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Tuning the Reaction Site for Enzyme-Free Primer-Extension Reactions through Small Molecule Substituents

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Abstract: The replication of genetic information relies on the template-directed extension of DNA primers catalyzed by polymerases. The active sites of polymerases accept four different substrates and ensure fidelity and processivity for each of them. Because of the pivotal role of catalyzed primer extension for life, it is important to better understand this reaction on a molecular level. Here we present results from primer-extension reactions performed with chemical systems that show high reactivity in the absence of polymerases. Small molecular caps linked to the 5′-terminus of templates are shown to enhance the rate and selectivity of primer extension driven by 2-methylimidazolides as activated monomers for any of the four different templating bases (A, C, G, and T). The most consistent effect is provided by a stilbene carboxamide residue, rather than larger aromatic or aliphatic substituents. Up to 20-fold rate enhancements were achieved for the reactions at the terminus of the template. The preference for a medium size cap can be explained by competing interactions with both the oligonucleotides and the incoming deoxynucleotide. The data also show that there is no particularly intractable problem in combining promiscuity with fidelity. Exploratory experiments involving a longer template and a downstream-binding strand with a 5′-cap show up to 38-fold rate acceleration over the same reaction templated by a single overhanging nucleotide.

Keywords: DNA replication · molecular recognition · nucleotides · oligonucleotides · sequence determination

Introduction

Replication and transcription of genetic information rely on the step-wise extension of a complementary strand, directed by a templating strand that engages in Watson–Crick base pairing with incoming nucleoside triphosphates (Figure 1a). The same reaction has been harnessed for key biomedical applications, such as the polymerase chain reaction (PCR),[1] sequencing by the chain terminator method,[2] or genotyping via primer extension.[3] For any of the processes named, enzymatic catalysis by a polymerase is required. Polymerases are fascinating enzymes, as they show promiscuity and fidelity at the same time. They are promiscuous by accepting four different substrates (dATP, dCTP, dGTP, and dTTP), and they show high fidelity by always incorporating the matched nucleotide to the templating base, with error rates below 1 in 10^5 reactions, even without proofreading capability.[4,5]

It is believed that the level of sequence fidelity found in polymerase-catalyzed reactions cannot be the result of the selectivity of base pairing alone.[6] Non-Watson–Crick base pairs are known to form readily between nucleobases in solution.[7] Even when the two nucleotides are preorganized in a duplex, base pairing at the termini, where primer extension occurs, is of low fidelity.[8,9] How, then, do polymerases achieve their remarkable effect on template-directed primer extension? Is their effect predominantly based on shape complementarity, specific hydrogen bonds, electrostatic complementarity to the transition state, or π-stacking? The interplay of competing forces in complex biochemical systems limits the ability to answer this question conclusively. Well defined chemical model systems can provide useful insights into primer-extension reactions. Further, such chemical systems may eventually be evolved to a level that makes them useful for biomedical applications, such as genotyping single-nucleotide polymorphisms,[10] at a fraction of the cost of enzymatic reactions.

We have recently presented a molecular system for non-enzymatic primer-extension reactions (Figure 1b)[11] that extends work in the realm of prebiotic evolution in that it uses

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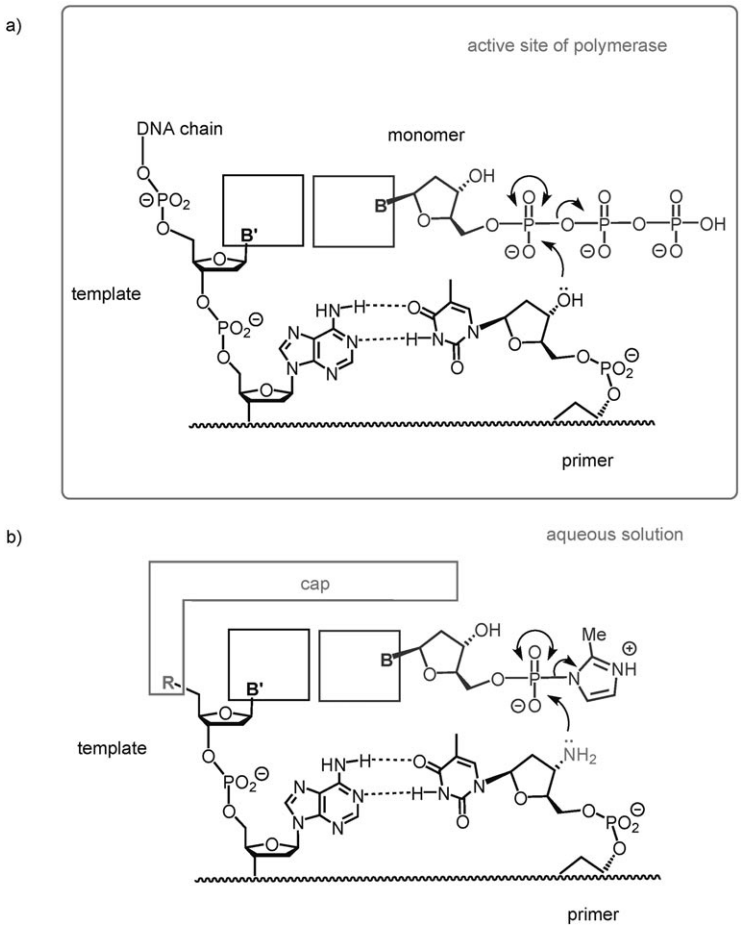


