Nonenzymatic Template-Directed Reactions on Altritol Oligomers, Preorganized Analogues of Oligonucleotides

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Abstract: Altritol nucleic acids (ANAs) are RNA analogues with a phosphorylated D-altritol backbone. The nucleobase is attached at the 2-(*S*)-position of the carbohydrate moiety. We report that ANA oligomers are superior to the corresponding DNA, RNA, and HNA (hexitol nucleic acid) in supporting efficient nonenzymatic template-directed synthesis of complementary RNAs from nucleoside-5′-phosphoro-2-methyl imidazolides. Activated ANA and HNA monomers do not oligomerize efficiently on DNA, RNA, HNA, or ANA templates.

Keywords: altritol nucleic acids · information transfer · oligomerizations · oligonucleotides · template synthesis

Introduction

Nonenzymatic synthesis of RNA oligomers from nucleoside 5′-phosphorimidazolides (Figure 1a) on RNA or DNA templates has been studied in detail.[1–14] Recently, we have reported nonenzymatic template-directed RNA synthesis on hexitol nucleic acids (HNAs, Figure 1b).[15–17] HNA oligomers, unlike the pRNAs studied by Eschenmoser and his coworkers,[18] form antiparallel duplexes with complementary DNA or RNA oligomers with structures that closely resemble that of the A form of double-stranded nucleic acids.[19–21] In general, HNA templates are superior to DNA and RNA templates with respect to efficiency and regioselectivity.[15–17]

Altritol nucleic acids (ANAs) are novel RNA analogues with a phosphorylated D-altritol backbone and a nucleobase at the 2-(*S*)-position of the carbohydrate residue (Figure 1b).[22] They can be considered as HNA analogues that have an additional hydroxy group introduced into the six-membered hexitol ring. In a duplex, this group is directed into the minor groove and contributes to stability of the duplex by increasing the hydration of the groove.[23] The hydroxy group may also help to pre-organize a helical single-stranded structure that is optimal for the formation of the A-type double helix.[23] ANA – RNA and ANA – DNA duplexes are more stable than the corresponding HNA hybrids.[23]

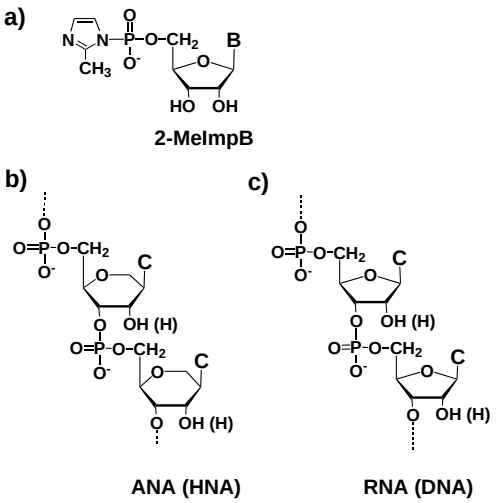

Figure 1. a) Structure of activated nucleoside 5′-phosphates (2-MeImpB), where B can be A, G, C, or U; b) structures of altritol nucleic acid (ANA) and hexitol nucleic acid (HNA) oligomers; c) structures of RNA and DNA oligomers.

In this work we compare the oligomerization of guanosine 5′-phosphoro-2-methylimidazole (2-MeImpG) on decacytidylate templates of ANA, HNA, RNA, and DNA. We also report experiments on information transfer from ANA heterosequences to RNA by nonenzymatic template-directed synthesis. The reaction conditions were chosen to facilitate direct comparison with previously published results. Unsuccessful attempts to generate long oligomers from activated hexitol and altritol monomers on various templates are also briefly reported.

