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A Non-Enzymatic, DNA Template-Directed Morpholino Primer Extension Approach

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Non-enzymatic RNA- and DNA-template-directed synthesis of oligonucleotides has been a focus of research for many years, particularly in the context of prebiotic chemistry. Notably, 5′-phosphorimidazolide-activated nucleotides can be assembled on nucleic acid templates to produce complementary oligonucleotide products.[1–3] In addition, modified systems including PNA,[4] have been used as templates for the non-enzymatic assembly of complementary oligonucleotides, by using similar 5′-activated nucleotides.[5] More recently, helper strands have been used to increase the selectivity and rates of non-enzymatic DNA primer extension reactions,[6] allowing detection of single-nucleotide polymorphisms with 5′-activated nucleotides labelled with fluorophores.[7] One problem associated with the use of 5′-activated nucleotides in the template-directed, non-enzymatic assembly of oligonucleotides is the fact that the formation of the phosphodiester bond is kinetically controlled and essentially irreversible. Of course nature has overcome this problem through the evolution of polymerases with additional hydrolytic proofreading function. However, in the absence of a proofreading polymerase, mismatches inevitably accumulate and can lead to complex mixtures of truncated products. In the case of ribonucleotides the possibility of forming 2′→5′ phosphodiester bonds can further complicate product analysis.[8] To overcome problems associated with the formation of phosphodiester bonds, thermodynamically controlled imine formation has been explored. For example, thymidine monomers with 5′-NH_2 and 3′-CH_2CHO groups were assembled on a $(dA)_8$ template and the imine-linked oligomers were trapped by $NaCNBH_3$ to give a polyamine product.[9] Whilst this approach has not been extended to include the other three nucleoside monomers (A, C and G), similar reductive amination chemistry was used to effect DNA-template-directed assembly of PNA tetramers, with *N*-terminal free amino and *C*-terminal aldehyde groups.[10]

In this paper we introduce an alternative approach towards the non-enzymatic transcription of DNA into complementary "non-natural" morpholino-amide oligomers (Scheme 1a). This system was investigated because a range of morpholine-based oligonucleotide mimics have been shown to form stable duplexes with complementary nucleic acids.[11] Also, unlike the well known phosphorodiamidate morpholino oligonucleotides (PMOs), which are now widely used antisense agents,[12] the amide-linked morpholino oligomers can potentially be derived through a reductive amination extension reaction between a 5′-glycinyl monomer or oligomer and the dialdehyde products formed from periodate oxidation of ribonucleosides (Scheme 1a).[13] Whilst a wide range of morpholines has been prepared, mainly under anhydrous conditions, from ribose derivatives through periodate oxidation followed by reductive amination, typically the reported yields are modest.[14] To address this issue, we explored the oxidation of ribonucleosides and subsequent reductive amination with benzylamine in aqueous buffer solution under conditions that would be compatible with DNA-template-directed synthesis. Reactions with equimolar ratios of dialdehydes and amines give modest yields of products. However, if an excess (5 mol equiv) of dialdehyde is formed in situ from ribonucleosides (rA, rT, rC and rG) with $NaIO_4$ and the amine added immediately, along with $NaCNBH_3$, then *N*-benzyl morpholine products can be produced in nearly quantitative yield based on the amine (Scheme 2a). In fact, by using this one pot procedure, excellent yields can be achieved with as little as two molar equivalents of the dialdehyde relative to benzylamine. Presumably the low yields previously reported are based on the dialdehyde and can be accounted by the decomposition, including over-oxidation, of the reactive intermediates derived from treatment of ribonucleosides with periodate.[15] In light of this observation the DNA-template-directed morpholino extension of a synthetic primer was explored, by using an excess of the ribonucleoside-derived dialdehydes (Scheme 1b).