Figure 1. Schematic drawing of template-directed primer-extension reactions, a) in the active site of a polymerase with a deoxynucleotide triphosphate as monomer, and b) in aqueous solution with an imidazolide of a deoxynucleotide as monomer, an amino-terminal primer, and a small molecule substituent as cap.

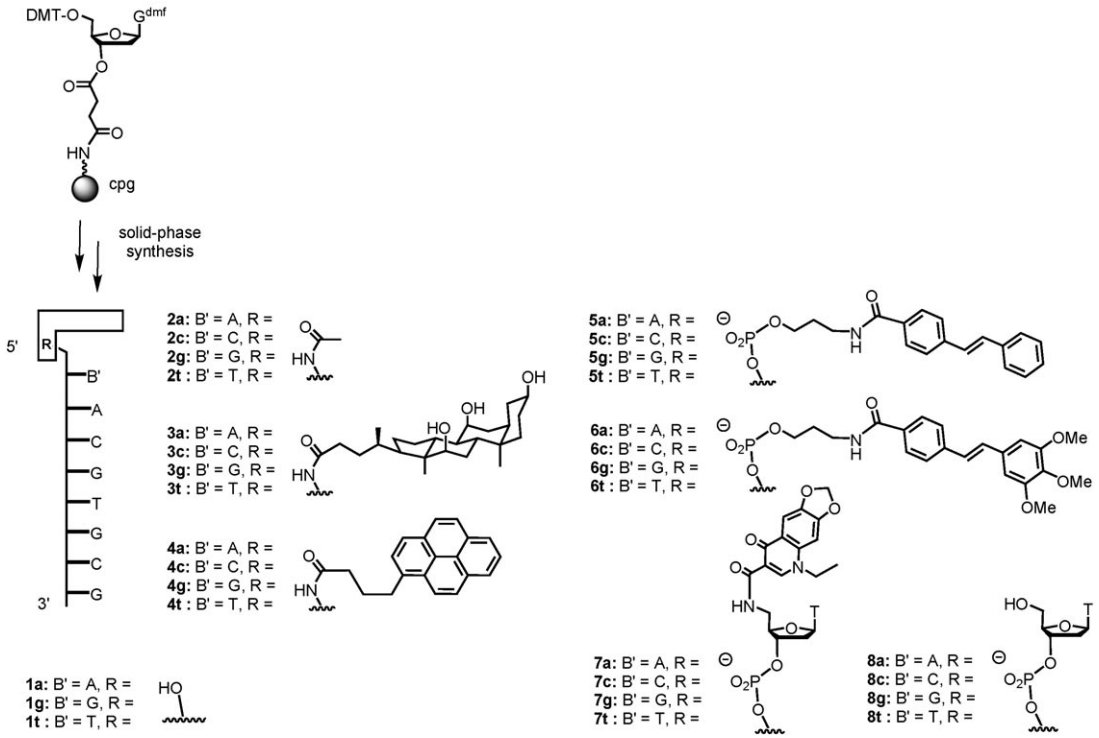
2-methylimidazolides of deoxynucleotides as activated monomers.[12,13] Methylimidazolides are known to undergo template-directed oligomerization in aqueous buffer.[14] While initially developed for reactions with the 2′/3′-diol of ribonucleotides,[15] they have been shown to react more rapidly with amines.[16] The non-enzymatic reactions between methylimidazolides and 3′-amino-terminal primers are therefore sufficiently fast to allow for assays on the time scale of days, rather than weeks.[11] Our molecular system uses covalently appended small molecules that can act as "caps" to modulate the chemical environment for primer-extension reactions. Competitive reactions, involving all four activated nucleotides, are monitored by quantitative MALDI-TOF mass spectrometry,[17] to measure both rates and fidelity of the template-directed reactions.