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Results

Oligomerization of 2-MeImpG on ANA, HNA, RNA, and DNA C_{10} templates: In the absence of a template, an aqueous solution of 2-MeImpG yields as products only dimers and smaller amounts of trimers. The results obtained with an HNA, RNA, or DNA C_{10} template (Figure 2a–c) are analogous to those previously reported.[16] The major peaks

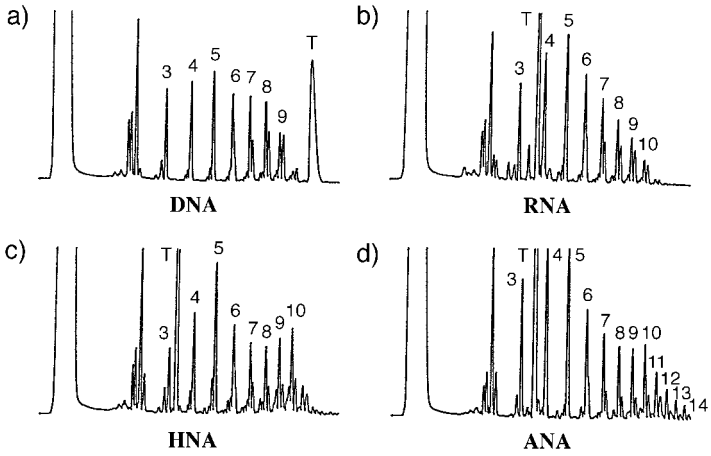

Figure 2. Elution profiles from an RPC5 column of the products from the oligomerization of 2-MeImpG on an ANA (d), HNA (c), RNA (b), and DNA (a) C_{10} templates after 14 days. The numbers above the peaks indicate the length of the all 3′–5′-linked oligo(G)$_n$ products, T indicates the template.

on the HPLC profiles correspond to all 3′-5′-linked oligo(G)$_n$s ranging in length from the dimer to the 10-mer; minor peaks correspond to oligomers that include 2′-5′-phosphodiester bands and/or 5′-terminal pyrophosphate caps. An ANA C_{10} template catalyzes efficient oligomerization of 2-MeImpG, generating significant amounts of G_n oligomers up to 14-mer (Figure 2d). The major products up to G_{10} formed on an ANA C_{10} template were shown by co-chromatography to be identical with oligo(G)$_n$s synthesized in a reaction on an RNA C_{10} template. The slow and non-regiospecific addition of the last G residue on RNA and DNA templates has been attributed to growing instability of the template–substrate double-helix as the 5′-terminus of the template is approached.[7, 8] Our present results show that ANA and HNA templates permit efficient chain elongation all the way to the 5′-terminus of the template.

In addition to oligomers up to G_{10}, we detected oligomers G_{11}–G_{14} among the products formed after two weeks on an ANA C_{10} template (Figure 2d). After four weeks, G_n oligomers up to the 25-mer were formed in appreciable yields (data not shown). We believe that this is due to "sliding" on the template, an effect which was described earlier.[7] We do not understand why sliding is stronger for an ANA template than for the corresponding DNA, RNA, or HNA templates.

An HNA D-C_{10} template is more enantioselective than the corresponding DNA or RNA template.[15] This finding provides a partial answer to the problem of enantiomeric cross-inhibition.[24, 25] An ANA D-C_{10} template has the same enantioselectivity as an HNA D-C_{10} template under our standard experimental conditions (data not shown).

Information transfer from ANA and HNA oligomers to RNA

A) Extension of a ^{32}p(dG)$_3$G primer on C_4XC_4 (X = G, U/T or A) ANA and HNA templates: The product distributions in the reactions of ^{32}P-labeled p(dG)$_3$G with 2-MeImpG or an equimolar mixture of 2-MeImpG with 2-MeImpC, 2-MeImpA, or 2-MeImpU on C_4XC_4 (X = G, U/T or A) ANA and HNA templates are shown in Figure 3. Significant