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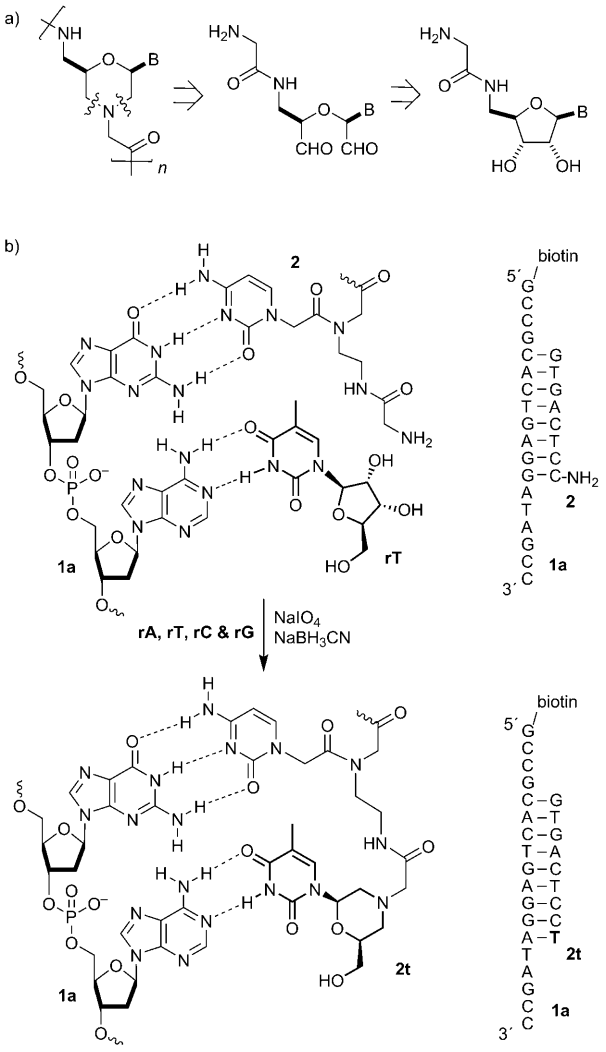
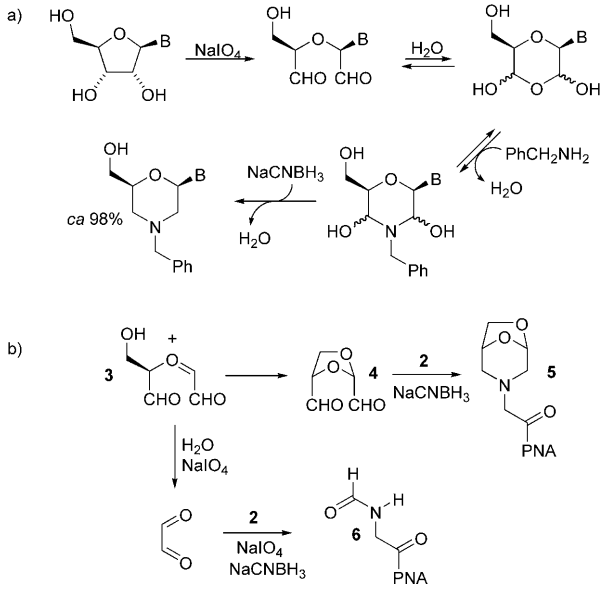


Scheme 1. a) Retrosynthetic disconnection of morpholino amide oligomers. b) The morpholino primer extension strategy. DNA template **1a**, hybridised with a PNA primer **2**, selectively binds complementary ribonucleoside rT, by Watson–Crick base paring. Subsequent periodate oxidation results in the dialdehyde, which reacts with the N-terminal glycine PNA primer **2** followed by in situ $NaCNBH_3$ reduction to give the morpholine extension product **2t**.

For synthetic expediency a PNA primer with an N-terminal glycinyl group, NH_2-Gly-PNA(CCT CAG TG)-$CONH_2$ (**2**), was prepared by using standard Boc-PNA protocols. Four DNA-template sequences were also designed: biotin-5′-GCC GCA CTG AGG **B**TA GCC-3′ where B is A, T, C or G (**1a**, **1t**, **1c** or **1g**). Each template possesses a complementary antiparallel PNA hybridisation sequence (underlined), with a variable base opposite the PNA *N*-glycinyl extension site (Scheme 1 b). The 5′-biotin label is also included to aid capture of the template and extension products. Initially, the non-competitive morpholino extension reactions with PNA **2** and dialdehydes derived from rC, rT, rA and rG were investigated, in the absence of a DNA template, by using the optimised in situ reductive amination procedure described above. The subsequent MALDI MS experiments