In our earlier work,[11] we were able to show that bile acid residues linked to the 5′-terminus of the template can enhance the rate of primer extension up to 6.5-fold. Here we show that rate increases of up to 19.6-fold are achievable with a stilbene as cap. The stilbene substituent accelerates the primer extension for all four templating bases. Further, we present results from exploratory studies involving a three-strand system, where a cap is presented by a third, downstream-binding strand. With this system, rate enhancements of up to 38-fold over the cap-free primer extension at the terminus of the template were achieved. The latter results show that the concept of using small molecule substituents to accelerate non-enzymatic primer extension can also be applied to reactions with unmodified, potentially biogenic, template DNA.

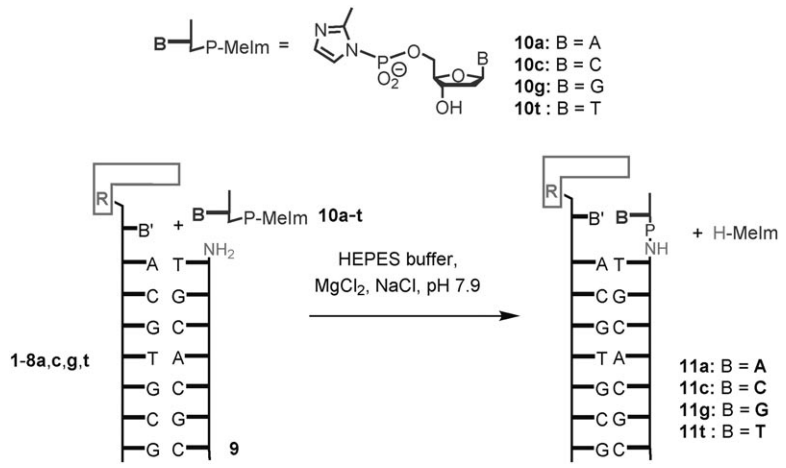
Results

A total of 31 different templates were prepared, starting from commercially available controlled pore glass (cpg) loaded with the first nucleoside (Scheme 1). This included seven control compounds, either with an unmodified 5′-terminus (**1a**,**g**,**t**) or with a 5′-acetamido group (**2a–t**). Control "**1c**" could not be used, since it has the same mass as one of the templates (**11g**, see Scheme 2), which would lead to a peak overlap in MALDI spectra. Eight templates with 5′-acylamido substituents were prepared, four with a cholic acid residue (**3a–t**), the lead structure from our earlier studies,[11] and four with a pyrenylbutyramide residue (**4a–t**). Pyrene is known to stack well on base pairs,[18] and has been studied extensively as an intercalator and base-pair surrogate.[19] Further, three sets of templates were prepared in which the 5′-substituent is linked to the templating strand through a phosphodiester linkage. Compounds **5a–t** and **6a–t** feature stilbene carboxamides as caps.[20] Stilbenes are known to bridge the termini of DNA duplexes, both when linked to either of the strands,[20] and when linked to one strand only.[21] For a DNA duplex with singly appended trimethoxystilbenes, increased base-pairing fidelity has been demonstrated,[20] and a three-dimensional structure has been solved that explains this effect.[22] Templates **7a–t** were prepared that feature a composite cap at their 5′-terminus, which consists of a thymidine residue and a 5′-appended oxolinic acid residue. A high-resolution structure of a DNA hexamer has shown that the composite cap can bridge termini.[23] The final set of templates, **8a–t**, are unmodified control strands that contain the 5′-thymidine residue of the composite cap, but lack the quinolone.

The terminal regions of duplexes capped by cholic acid, the trimethoxystilbene, and the composite cap are shown in Figure 2. These should be structurally similar to the products formed in single-nucleotide-extension reactions in the presence of these caps. The non-enzymatic primer-extension reactions themselves are shown in Scheme 2. Primer **9** is allowed to react with any of the four imidazolides **10a–t** present in an aqueous buffer to give extended primers **11a–t** in the presence of one equivalent of any of the 31 templates shown in Scheme 1. Rate constants for each of the four competing reactions (with **10a**,**c**,**g**,**t**) were obtained by fitting monoexponential functions to the kinetics obtained from MALDI spectra at stated intervals. In every case, experiments included control assays with unmodified or 5′-ace-



Scheme 1.