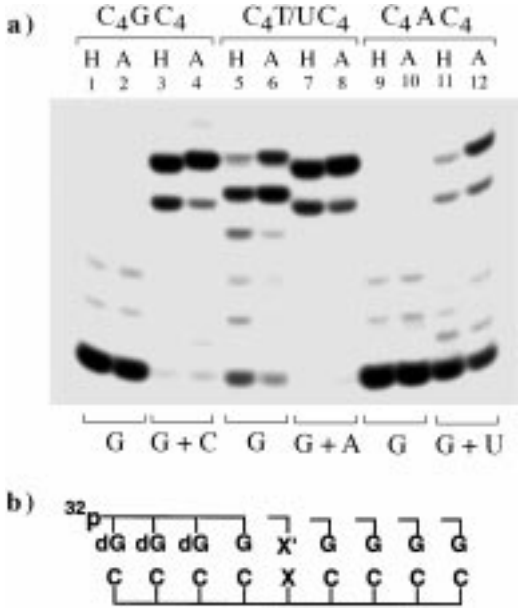

Figure 3. a) Extension of a ^{32}p(dG)$_3$G primer on ANA and HNA templates C_4XC_4 (X = G, U/T or A) after five days; A represents an ANA template, H represents a HNA template, G represents 2-MeImpG, G + C represents an equimolar mixture of 2-MeImpG and 2-MeImpC, etc. The fastest-moving band in the diagram corresponds to the ^{32}p(dG)$_3$G primer. b) Schematic representation of the primer ^{32}p(dG)$_3$G extension reaction with 2-MeImpG and 2-MeImpX′ on a C_4XC_4 template, X = G, U/T or A, and X′ is the complement of X.

extension of the primer p(dG)$_3$G with 2-MeImpG alone does not take place on C_4GC_4 or C_4AC_4 templates in either the ANA or the HNA series (Figure 3, lanes 1 and 2 and 9 and 10). In the presence of a C_4U/TC_4 template conversion of 90% (ANA template) and 60% (HNA template) of the primer to products p(dG)$_3$G(G)$_n$ up to octamer is observed (Figure 3, lanes 5 and 6), presumably due to G-U/T wobble pairing.[6]

The extension of the primer p(dG)$_3$G with an equimolar mixture of 2-MeImpG and 2-MeImpC in the presence of a C_4GC_4 ANA or HNA template leads to conversion of more than 95% of the primer to p(dG)$_3$GC(G)$_4$ and p(dG)$_3$GC(G)$_3$ products (Figure 3, lanes 3 and 4). However, the ratio between p(dG)$_3$GC(G)$_4$ and p(dG)$_3$GC(G)$_3$ products is about 9:1 in the case of a C_4GC_4 ANA template and only 1:1 in the case of a C_4GC_4 HNA template. The extension of the primer p(dG)$_3$G with an equimolar mixture of 2-MeImpG and 2-MeImpA on a C_4UC_4 ANA template or a C_4TC_4 HNA template leads to conversion of more than 95% of the primer to a mixture of p(dG)$_3$GA(G)$_4$ and p(dG)$_3$GA(G)$_3$ (Figure 3,

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lanes 7 and 8). The ratio between $p(dG)_3GA(G)_4$ and $p(dG)_3GA(G)_3$ products is about 9:1 in the case of a C_4UC_4 ANA template and about 1:1 in the case of a C_4TC_4 HNA template. Oligomers $p(dG)_3G(G)_n$ $(n=1-4)$ which are obtained with 2-MeImpG alone on C_4U/TC_4 ANA or HNA templates (see above) are not observed when a mixture of 2-MeImpG and 2-MeImpA is used (compare lanes 5–8 in Figure 3). The extension of the primer $p(dG)_3G$ with an equimolar mixture of 2-MeImpG and 2-MeImpU on a C_4AC_4 ANA template leads to conversion of about 30% of the primer to $p(dG)_3GU(G)_4$ and $p(dG)_3GU(G)_3$ products with a ratio of 3:2, while a C_4AC_4 HNA template catalyzes conversion of about 15% of the primer into $p(dG)_3GU(G)_4$ and $p(dG)_3GU(G)_3$ products with a ratio of 2:3 (Figure 3, lanes 11 and 12).