Scheme 2. a) Model periodate oxidation and reductive amination reactions with ribonucleosides 5-methyluridine (rT), adenosine (rA), cytidine (rC) or guanosine (rG) (1.93 mmol), $NaIO_4$ (3.9 mmol), benzylamine (0.39 mmol), $NaCNBH_3$ (7.7 mmol), in KH_2PO_4 buffer (30 mL, 500 mM, pH 7.0) for 6 h give morpholine products in about 98% yield based on benzylamine. Reactions are likely to proceed via a dialdehyde, hydrated cyclic acetal, carbanolamine and other intermediates. b) Possible degradation products resulting from depurination and overoxidation of ribonucleosides. MALDI MS: *m*/*z*: calcd for **5**: 2317.2; found: 2317.0 $[M+H]^+$; calcd for **6**: 2247.1; found, 2247.3 $[M+H]^+$.

approved the formation of PNA–morpholino extension products (**2a**, **2t**, **2c** and **2g**). However, the excess of salt from the buffer solution and other reagents complicated the quality of the spectra. To overcome this, the reactions were repeated with the PNA hybridised to the four templates. After reductive amination with the complementary nucleoside dialdehyde, the resulting primer extension products remained hybridised to the DNA template. The DNA/PNA complex could then be captured on avidin agarose resin and the resin washed extensively to remove excess salts and reagents. Denaturation of the primer extension products from the DNA templates, at 85 °C, resulted in samples that gave very clean and reproducible MALDI spectra, which were consistent with calculated masses of the extension products **2a**, **2t**, **2c** and **2g** (Figure S1 in the Supporting Information). By using NH_4F for preparing the MALDI matrix it was also possible to gain reproducible and accurate MALDI spectra free of Na^+ and K^+ adducts.[16] Moreover, the intensities of the $[M+H]^+$ ions in the MALDI MS were proportional to the ratio of the extension products, presumably because the protonated product ions have similar global ionisation efficiency.[17]

Internally calibrated MALDI MS[17] was therefore adopted as the method of choice for determination of product ratios from competitive template extension reactions. Accordingly, an equimolar mixture of rA, rT, rC and rG was oxidized with periodate and the mixture was incubated with