Scheme 2.

tylated templates to ensure high accuracy and reproducibility for the rate constants. Rate constants in Tables 1 and 2 are given relative to the slowest of the control reactions with unmodified templates, namely that with template **1t**, as absolute rate constants can vary by up to a factor of two, depending on the history of the imidazolides and the exact room temperature. Calibration data for quantitation by MALDI-TOF MS are given in the Supporting Information. The fidelity of the reactions is expressed as the ratio between the rate of incorporation for the complementary nucleotide and the rate for incorporation of the closest competitor among the mismatched nucleotides. This ratio is important when using the primer-extension reactions for genotyping, as it defines whether an unambiguous base call can be made or not. Since the acetamido group of the templates of general structure **2** should not have an appreciable effect on the primer-extension reaction, the comparison of the rates for reactions templated by **1a–t** and those templated by **2a–t** provides an additional impression of the reproducibility of the results.

All cap-bearing templates give rate accelerations and increase the fidelity of the non-enzymatic primer-extension reactions, when compared with controls of general structure **1** and **2** (Table 1). Among the five caps tested, pyrenylbutyramide consistently gave the smallest effect, resulting in rates with templates of general structure **4** between 1.9- and 3.1-fold higher than those of the control with the same templating base. For the templates with strongly base-pairing nucleobase at the extension site (those of the **g** and the **c** series), cholic acid (general structure **3**) was the next better cap, with $\leq$6.6-fold rate increases. For the weakly base-pairing templates (**a** and **t** series), the cholic acid cap was a little

Table 1. Results from primer-extension reactions.

Template	$k^{A[a]}$	$k^{C[a]}$	$k^{G[a]}$	$k^{T[a]}$	$t_{1/2}^{pr}$ [h][b]	Rel. signal[w/sc[c]]	Acceleration[d]
1a[e]	1.1	0.5	0.4	**1.1**	11.5	1.1	1.0
2a	1.0	0.6	0.7	**1.2**	10.2	1.2	1.1
8a	1.0	0.4	0.6	**1.8**	9.4	1.8	1.6
4a	1.3	0.5	0.8	**3.4**	6.0	2.7	3.0
6a	0.8	0.3	1.0	**4.9**	5.0	4.9	4.4
3a	1.1	0.8	0.9	**5.5**	4.2	5.0	4.9
7a	1.4	0.7	1.2	**7.9**	3.1	5.7	7.1
5a	1.9	1.0	1.9	**21.6**	1.3	11.4	19.6
2c	0.7	0.3	**1.8**	0.3	11.5	2.7	1.0
4c	0.8	0.4	**2.3**	0.4	9.5	3.1	1.3
8c	0.6	0.3	**2.4**	0.3	10.1	4.4	1.3
3c	0.6	0.5	**6.6**	0.3	4.3	10.7	3.7
6c	1.5	0.5	**12.0**	0.9	2.4	8.0	6.7
5c	1.3	0.7	**18.0**	0.6	1.7	14.1	10.0
7c	1.1	0.7	**22.6**	0.6	1.4	20.4	12.6
1g	0.7	**3.4**	0.5	0.5	6.8	4.7	1.0
2g	0.7	**3.6**	0.5	0.6	6.6	5.2	1.1
8g	0.6	**3.8**	0.5	0.5	6.5	6.5	1.1
4g	1.2	**7.5**	0.5	0.9	3.5	6.5	2.2
3g	0.9	**15.3**	0.5	1.2	1.9	13.0	4.5
6g	1.8	**18.4**	0.5	1.5	1.6	10.3	5.4
5g	2.2	**25.6**	0.5	2.4	1.1	10.7	7.5
7g	0.9	**31.2**	0.5	1.7	1.0	19.0	9.2
2t	**0.8**	0.3	0.3	0.3	22.1	2.5	1.0
1t	**1.0**	0.3	0.3	0.3	18.9	3.3	1.3
8t	**1.1**	0.3	0.3	0.3	18.3	3.6	1.4
4t	**1.9**	0.4	0.5	0.4	11.1	3.7	2.4
6t	**5.2**	0.4	0.9	0.2	5.3	6.1	6.6
3t	**5.9**	0.6	0.8	0.3	4.6	7.2	7.4
7t	**6.1**	0.5	1.2	0.3	4.4	5.2	7.7
5t	**7.3**	0.5	1.1	0.5	3.7	6.8	9.2

[a] Relative rate constants for the formation of extension products; values for matched case in bold. [b] Half life times for the disappearance of primer. [c] Ratio of k^{winner} to $k^{strongest\ competitor}$. [d] Relative increase in rate for the reaction leading to the formation of matched product. [e] Strands of a given templating base are ordered according to rates of desired extension reaction.