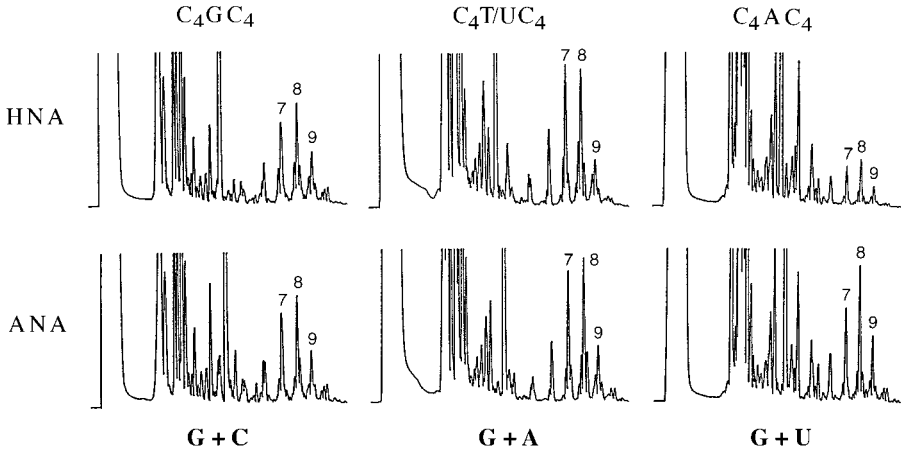

Figure 4. Elution profiles from an RPC5 column of the products from the oligomerization on ANA and HNA C_4XC_4 templates (X = G, U/T or A) an equimolar mixture of 2-MeImpG and 2-MeImpC **(G + C)**, an equimolar mixture of 2-MeImpG and 2-MeImpA **(G + A)**, or an equimolar mixture of 2-MeImpG and 2-MeImpU **(G + U)**. The reaction time was 14 days. The numbers above the peaks indicate the length of the all 3′–5′-linked oligoribonucleotide products.

The presence of predominantly 3′–5′ internucleotide bonds upstream and downstream of the X residue in $p(dG)_3GX(G)_n$ primer extension products (X = C, A or U) was confirmed by RNase hydrolysis. The CpG and UpG internucleotide bonds in $p(dG)_3GC(G)_n$ and $p(dG)_3GU(G)_n$ products were cleaved with more than 90% efficiency by RNase A, an enzyme which cleaves 3′–5′ internucleotide bonds after C and U residues. The ApG internucleotide bond in $p(dG)_3GA(G)_n$ oligomers was cleaved with more than 90% efficiency with RNase U_2, an enzyme which cleaves 3′–5′ internucleotide bonds after A residues. The GpX internucleotide bonds in $p(dG)_3GX(G)_n$ products were cleaved with RNase T_1, an enzyme which cleaves 3′–5′ internucleotide bonds after G residues, with more than 90% efficiency, for X = U, C or A.

B) Oligomerization of activated mononucleotides on C_4XC_4 (X = G, U/T or A) ANA and HNA templates: The presence of a C_4GC_4 or a C_4AC_4 ANA or HNA template leads to oligomerization of 2-MeImpG alone to give large amounts of G_4 and small amounts of G_5. The presence of a C_4UC_4 ANA or C_4TC_4 HNA template leads to oligomerization of 2-MeImpG to give products up to G_9 (data not shown). An equimolar mixture of 2-MeImpG and 2-MeImpC in the presence of a C_4GC_4 ANA or HNA template leads to the generation of G_4CG_n products up to a nonamer (Figure 4, left). An equimolar mixture of 2-MeImpG and 2-MeImpA in the presence of a C_4UC_4 ANA or C_4TC_4 HNA template leads to very efficient generation of G_4AG_n products up to a nonamer (Figure 4, center). Oligomers G_n which were obtained in the presence of 2-MeImpG alone on C_4UC_4 ANA or C_4TC_4 HNA templates are not observed when a mixture of 2-MeImpG and 2-MeImpA is used. An equimolar mixture of 2-MeImpG and 2-MeImpU in the presence of a C_4AC_4 ANA template leads to formation of G_4UG_n products up to a nonamer but on a C_4AC_4 HNA template the yield of these products is reduced by a factor of about two (Figure 4, right).

Reaction of activated altritol and hexitol monomers on ANA, HNA, RNA, and DNA C_{10} templates: The HPLC elution profiles of products formed from activated hexitol monomers (2-MeImpHG, Figure 5, left) and from activated altritol monomers (2-MeImpAG, Figure 5, right) on ANA and RNA C_{10} templates are shown in Figure 5. The results obtained on HNA and DNA C_{10} templates are almost identical to the results obtained on ANA and RNA templates, respectively (data not shown). The oligomerization of 2-MeImpHG and 2-MeImpAG is template dependent, but