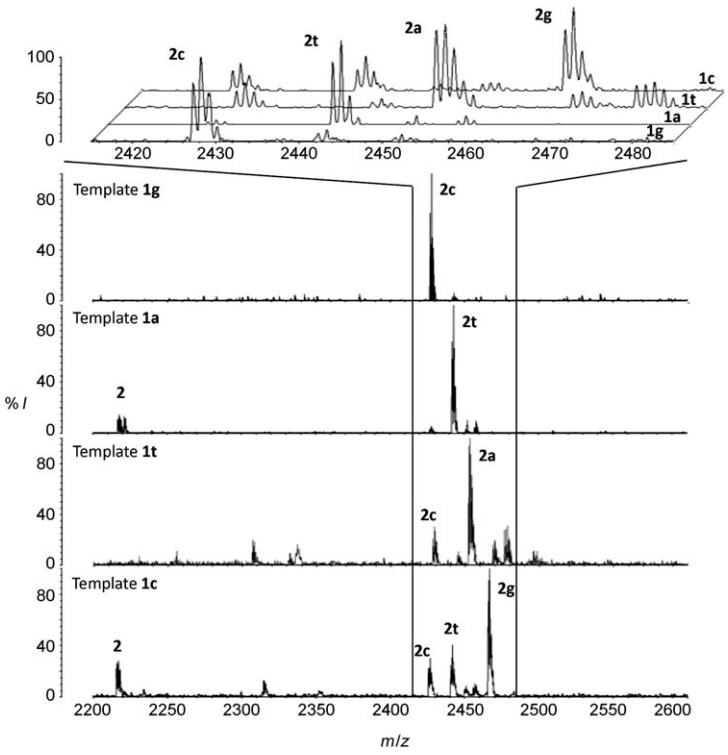


Figure 1. MALDI MS spectra of competitive primer extension reactions. PNA **2** (1.0 nmol) and the biotinylated DNA template **1a**, **1t**, **1c** or **1g** (2.5 nmol) were annealed for 1 h in NaH_2PO_4 buffer solution (50 μL, 250 mM, pH 7.0). A mixture of the ribonucleosides rA, rT, rC and rG (0.625 μmol of each) was added and the mixture was equilibrated for 4 h in NaH_2PO_4 buffer solution (100 μL, 250 mM, pH 7.0), before addition of $NaIO_4$ (2.5 μmol) and then $NaCNBH_3$ (2.5 μmol) each separately dissolved in NaH_2PO_4 buffer solution (50 μL, 250 mM, pH 7.0). MALDI MS: *m/z*: calcd for **2c**: 2428.3; found: 2428.4 $[M+H]^+$; calcd for **2t**: 2443.3; found 2443.2 $[M+H]^+$; calcd for **2a**: 2452.3; found: 2452.4 $[M+H]^+$; calcd for **2g**: 2468.3; found: 2468.5 $[M+H]^+$.

each of the four DNA templates and primer **2**. Reductive amination, work up and MALDI MS analysis, showed that the extension products **2c**, **2t** and **2a** were clearly the major products obtained from complementary DNA templates **1g**, **1a** and **1t**, respectively (Figure S2 in the Supporting Information). However, only 34% of the **2g** extension products was obtained from the complementary template **1c**, with mismatch products **2c** and **2t** also present in 27 and 33%, respectively. It is also evident from the competitive reaction with the template **1c** that two major side products $m/z=$ 2317 and 2247 as well as unreacted primer **2** are present. The mass of the side product $m/z=2317$ is consistent with depurination of guanosine (rG), or its oxidation product, to generate an oxonium ion **3** that can cyclise to give the 5-membered acetal **4** that under conditions of reductive amination results in primer *N*-capped side product **5** (Scheme 2b). The mass of the other side product, $m/z=2247$, is consistent with an *N*-formyl PNA **6**, which could be derived from reaction of **2** with glyoxal or alternative products of nucleoside overoxidation.[15] Indeed, when PNA primer **2** was reacted with glyoxal, periodate and $NaCNBH_3$ a product of $m/z=2247$ is similarly formed (Figure S3 in the Supporting Information). The low selectivity of template **1c**, combined with greater intensity of side products **5** and **6** (Figure S2 in the Supporting Information), could thus be explained by the greater propensity of guanosine and/or its oxidation products to depurinate and overoxidise during templating. Nevertheless, by decreasing the amount of $NaIO_4$ used in the initial oxidation to one equivalent relative to the ribonucleosides it was possible to reduce formation of the overoxidation side product **6** to a large extent. A series of experiments were undertaken to optimise conditions with the problematic template **1c** and to further explore how sequence selectivity is governed during the morpholino primer extension reaction. Firstly, equilibration of the ribonucleosides with the primer–template complex (**2**–**1c**) prior to oxidation was explored. Accordingly, a mixture of the four ribonucleosides was incubated with the primer–template complex and $NaIO_4$ was added after a period of 0, 2 and 4 h. MALDI MS spectra, subsequent following reductive amination, clearly showed that the longer the equilibration time the greater the selectivity, with the proportion of the complementary product **2g** increasing to 55% (Figure S4 in the Supporting Information). No further increase in selectivity was observed when equilibration was extended beyond 4 h. Conversely, if the ribonucleosides are treated with $NaIO_4$ for 2 h prior to incubation with the primer–template complex (**2**–**1c**), essentially all selectivity is lost (Figure S4 in the Supporting Information). Moreover, the side product **5** becomes prominent, supporting the idea that ribonucleoside oxidation products are more prone to depurination.