Table 2. Kinetic parameters for extension reactions with helper oligonucleotides.

Oligomers	$k^{A[a]}$	$k^{C[a]}$	$k^{G[a]}$	$k^{T[a]}$	$t_{1/2}^{pr}$ [h][b]	Rel. signal[w/sc[c]]	Acceleration[d]
1a, **9**	0.9	0.6	0.7	**1.1**	9.7	1.2	1.0
12a, **9**	0.3	1.8	0.8	**9.2**	2.6	5.4	8.5
12a, **13**, **9**	1.1	1.8	1.9	**9.9**	2.2	5.4	9.0
12a, **14**, **9**	1.1	2.8	2.5	**42.1**	0.7	15.3	38.4
1g, **9**	1.1	**3.4**	0.7	0.4	2.9	3.1	1.0
12g, **9**	1.5	**21.8**	1.0	0.6	0.6	14.6	6.5
12g, **13**, **9**	2.3	**23.0**	1.7	0.9	0.6	9.8	6.8
12g, **14**, **9**	1.9	**45.8**	1.4	1.3	0.3	24.2	13.5

[a] Relative rate constants for the formation of extension products; values for matched case in bold. [b] Half life times for the disappearance of primer. [c] Ratio of k^{winner} to $k^{strongest\ competitor}$. [d] Relative increase in rate for the reaction leading to the formation of matched product.

better than the trimethoxystilbene (general structure **6**). Two caps consistently gave the strongest effect on the primer-extension reactions: the stilbene carboxamide residue (general structure **5**) and the composite cap of templates **7a–t**. The former was the best performer for the weakly base-pairing templates (A and T as templating bases), whereas the latter gave the most pronounced rate acceleration for the templates presenting a C or a G as templating base. If one considers the templates with an extra thymidine residue at the 5′-position (**8c**,**g**) as the relevant controls for the composite cap templates, the small lead for the composite cap is negligible, even for the strongly base-pairing templates. This makes the stilbene carboxamide residue of templates **5a–t** the "winner" of the assays performed. The strongest rate increase is that for A as templating base, where it reached a factor of 19.6-fold over the control reaction.

The selectivity of the incorporation correlates surprisingly well with the rate enhancements, with relatively few exceptions. The increase in ratio between incorporation of the complementary nucleotide and the next best competitor (penultimate column, Table 1), defining the "signal-to-noise" in practical applications, roughly parallels the increase in rate for incorporation of the correct nucleotide. The exceptions are reactions templated by a thymine. Clearly the most selective reactions are those for strongly base-pairing nucleobases (C and G) occurring in the presence of the composite cap, with ratios between matched incorporation and most prominent of the misincorporation reactions of approximately 20:1 (Figure 3 and Table 1).

Finally, we performed an exploratory study on the effect of a downstream-binding strand with or without cap on the rate and fidelity of the primer-extension reaction involving primer **9** (Scheme 3). Downstream-binding oligonucleotides, also called "helper oligonucleotides" bind in the 5′-region of the template such that they leave only the templating nucleotide free.[10] Together with the overhang of the template they thus form a high molecular weight cap for primer-extension reactions. In the system tested (Scheme 3), two different templates (**12a**,**g**) were employed, the equivalent of two different alleles of a single-nucleotide polymorphism (SNP). The templates feature a ten nucleotide overhang. The unmodified helper oligonucleotide **13** binds to the nonamer region downstream from the templating nucleotide. Helper oligonucleotide **14** is an octamer that contains a pyrenylmethylpyrrolidinol cap instead of the 5′-terminal deoxycytidine residue. This type of pyrenyl cap came up as a hit in a recent combinatorial study on caps for hybridization probes.[9] It has the advantage of being commercially available (Glen Research, Sterling, VA, USA, see: www.glenres.com), so that this molecular system can be readily generated by laboratories without special synthetic effort, using automated chain assembly. Much like the stilbene of **5a–t**, the pyrenyl ring system of **14** is also very lipophilic.