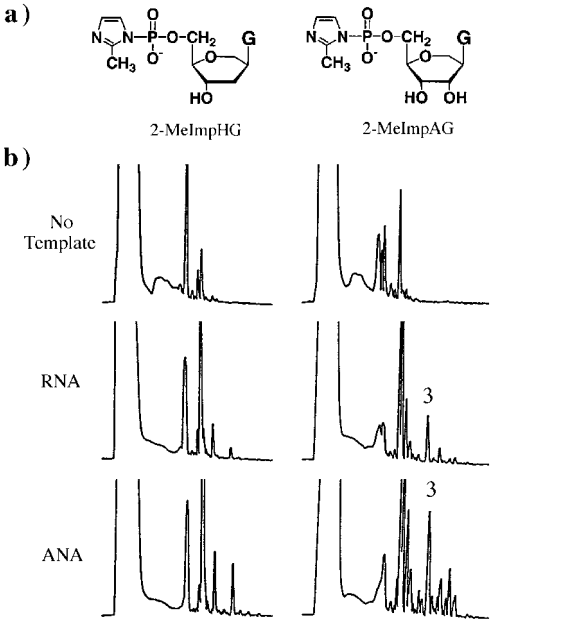

Figure 5. a) Structures of 2-MeImpHG and 2-MeImpAG. b) Elution profiles from an RPC5 column of the products from the oligomerization of 2-MeImpHG and 2-MeImpAG on an ANA or an RNA C_{10} template after seven days. The ANA and RNA C_{10} templates were cleaved with RNase A before analysis.

formation of oligomers longer than 4-mer in detectable amounts does not occur. The material obtained after oligomerization of 2-MeImpAG on an ANA C_{10} template and designated as peak "3" in the Figure 5 was collected and dialyzed. Hydrolysis by alkaline phosphatase and Zr^{4+} ions[16] shows that this material is a mixture of altritol oligomers pGpGpG and GppGpG in a ratio of about 3:2.

In further experiments we have found that activation of altritol and hexitol monomers with imidazole or 2-ethylimidazole does not lead to the formation of oligomers on any of the templates we tested. Furthermore, Pb^{2+}-catalyzed oligomerization[16, 26] is not successful in the case of activated altritol and hexitol monomers (data not shown).

Attempts to extend a ^{32}P-labeled $p(dG)_3G$ primer with 2-MeImpHG or 2-MeImpAG on a C_{10} DNA, RNA, ANA, or HNA templates led to the addition of one monomer to the primer with almost 100% efficiency. However, no longer oligomers were detected other than that in which one additional monomer was attached to the 5′-end, generating a pyrophosphate-capped product (data not shown).

Discussion

The results reported above confirm that HNA templates are superior to DNA and RNA templates in terms of efficiency and regiospecificity. We now find that the properties of ANA templates are qualitatively similar to those of HNA templates, but that ANA templates are quantitatively superior whenever differences can be detected. The difference is most marked for the least efficient steps in template-directed elongation, the addition of the last nucleotide in the oligomeric product on C_4XC_4 templates and the incorporation of a U residue opposite an A residue on the C_4AC_4 templates. In previously published work[6, 17, 27, 28] the incorporation of U opposite an A on a template has proved to be significantly less efficient than the corresponding incorporations opposite U or G. It is striking that copying proceeds past an A residue in ANA as efficiently as it proceeds past the other bases (Figure 4, center).

A more detailed comparison of the incorporation of U opposite A on an ANA template reveals an interesting difference between extension of a $(dG)_3G$ primer and synthesis of larger oligomers from activated ribonucleotide monomers. In the latter case, as we have seen, an A residue in the template is not a block for primer extension. However, addition of a U residue to the primer opposite A is significantly less efficient than other primer extension reactions. This indicates that G_4 is a much more efficient primer than $(dG)_3G$ with respect to addition of a U residue opposite A on an ANA template. A related effect on DNA and RNA templates has been reported.[13, 14] The authors suggested that the structure of the primer–template duplex has a substantial effect on the efficiency of the reaction, and that the duplexes with the A structure of nucleic acid double helices promote the most efficient primer extension.