A second series of experiments were carried out to determine if selectivity is affected by the length of time that the dialdehydes are allowed to equilibrate with the primer–template complex, prior to reduction with $NaCNBH_3$. In this case the template **1g** was used, which had already been shown to give very good selectivity for primer extension product **2c**. Accordingly, the ribonucleoside mixture and the primer–template complex (**2**–**1g**) were equilibrated for 4 h, before the reaction was initiated with $NaIO_4$. The $NaCNBH_3$ was then added after a period of 0, 2, or 4 h. From this it was apparent that increasing the time before addition of $NaCNBH_3$ from 0 to 4 h decreases selectivity for the **2c** product from 82 to 66%, respectively, and also increases the yield of side products **5** and **6** (Figure S5 in the Supporting Information). Thus, it is clear that template selectivity is governed by non-covalent interaction of the ribonucleosides with the primer–template complex. Furthermore, extended equilibration of the ribonucleoside oxidation products with the primer–template complex prior to $NaCNBH_3$ reduction of the covalent imine intermediates serves to reduce selectivity and increases side-product formation. Fully optimised conditions established a full set of primer extension reactions that were carried out with the four ribonucleosides on the templates **1g**, **1a**, **1t** and **1c** resulting in the formation of the extension products **2c**, **2t**, **2a** and **2g** with selectivities of 95, 86, 70 and 55%, respectively (Figure 1 and Table 1). Selectivities are high except for extension with guanosine.

Table 1. Product ratios for competitive primer extension reactions.

Template	Ribonucleosides[a] **2c/2t/2a/2g**	NMPs[b] **2c^p/2u^p/2a^p/2g^p**	Biased NMPs[c] **2c^p/2u^p/2a^p/2g^p**
1g	**95**:5:0:0	**97**:0:3:0	**94**:0:5:0
1a	5:**86**:9:0	0:**77**:19:4	0:**85**:14:0
1t	16:5:**70**:10	13:13:**72**:2	0:0:**93**:7
1c	17:23:5:**55**	18:18:0:**64**	7:7:0:**86**

In addition to depurination, we observed that guanosine has low solubility and can form hydrogels on oxidation with $NaIO_4$, presumably due to Na^+-mediated G-quadruplex formation.[18] These factors may contribute to lowering the effective concentration of free guanosine for template-directed extension. Guanosine 5′-monophosphate (GMP), on the other hand, is considerably more soluble in aqueous media and does not form a hydrogel on oxidation with $NaIO_4$. Therefore primer extension reactions were repeated by using the ribonucleoside 5′-monophosphates (adenosine 5′-monophosphate (AMP), uridine 5′-monophosphate (UMP), cytosine 5′-monophosphate (CMP) and GMP). Under identical conditions the extension reactions with ribonucleoside 5′-monophosphates gave selectivities of 97, 77, 72 and 64% for the primer extension products **2c^p**, **2u^p**, **2a^p** and **2g^p**, respectively (Figure S6 in the Supporting Information and Table 1). Notably, the selectivity for the template **1c**, although still lower than the other templates, increases from 55 for guanosine to 64% for GMP. The 5′-phosphoryl group presumably prevents formation of side product **5**. However, depurination and further oxidation still resulted in the side product **6**, which is most evident for reactions with the template **1c**. To further explore if degradation of the GMP oxidation products results in a lower effective concentration of the GMP-derived dialdehyde, the concentrations of the ribonucleoside 5′-monophosphates was biased from equimolar to 1.0:0.8:0.8:1.2 (AMP/UMP/CMP/GMP), in favour of GMP. This led to an increase in selectivity of 94, 85, 93 and 86% for the primer extension products **2c^p**, **2u^p**, **2a^p** and **2g^p**, respectively (Figure 2 and Table 1). This means for the first time template **1c** shows selectivity that is on par with the other templates. Indeed, these selectivities compare well with the best selectivities observed with similar non-enzymatic primer extension reactions by using standard 5′-activated nucleotides,[6,7] which had been developed and optimised over 30 years since Orgel first introduced this chemistry.[1] In addition, high selectivities are obtained with the morpholino approach without the aid of a "helper" strand.[6,7]