Table 2 compares the rate constants and selectivities for reactions with the template (**12a**,**g**) either in the presence or absence of **13** and **14** with those of templates **1a** and **1g**. With the weakly base-pairing nucleobase of **12a**, the selectivity is modest, but the overhang has a substantial rate-en-

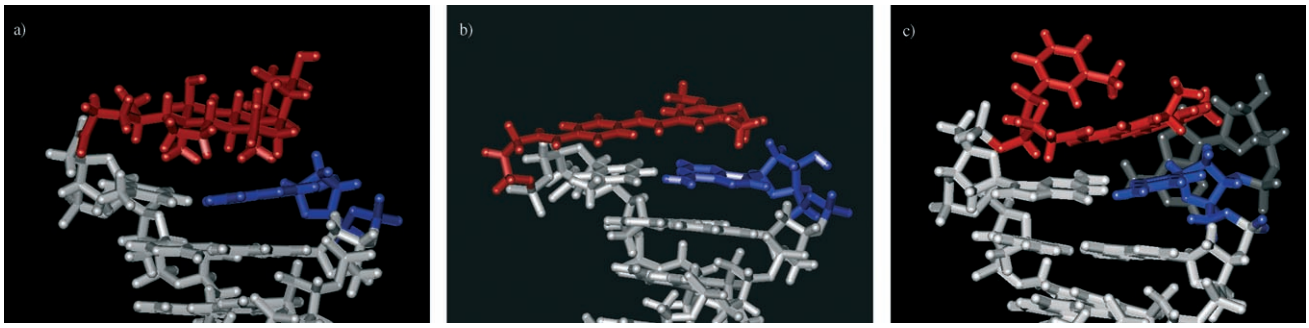


Figure 2. Details of solution structures of duplexes with caps found in templates **3**, **6**, and **7**; a) terminal region of duplex (chl-TGCGCA)$_2$,[24] b) terminal region of duplex (TMS-TGCGCA)$_2$,[22] and c) terminal region of duplex (OA-TGCGCA)$_2$.[23] Color code: caps, red; 3′-terminal residues of the duplex, blue; other residues of duplex, white; dangling residue, gray. Please see first paragraph of the Experimental Section for abbreviations.

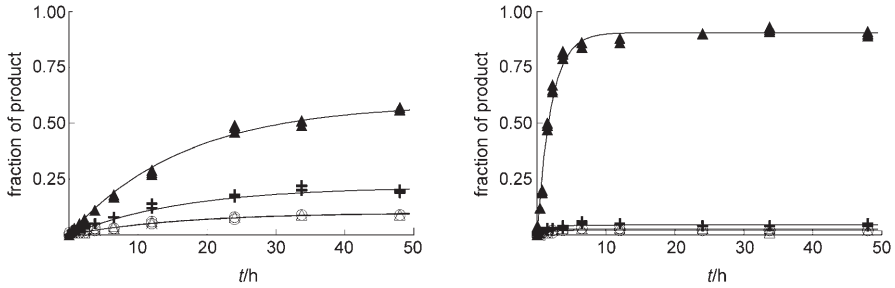
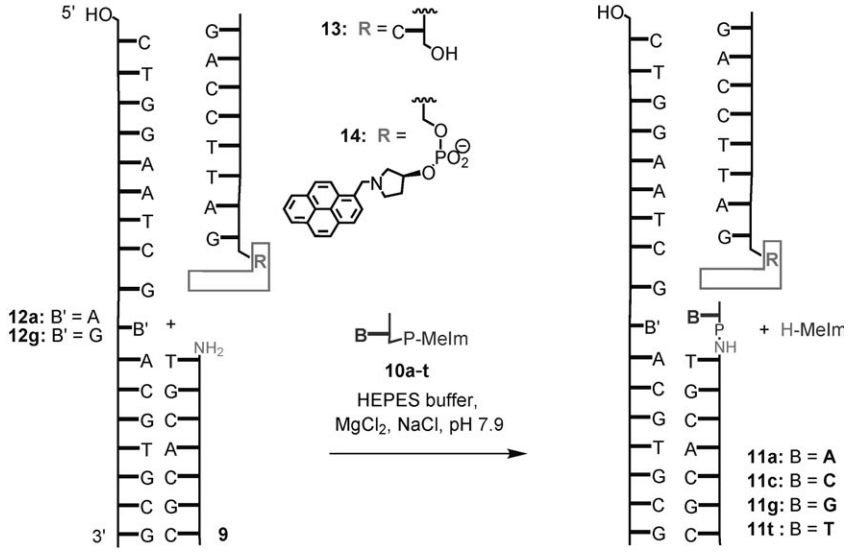


Figure 3. Representative kinetics of templated primer-extension reactions (compare Scheme 2). Shown are the kinetics of the control reaction with **2c** (left) and the reaction templated by **7c** (right). The graphs show mono-exponential fits to data obtained by quantitative MALDI-TOF MS. Crosses are for **11a**, open triangles for **11c**, filled triangles for **11g**, and open circles for **11t**.