Our experiments do not establish the reason for the superiority of ANA templates. However, they are consistent with the suggestion that pre-organization of a single-stranded oligomer into a helix the structure of which approximates to the A structure of DNA and RNA strongly favors template activity. There is independent evidence that HNAs and ANAs are extensively pre-organized into such a structure.[19, 20, 23]

It is perhaps surprising that activated HNA and ANA monomers are not good substrates in template-directed reactions, given that HNA and ANA oligomers are excellent templates. This finding demonstrates that the stability of a double-helical structure based on nucleotide analogues provides no guarantee that template-directed synthesis from monomers will be favored. The success of template-directed reactions depends on a favorable alignment of an hydroxy group of a primer with an activated phosphate group of a monomer. Stability of the double-helical product does not guarantee that the primer and the activated monomer will align correctly. The failure of template reactions in systems where the B form of DNA is stable illustrates this point.[13, 14, 16]

The results presented above reinforce the suggestion of Göbel and co-workers[13, 14] that the work of Wu et al[2, 5, 6] may represent a somewhat too pessimistic view on the prospects for nonenzymatic replication of RNA. In many earlier experiments, as in those reported above, the primer consisted of an oligodeoxynucleotide terminated by a single ribonucleotide residue and the template was an oligodeoxynucleotide. With this arrangement, the template–primer double helix may not adopt an A conformation around the 3′-terminus of the primer.[13, 14] Therefore, the primer extension reaction may be inhibited. Clearly, more detailed information transfer experiments with RNA primers and templates are now necessary, and they are underway.

Conclusion

HNA and ANA are nucleic acid analogues that are pre-organized to form double-helical complexes with an A-like nucleic acid structure. The superiority of these analogues to DNA and RNA as templates is thought to be due to this pre-organization. The superiority of ANA over HNA as a template correlates with the greater stability of ANA–RNA double helices.[23] This provides further evidence that template-directed reactions of nucleoside-5′-phosphoro-2-methylimidazolides are restricted to the A-type nucleic acid structure. [13, 14, 16]

The failure of activated monomers of the HNA and ANA to undergo efficient template-directed oligomerization emphasizes that not all monomers that form stable double-helical polymers are likely to undergo efficient template-directed oligomerization. Replication may be more demanding than helix formation.

Experimental Section

Unless otherwise noted, all chemicals were reagent grade, were purchased from commercial sources, and were used without further purification. Nucleotide 5′-phosphoro-2-methylimidazoles (2-MeImpB, B = G, C, A, U) were obtained by a published method[3] in at least 95% yield. 2-MeImpHG or 2-MeImpAG were obtained from corresponding monomers by a

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published method[3] in at least 90% yield. The oligodeoxyribonucleotides were synthesized and purified as described.[29] The altritol and hexitol nucleic acid templates and nucleosides were synthesized and purified as described.[20, 22, 23] The monophosphates of the altritol and hexitol nucleic acid nucleosides were synthesized according to the method of Yoshikawa et al.[30]

pAG: ^{31}P NMR (D_2O): $\delta =$ 1.870 (s); ^{13}C NMR (D_2O):$\delta = 159.52$ (C-6), 154.32 (C-2), 152.43 (C-4), 139.34 (C-8), 116.22 (C-5), 76.47 (d, $^3J_{c,P} =$ 7.8 Hz, C-5′), 68.22 (C-3′), 65.20 (C-4′), 64.61 (br., C-6′), 64.12 (C-1′), 55.34 (C-2′); ^{1}H NMR (D_2O): $\delta = 3.897$ (dd, 1H, $J_{4',3'} = 3.2$ Hz, $J_{4',5'} =$ 8.2 Hz, H-4′), 4.032 (dt, 1H, $J_{5',4'} =$ 8.1 Hz, $J_{5',6'a+b} =$ 3.8 Hz, H-5′), 4.185 (dd, 2H, $J_{6',5'} =$ 3.9 Hz, $J_{6',P} = 5.6$ Hz, H-6′$_{a+b}$), 4.273 (d, 2H, $J =$ 3.2 Hz, H-1′), 4.362 (dd, 1H, $J_{3',2'} = 4.7$ Hz, $J_{3',4'} = 3.3$ Hz, H-3′), 4.561 (dt, 1H, $J_{2',3'} =$ 4.6 Hz, $J_{2',1'a+b} = 3.2$ Hz, H-2′), 8.120 (s, 1H, H-8).