In summary, we have developed a new non-enzymatic DNA-template-directed synthesis approach that delivers morpholino nucleoside extension of a synthetic PNA primer with high sequence selectivity that is similar to those observed with the well established prebiotic chemistry inspired approaches.[6,7] Initially, we envisaged that exchange of the ribonucleoside dialdehydes with the *N*-terminal amino group of the PNA primer would control selectivity following the principles of covalent dynamic combinatorial chemistry.[19] However, this proved not to be the case as extended equilibration of the dialdehydes with the template–primer complex actually reduces selectivity. Instead, extending the equilibration time of the ribonucleosides with the template–primer complex prior to oxidation, was shown to increase selectivity in accordance with the Watson–Crick base-pairing rules. Presumably the subsequent oxidation of the ribonucleoside results in the formation of stable covalent adducts with the *N*-terminal amino group of the PNA primer, that do not exchange significantly before in situ reduction. From a practical perspective this approach could be further developed for use in the detection of single-nucleotide polymorphisms (SNPs). Finally, research in prebiotic chemistry has focused on alternative genetic systems, which may have acted as templates for the first synthesis of today's genetic material.[4,20] In contrast, the work described in this paper seeks to address the problem of how natures genetic information could be transcribed into the sequence of non-natural or abiological polymers.[21]

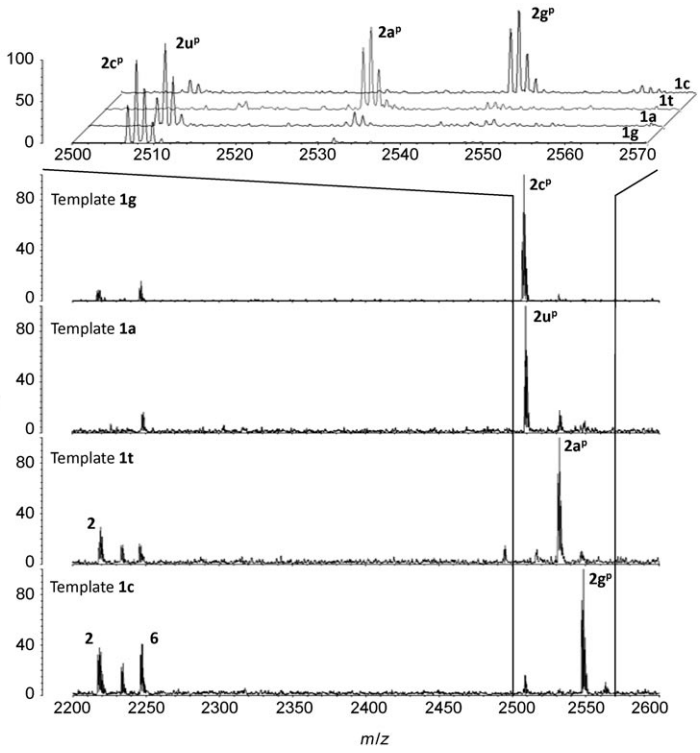


Figure 2. MALDI MS spectra of competitive primer extension reactions with biased ribonucleoside 5′-monophosphates. PNA **2** (1.0 nmol), biotinylated the DNA template **1a**, **1t**, **1c** or **1g** (2.5 nmol) were annealed for 1 h in NaH_2PO_4 buffer solution (50 μL, 250 mM, pH 7.0). AMP (0.625 μmol), UMP (0.50 μmol), CMP (0.50 μmol) and GMP (0.75 μmol) were added and the mixture was equilibrated for 4 h in NaH_2PO_4 buffer solution (100 μL, 250 mM, pH 7.0), before addition of $NaIO_4$ (2.38 μmol) and then $NaCNBH_3$ (2.5 μmol) each separately dissolved in NaH_2PO_4 buffer solution (50 μL, 250 mM, pH 7.0). MALDI MS *m*/*z*: calcd for **2c^p**: 2508.3; found: 2508.0 $[M+H]^+$; calcd for **2u^p**: 2509.3; found: 2509.1 $[M+H]^+$; **2a^p**: 2532.3; found: 2532.5 $[M+H]^+$; **2g^p**: 2548.3; found: 2548.7 $[M+H]^+$.

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Keywords: DNA · morpholino · non-enzymatic · PNA · ribonucleosides · primer extension

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