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Efficient guanidination of the phosphate linkage towards cationic phosphoramidate oligonucleotides

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Abstract—An efficient postsynthesis method of guanidination of oligonucleotides was employed to introduce several guanidinium groups into internucleotide phosphoramidate linkages. The amino functions of aminobutylphosphoramidate links were converted to guanidine butylphosphoramidates using a solution of *O*-methylisourea hemisulfate in aqueous ammonia, in a short reaction time. The synthesis of various fully guanidylated oligonucleotides was successfully performed to provide a new class of cationic phosphoramidate oligonucleotides.
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The past ten years have witnessed an explosion in the design of nucleic acid analogues to improve antisense or antigene properties of natural oligonucleotides (ON). The binding affinity to DNA or RNA complementary targets while maintaining fidelity of base recognition, resistance to degradation by nucleases, and effective cellular penetration are requisite parameters for therapeutic applications. In order to obtain efficient analogues, numerous modifications were introduced in nucleobase, sugar or phosphodiester backbone moieties.[1,2]

Among all the possibilities, one way to increase duplex and triplex stabilities is to reduce the overall negative charge number of modified ON to decrease the negative charge to charge repulsion between the two or three strands of the complexes. In recent years, approaches involving the incorporation of positive charges in ON have been developed.[3–6] In particular, the guanidinium group which is highly basic (pK_a 12.5) introduces a positive charge over a wide pH range and is able to form intermolecular contacts mediated by H-bonding or electrostatic interactions.

The syntheses of a few guanidinium derivatives of ON have been described in the literature. The deoxyribonucleic guanidine (DNG) oligomers[7,8] which were designed with a guanidinium backbone in place of phosphate groups form duplex and triplex structures with DNA or RNA that are much more stable at physiological ionic strength than the corresponding unmodified structures. Pedroso and co-workers[9] prepared modified ON containing 4-guanidino-2-pyrimidinone nucleosides as new analogues of protonated cytosine in the third strand in order to increase triplex stability at neutral pH. Similarly, the guanidino G-clamp[10] was designed as a cytosine analogue that forms five hydrogen bonds to guanosine. More recently, the guanidinium group was introduced at the 5-position of uracil in ON for duplex and triplex stabilization.[11] The 2′-position of a nucleotide was also targeted to incorporate guanidinium groups at the end of an hexyl linker.[12] However, the postsynthesis conversion of a primary amino function into a guanidinium group by treatment

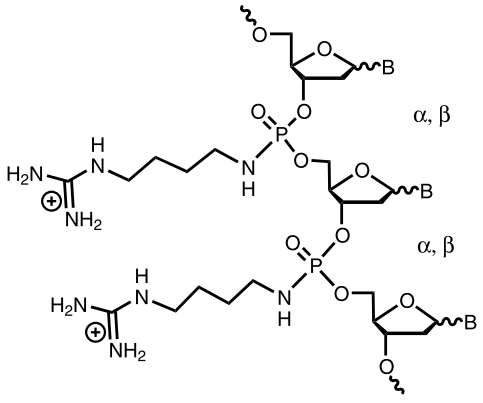

Figure 1. New cationic analogues: guanidinobutylphosphoramidate (PNHBuGua) β- or α-oligonucleotides.

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of these ON containing 5-(1-propargylamino)-2′-dU[11] or 2′-*O*-aminohexyl nucleosides[12] with 1*H*-pyrazole-1-carboxamidine hydrochloride was not quantitative. As a result, only one or a very few guanidinium groups have been positioned in these modified ON.