Scheme 3.

hancing effect. This may be due to the formation of non-covalent assemblies between monomers and the template at the monomer concentration employed. The formation of non-covalent assemblies has been considered in work on the kinetics of non-enzymatic replication.[25] This is confirmed by the very modest rate enhancement in the presence of **13**, the downstream-binding oligonucleotide. At lower monomer concentration, where non-covalent helices are less stable, helper oligonucleotides have a more pronounced effect.[10] Faster reactions in the interior of templates, compared with the termini, are known from other reports on non-enzymatic replication.[26]

In the three-strand system involving **14** with its pyrenylmethylpyrrolidinol cap, the fastest reactions were measured of all systems studied in this work. For the fastest case, template **12g**, full primer conversion can be achieved in less than one hour (Figure 4). The cap increases the rate for the correctly appended monomer over the rate with unmodified helper **13** four-fold for the A-template and two-fold for the G-template. Further, excellent selectivities are observed with the incorporation of the most easily incorporated competitor well below 10% for either template

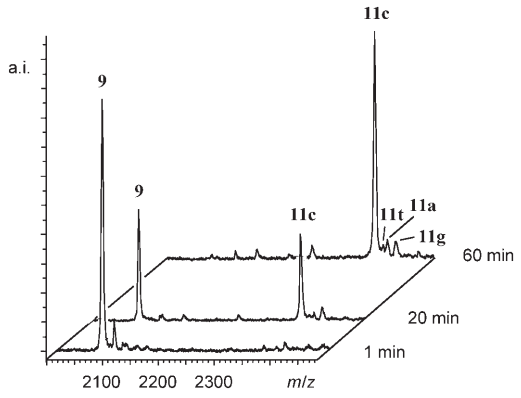


Figure 4. Selected MALDI-TOF mass spectra for the extension of **9** in the presence of template **12g** and the downstream-binding oligomer **14** (see Scheme 3 for sequences).

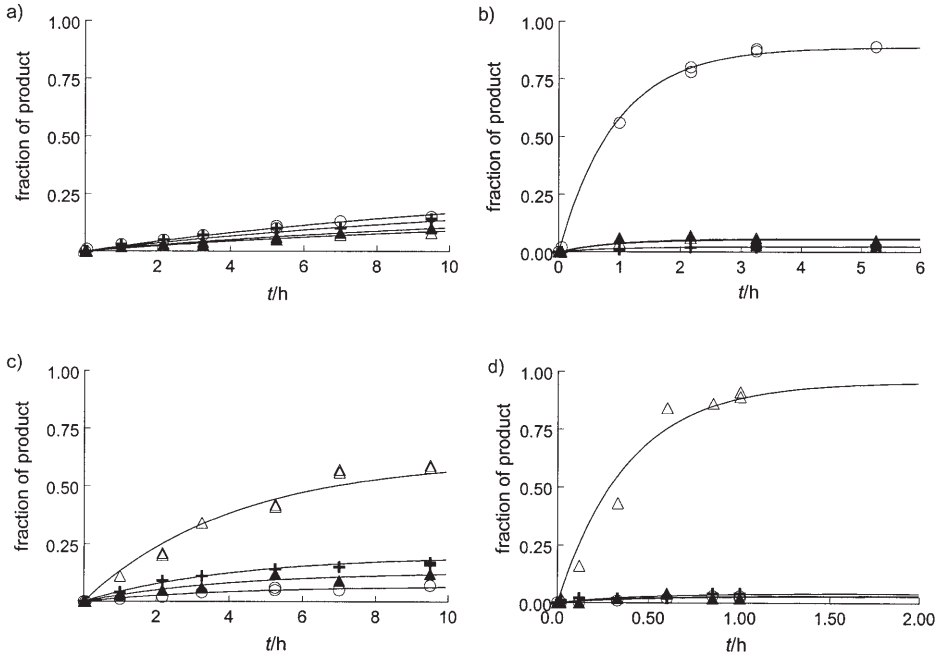


Figure 5. Kinetics for primer-extension reactions templated with or without an overhang in the template and a capped downstream-binding oligonucleotide: a) templated by **1a**; b) templated by **12a** and **14**; c) templated by **1g**; d) templated by **12g** and **14**. Kinetics are based on mass spectra such as those shown in Figure 4. +: primer-extension product **11a**, △: **11c**, ▲: **11g**, and ○: **11t**. Note that the time axes differ between the individual plots.

in the cases where the pyrenyl cap is presented at the 5′-terminus of the downstream-binding oligonucleotide. A comparison of the kinetics for templates **1a**, **12a**, **14** and **1g**, **12g**, **14** are shown in Figure 5.