pHG: ^{31}P NMR (D_2O): $\delta = 1.843$ (s); ^{13}C NMR (D_2O): $\delta = 159.44$ (C-6), 154.25 (C-2), 152.95 (C-4), 139.55 (C-8), 116.22 (C-5), 81.60 (d, $^3J_{c,P} =$ 8.1 Hz, C-5′), 68.92 (C-1′), 64.90 (d, $J = 4.3$ Hz, C-6′), 61.97 (C-4′), 51.13 (C-2′), 35.35 (C-3′); ^{1}H NMR (D_2O): $\delta = 1.935$ (m, 1H, H-3′a), 2.338 (br. d, 1H, $J = 12.0$ Hz, H-3′b), 3.500 (br., 1H, H-4′), 3.699 (br., 1H, H-5′), 3.929 (d, 1H, $J = 12.5$ Hz, H-1′A), 4.010 (br., 1H, H-6′a), 4.057 (br., 2H, H-6′b and H-2′), 4.206 (d, 1H, $J = 12.5$ Hz, H-1′b), 8.000 (s, 1H, H-8).

The oligomerization reactions of D-2-MeImpG (or its mixture with equal amount of L-2-MeImpG) on all-D DNA, RNA, HNA, or ANA C_{10} templates were run for 14 days at 0 °C in 0.2 M 2,6-lutidine-HCl buffer (pH 7.9 at 25 °C) containing 1.2 M NaCl, 0.2 M $MgCl_2$, 0.5 mM of a template and 0.05 M 2-MeImpG (or 0.05 M D-2-MeImpG and 0.05 M L-2-MeImpG). The oligomerization reactions of 2-MeImpHG or 2-MeImpAG on DNA, RNA, HNA, or ANA C_{10} templates were run for seven days at 0 °C in 0.2 M 2,6-lutidine-HCl buffer (pH 7.9 at 25 °C) containing 1.2 M NaCl, 0.2 M $MgCl_2$, 0.5 mM of a template and 0.1 M 2-MeImpHG (or 2-MeImpAG). Reaction conditions for the oligomerization of 2-MeImpG (or its mixture with equal amount of 2-MeImpC, 2-MeImpA, 2-MeImpU) on HNA or ANA C_4XC_4 templates were chosen to permit comparison with earlier published work.[28, 29] One set of reactions was run for 14 days at 0 °C in 0.2 M 2,6-lutidine-HCl buffer (pH 7.9 at 25 °C) containing 1.2 M NaCl, 0.2 M $MgCl_2$, 0.5 mM of a template and 0.1 M 2-MeImpG. In the second set of reactions the solution described above contained not only 0.1 M 2-MeImpG but also 0.1 M of the activated nucleotide complementary to X in the corresponding C_4XC_4 template. The reaction mixtures were analyzed by HPLC on an RPC5 column as previously described.[29] An RNA C_{10} template was cleaved with RNase A before analysis as described.[3] We used the same conditions to cleave an ANA C_{10} with RNase A.

Reaction conditions for $p(dG)_3G$ primer extension with 2-MeImpG (or its mixture with an equal amount of 2-MeImpC, 2-MeImpA, or 2-MeImpU) on different templates were again chosen to permit comparison with earlier published work.[28, 29] One set of reactions was incubated for five days at 0 °C in 0.2 M 2,6-lutidine buffer (pH 7.9 at 25 °C) containing 1.2 M NaCl, 0.2 M $MgCl_2$, 20 μM of a template, 20 nM of the primer, and 50 mM 2-MeImpG, 2-MeImpHG, or 2-MeImpAG. In the second set of reactions the solution described above contained not only 50 mM 2-MeImpG but also 50 mM of the activated nucleotide complementary to X in the corresponding C_4XC_4 template. The reaction mixtures were analyzed by electrophoresis in 20% PAG containing 8 M urea as previously described.[28, 29] The reaction mixtures were desalted on Nensorb columns (Nen DuPont) prior to RNase digestion. Digestion with RNase T_1 (10U; Sigma), RNase U_2 (10U; Pharmacia) and RNase A (5U; Sigma) was carried out in 12 μL of 25 mM Na-citrate buffer containing 1 mM EDTA, 6 M urea and about 1 nM of total primer extension products. The reaction mixture was incubated at 50 °C for 30 min at pH 5.0 (RNases T_1 and A) or pH 3.5 (RNase U_2). The reaction mixtures were analyzed by electrophoresis in 20% PAG containing 8 M urea.[28, 29]

Acknowledgments

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