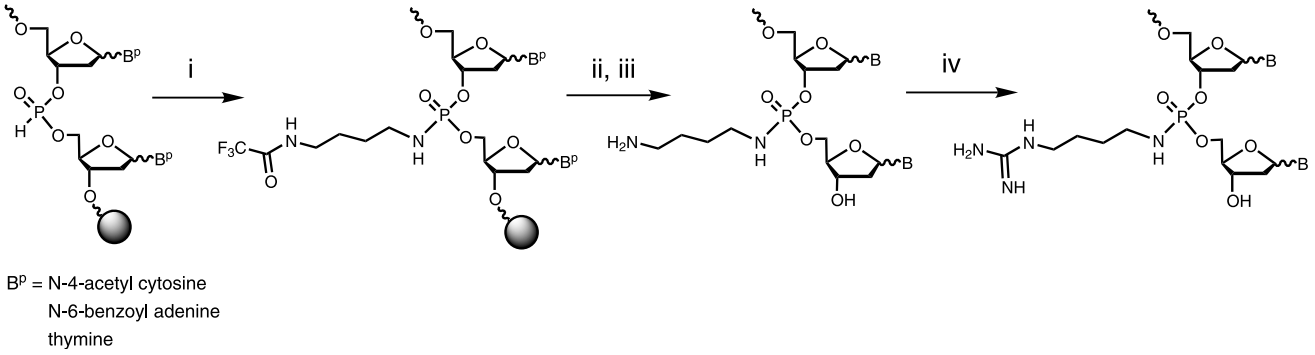
Our ongoing research is focused on the development of β- and α-anomeric ON in which the phosphodiester linkages are replaced by phosphoramidate diester linkages (PNHR).[13] Our goal in this area is to develop cationic PNHR α-oligonucleotides and particularly guanidylated ON (PNHBuGua) (Fig. 1) in order to prevent nuclease degradation and to improve hybridization with nucleic acid targets. Furthermore, the positive charges of guanidinium should help cell membrane permeation through possible electrostatic attractions of the oligonucleotides to the negatively charged phosphate groups of the cell surface. Here we report a very efficient postsynthesis method of guanidination of aminoalkylphosphoramidate internucleotide linkages to easily obtain ON with a large number of guanidinium moieties (Fig. 1).

First, as a model, we synthesized one fully aminobutylphosphoramidate ($PNHBuNH_2$) β-dT_{12} (ON 1, Table 1) which was solid-phase assembled using *H*-phosphonate chemistry.[14] At the end of the elongation, all the *H*-phosphonate diester linkages were manually oxidized with anhydrous CCl_4 in the presence of pyridine and *N*-1-trifluoroacetylbutyldiamine[15] (Scheme 1). In a first attempt, we have used diaminobutane in large excess without amine protection and we observed by HPLC analysis the presence of two side products which were further characterized by MALDI-TOF mass spectrometry (MS). The minor peak corresponds to a compound (m/z_{found} 8627.37) resulting from an intermolecular reaction with a butyl bridge between two ON strands whereas the major side product with a m/z_{found} of 4271.1 results from an intramolecular reaction with a butyl bridge between two adjacent phosphorus atoms. Thus, one primary amino function of the diaminobutane has been protected with trifluoroacetyl, 9-fluorenylmethoxycarbonyl or monomethoxytrityl groups. Among these protecting groups, the trifluoroacetyl group was chosen as it can be conveniently removed with the final ammonia treatment of the ON.

Table 1. Sequence of modified oligonucleotides synthesized

ON	Anomeric configuration	Sequence 5′→3′	Cationic phosphoramidates internucleotide linkages	MALDI-TOF MS (+) m/z_{calcd}	MALDI-TOF MS (+) m/z_{found}
1	β	T+T+T+T+T+T+T+T+T+T+T+T	(+) $PNHBuNH_2$[a]	4360.8	4359.4
2	β	T+T+T+C+T+T+C+C+T+C+T+T	(+) $PNHBuNH_2$	4300.8	4299.2
3	α	T+T+C+T+C+C+T+T+C+T+T+T	(+) $PNHBuNH_2$	4300.8	4299.9
4	α	T+C+T+T+A+A+C+C+C+A+C+A	(+) $PNHBuNH_2$	4321.8	4319.4
5	β	T*T*T*C*T*T*C*C*T*C*T*T	(*) PNHBuGua[b]	4763.2	4764.6
6	α	T*T*C*T*C*C*T*T*C*T*T*T	(*) PNHBuGua	4763.2	4766.9
7	α	T*C*T*T*A*A*C*C*C*A*C*A	(*) PNHBuGua	4784.3	4782.2

[a] Aminobutylphosphoramidate backbone.
[b] Guanidinobutylphosphoramidate backbone.



Scheme 1. Synthesis of guanidinobutylphosphoramidate α- or β-ON. *Reactions conditions* (1 μmol scale): (i) 10% *N*-1 trifluoroacetyldiaminobutane in carbon tetrachloride–pyridine (8/2 v/v) for 10 min at rt, (ii) 40% aq. CH_3NH_2/conc. aq. NH_3 (1/1), 55°C, 45 min, (iii) cation exchange HPLC purification, (iv) 160 μl of freshly prepared solution of 100 mg *O*-methylisourea hemisulfate in 100 μl water added to 250 μl of solution ON in 15% aq. NH_3, 65°C, 45 min.

Furthermore *N*-1-trifluoroacetylbutyldiamine[15] can be easily obtained as a very dried powder to minimize side PO oxidation of the ON. The $PNHBuNH_2$ heteropolymers 2–4 were synthesized in the same manner as the model β-dT_{12}.

