **1998**, *120*, 8531–8532.
27. Gerlt, J. A. *Compr. Nat. Prod. Chem.* **1999**, *5*, 5–29.
28. Gerlt, J. A. *Chem. Rev.* **1987**, *87*, 1079–1105.
29. Perreault, D. M.; Cabell, L. A.; Anslyn, E. V. *Bioorg. Med. Chem.* **1997**, *5*, 1209–1220.
30. Dixon, R. P.; Geib, S. J.; Hamilton, A. D. *J. Am. Chem. Soc.* **1992**, *114*, 365–366.
31. De Guzman, R. N.; Turner, R. B.; Summers, M. F. *Biopolymers* **1998**, *48*, 181–195.
32. Rich, A. *Chem. Bond* **1992**, 31–86.
33. Weiss, M. A.; Narayana, N. *Biopolymers* **1998**, *48*, 167–180.
34. Weiss, M. A. *Nature Struct. Biol.* **1998**, *5*, 329–333.
35. Luedtke, N. W.; Baker, T. J.; Goodman, M.; Tor, Y. *J. Am. Chem. Soc.* **2000**, *122*, 12035–12036.
36. Dempcy, R. O.; Almarsson, O.; Bruice, T. C. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 7864–7868.
37. Doronina, S. O.; Behr, J.-P. *Tetrahedron Lett.* **1998**, *39*, 547–550.
38. Robles, J.; Grandas, A.; Pedroso, E. *Tetrahedron* **2001**, *57*, 179–194.
39. Wilds, C. J.; Maier, M.; Tereshko, V.; Manoharan, M.; Egli, M. *Angew. Chem., Int. Ed.* **2002**, *41*, 115–117.
40. Barber-Poec'h, I.; Manoharan, M.; Cook, P. D. *Nucleosides Nucleotides* **1997**, *16*, 1407–1410.
41. Manoharan, M.; Prakash, T. P.; Barber-Peoc'h, I.; Bhat, B.; Vasquez, G.; Ross, B. S.; Cook, P. D. *J. Org. Chem.* **1999**, *64*, 6468–6472.
42. Bernatowicz, M. S.; Wu, Y.; Matsueda, G. R. *J. Org. Chem.* **1992**, *57*, 2497–2502.