Deprotection of β-dT_{12} was performed with the standard aqueous ammonia treatment (5 h, 50°C) without any problems. The same treatment was applied to the ON 2 with cytosine and thymine as nucleobases and we noticed that a side transamidation reaction occurred (HPLC chromatogram and MALDI-TOF MS (positive mode), data not shown). In the mass spectrum, the observed mass difference of 104 Da between peaks at *m*/*z* 4403.2 and *m*/*z* 4299.2 was attributed to the presence of a remaining benzoyl group on the molecule. An extended ammonia treatment did not eliminate the extra peak at *m*/*z* 4403.2 which let us assume that this benzoyl group did not protect the *N*-4 position of cytosine. Indeed, once the trifluoroacetyl group was removed, the amine of the butyl chain can deprotect a neighbouring base such as dA or dC and in doing so, be locked up in a non-hydrolysable benzamide linkage (HPLC chromatogram, approximately 30% peak combined area). To avoid such a side reaction, the $PNHBuNH_2$ ON 3 and 4 were deprotected with methylamine/ammonia (1/1 v/v) (AMA treatment)[16] at 55°C for 45 min (Scheme 1). However, this treatment requires the use of deoxycytidine 3′-*H*-phosphonate synthons protected with the acetyl group instead of the benzoyl group to prevent transamination with the *N*-4 primary amine. After deprotection, the $PNHbuNH_2$ ON 1–4 were purified by cationic exchange HPLC and characterized by MALDI-TOF MS (Table 1, Fig. 2A and 2B).

Numerous methods for producing guanidine compounds from amines were reported in the literature. Most of them were achieved with small organic molecules and amino acids or peptides; the most recent methods consist in the reaction of various guanidylating agents such as aminoiminomethanesulfonic acids,[17] *S*-methylisothiourea,[18,19] acylthiourea,[20,21] *O*-methylisourea[22,23] or 1*H*-pyrazole-1-carboxamidine hydrochloride[23,24] with amino functions. Among these reagents widely used for guanidination of peptides, only the last one has been recently tested for nucleic acids.[11,12] In both cases, the modified ON contained one or two guanidinium derivatives, certainly because the conversion of primary amino functions into guanidinium groups by using 1*H*-pyrazole-1-carboxamidine hydrochloride was not quantitative: 90% after 5 h for one guanidylated residue introduced in the modified ON[12] and 50–60% isolated yield of modified ON with one or two guanidylated residues after an overnight reaction.[11] In our case, the use of 1*H*-pyrazole-1-carboxamidine hydrochloride was not suitable to quantitatively convert eleven amino functions in the $PNHBuNH_2$ ON 1–3 into guanidino functions.

Therefore, our goal was to develop an improved method for guanidination of nucleic acids. The complete conversion of lysines to homoarginines in peptides or in digests of proteins was recently reported to be achieved with *O*-methylisourea hemisulfate in a short reaction time.[25] Although this guanidination reaction with *O*-methylisourea has been known for many years,[26] researchers have worked on this conversion to optimise guanidination protocols for MALDI mass mapping of proteins.[25,27–29]

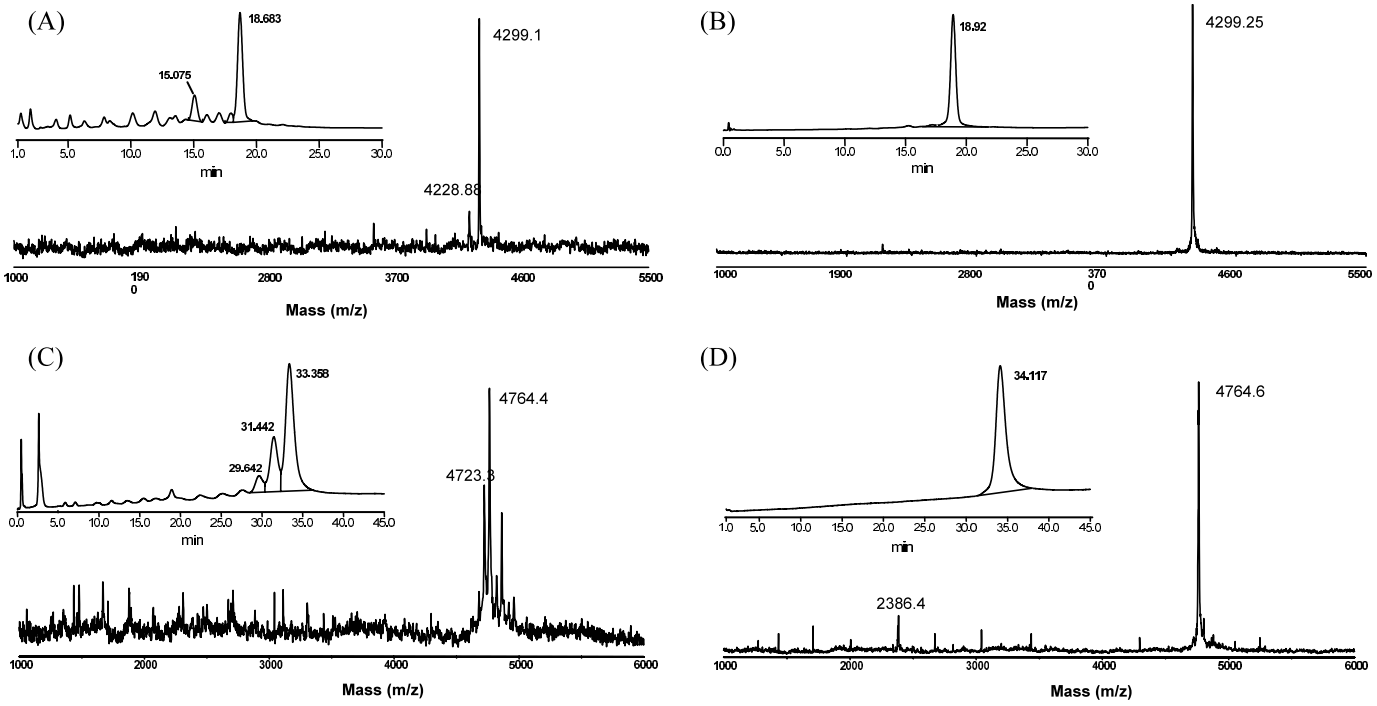


Figure 2. HPLC and MALDI-TOF MS analysis of: (A) crude synthesis mixture of $PNHBuNH_2$ ON 2; (B) purified ON 2; (C) crude synthesis mixture of PNHBuGua ON 5; (D) purified ON 5.

For guanidination of the purified $PNHBuNH_2$ ON 1–4, we applied the improved procedure described in the literature[25] with some minor modifications. The ON 1–4 (about 10 OD) were treated with a freshly prepared solution of *O*-methylisourea hemisulfate in aqueous ammonia (Scheme 1). The reaction mixtures were incubated for 45 min at 65°C in a water bath, then quenched at 0°C and finally partially dried in a speed-vac. The HPLC chromatogram and mass spectrum (Fig. 2C) of the crude guanidylated ON 5 show the presence of two extra peaks corresponding to an incomplete guanidination of one or two internucleotide linkages (approximately 33% peak combined area). The guanidination average yield by primary amino function was evaluated to 97% with ON 5 which contains eleven guanidinium residues. This yield is greatly higher than the guanidination yield obtained by using 1*H*-pyrazole-1-carboxamidine hydrochloride (90%)[12] and allows the introduction of several guanidinium groups in oligonucleotides. Furthermore, the side products were easily separated from the fully guanidylated compound 5 by cationic exchange HPLC (Fig. 2D). The same results were observed with ON 6 and 7. After purification and desalting through cartridges filled with polystyrene–divinylbenzene (PS/DVB) co-polymers, the desired ON 5–7 were obtained highly pure (Fig. 2D) and characterized by MALDI-TOF MS (Table 1).

The success of this guanidination reaction performed with ON strongly depends on reaction conditions. In particular, as already observed,[25] the use of *O*-methylisourea hemisulfate instead of *O*-methylisourea hydrogen sulfate was crucial for the complete conversion of amino groups into guanidine functions. Furthermore, a pH minimum of 10 and a temperature of 65°C are required for a total guanidination. Detrimental side effects, such as degradation of phosphoramidate backbones, were not observed certainly because the degradation is much slower than guanidination at this temperature. In several trials using a reaction time lower than 45 min, we found that the guanidination yield was less than 97%. Increasing the reaction time over 45 min did not improve this yield but induced degradation of ON.

In summary, we have presented an efficient method for postsynthesis conversion of primary amino functions into guanidinium groups, which was applied for the first time to modification of oligonucleotides. Several ON with eleven phosphoramidate internucleotide linkages ending with guanidinium moieties have been successfully prepared and characterized. Their binding properties to RNA and single- or double-stranded DNA and their ability to penetrate cells are being evaluated. In the same way, using this method, the conversion of primary amino groups to guanidinium groups on either the 2′-position of a nucleotide or the nucleobase is currently in process.

Acknowledgements

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References

1. Herdewijn, P. *Antisense Nucleic Acid Drug Dev*. **2000**, *10*, 297–310.
2. Kurreck, J. *Eur. J. Biochem*. **2003**, *270*, 1628–1644.
3. Bijapur, J.; de Keppler, M.; Bergqvist, S.; Brown, T.; Fox, K. R. *Nucleic Acids Res*. **1999**, *27*, 1802–1809.
4. Puri, N.; Majumdar, A.; Cuenoud, B.; Natt, F.; Martin, P.; Boyd, A.; Miller, P. S.; Seidman, M. M. *Biochemistry* **2002**, *41*, 7716–7724.
5. Chaturvedi, S.; Horn, T.; Letsinger, R. L. *Nucleic Acids Res*. **1996**, *24*, 2318–2323.
6. Dagle, J. M.; Weeks, D. L. *Nucleic Acids Res*. **1996**, *24*, 2143–2149.
7. Barawkar, D. A.; Bruice, T. C. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 11047–11052.
8. Reddy, P. M.; Bruice, T. C. *Bioorg. Med. Chem. Lett*. **2003**, *13*, 1281–1285.
9. Robles, J.; Grandas, A.; Pedroso, E. *Tetrahedron* **2001**, *57*, 179–194.
10. Wilds, C. J.; Maier, M. A.; Tereshko, V.; Manoharan, M.; Egli, M. *Angew. Chem., Int. Ed*. **2002**, *41*, 115–117.
11. Roig, V.; Asseline, U. *J. Am. Chem. Soc*. **2003**, *125*, 4416–4417.
12. Maier, M. A.; Barber-Peoc'h, I.; Manoharan, M. *Tetrahedron Lett*. **2002**, *43*, 7613–7616.
13. Laurent, A.; Naval, M.; Debart, F.; Vasseur, J.-J.; Rayner, B. *Nucleic Acids Res*. **1999**, *27*, 4151–4159.
14. Froehler, B. C. In *Methods in Molecular Biology. Protocols for Oligonucleotides and Analogs*; Agrawal, S., Ed.; Humana Press: Totowa, 1993; Vol. 20, pp. 63–80.
15. Agrawal, S.; Tang, J.-Y. *Tetrahedron Lett*. **1990**, *31*, 1543–1546.
16. Reddy, M. P.; Hanna, N. B.; Farooqui, F. *Tetrahedron Lett*. **1994**, *35*, 4311–4314.
17. Miller, A. E.; Bischoff, J. J. *Synthesis* **1986**, 777–779.
18. Bergeron, R. J.; McManis, J. S. *J. Org. Chem*. **1987**, *52*, 1700–1703.
19. Dumas, D. J. *J. Org. Chem*. **1988**, *53*, 4650–4653.
20. Poss, M. A.; Iwanowicz, E.; Reid, J. A.; Lin, J.; Gu, Z. *Tetrahedron Lett*. **1992**, *33*, 5933–5936.
21. Kim, K. S.; Qian, L. *Tetrahedron Lett*. **1993**, *34*, 7677–7680.
22. Callahan, J. F.; Ashton-Shue, D.; Bryan, H. G.; Bryan, W. M.; Heckman, G. D.; Kinter, L. B.; McDonald, J. E.; Moore, M. L.; Schmidt, D. B.; Silvestri, J. S.; Stassen, F. L.; Sulat, L.; Yim, N. C. F.; Huffman, W. F. *J. Med. Chem*. **1989**, *32*, 391–396.
23. Dal Pozzo, A.; Muzi, L.; Moroni, M.; Rondanin, R.; De Castiglione, R.; Bravo, P.; Zanda, M. *Tetrahedron* **1998**, *54*, 6019–6028.
24. Bernatowicz, M. S.; Wu, Y.; Matsueda, G. M. *J. Org. Chem*. **1992**, *57*, 2497–2502.
25. Beardsley, R. L.; Reilly, J. P. *Anal. Chem*. **2002**, *74*, 1884–1890.
26. Kimmel, J. R. *Methods Enzymol*. **1967**, *11*, 584–589.
27. Bonetto, V.; Bergman, A.-C.; Jörnvall, H.; Sillard, R. *Anal. Chem*. **1997**, *69*, 1315–1319.
28. Beardsley, R. L.; Karty, J. A.; Reilly, J. P. *Rapid Commun. Mass Spectrom*. **2000**, *14*, 2147–2153.
29. Keough, T.; Lacey, M. P.; Youngquist, R. S. *Rapid Commun. Mass Spectrom*. **2000**, *14*, 2348–2356.