Discussion

Polymerases put an α-helix over the base pair of the incoming deoxynucleotides and templating bases,[27] which, together with the remainder of the active site, fit the transition state for correct base pairs better than transition states involving mismatched base pairs. To achieve sufficient rates and selectivities with simpler, chemical entities, catalysts could attract the incoming nucleotides to the active site, they could electrostatically favor the attack of the 3′-nucleophile on the phosphate, and they could generate energetic hurdles that prevent the formation of products with mismatched base pairs. Polymerases are complex structures, which complicates the separation of the individual factors. Whether interactions that are predominantly van der Waals interactions, hydrogen bonding, dipol–dipol/dipol–quadrupol interactions, electrostatic interactions, or hydrophobic effects are responsible for the rate enhancements achieved with them over the background reaction is difficult to determine. Shape complementary has been emphasized by some, based on studies with isosters of nucleotides,[28] whereas others have highlighted the presence of universal hydrogen-bond acceptors[29] in polymerase-catalyzed reactions.[30]

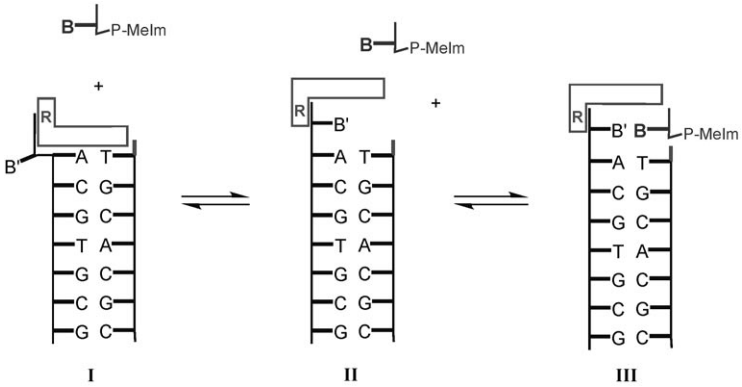
What may be learned about primer-extension reactions from the results presented here? None of the rate-accelerating caps displays cationic groups that could have been expected to stabilize the transition state of the extension reaction electrostatically. Further, there is no obvious shape complementarity that prevents incorporation of mismatched nucleotides. Except for the trimethoxystilbene and the cholic acid residue, which are known to "gauge" terminal base pairs through molecular interactions with terminal base pairs by packing against the deoxyribose of the target nucleotide,[22,24] neither cap has an obvious structural feature that prevents the formation of wobble base pairs and other possible mismatches. Neither the trimethoxystilbene nor the cholic acid residue is among the most selectivity-inducing caps in our study.

All caps have features that may help to retain the incoming nucleotide at the reaction site, though. This could mean that the ability to retain monomers at the reaction site is the dominant effect. Duplex stability has also been found to be the critical feature for achieving primer ligation,[31] a reaction related to primer extension. But, there is no clear correlation between the UV melting points of duplexes stabilized by the individual caps[8,9,20,23] and the relative rates measured in our assays. Also, there is no simple correlation between lipophilicity or π-stacking surface and rate enhancement. The two "winner" caps identified in the overhang-free system (**5** and **7**) differ greatly in size, shape, and repertoire

of functional groups. The pyrenyl cap of templates **4** features the largest aromatic ring system, but induces the smallest rate acceleration among the caps tested.

Perhaps the easiest comparison can be made for the caps of templates **3**–**6**. All of them are tethered to the 5′-terminus of the template via alkyl chains of comparable length. In terms of π-stacking surface, they differ quite substantially. The stilbenes contain two benzene rings separated by a vinyl bridge, whereas the pyrenyl substituent has a tetracyclic ring system, and the cholic acid does not have any aromatic moiety. The rate enhancing effect is greatest for the stilbene and the smallest for the pyrenyl cap. This shows that the general ability to engage in π-stacking is not a dominant factor for the acceleration of primer extension. Further, since the stilbene cap, rather than the trimethoxystilbene cap, has the stronger rate-accelerating effect among the stilbenes, even though the latter is known to have the stronger duplex-stabilizing interactions when employed as a cap,[20] it is clear that the capping ability alone (i.e., the ability to retain the incoming monomer) is not the governing factor.

One important difference between the active site of a polymerase and the caps tested here may help to explain our results. Due to the tightly folded structure of the polymerase, the active site of the enzyme features a stable cavity, preorganized to accept the substrates. The capping portions of the modified templates, on the other hand, are not fixed in space, allowing it to adopt a number of different conformations. This affects their ability to act as rate-enhancing moieties. Most likely, an equilibrium, in which capping of the monomer-template base pair competes with capping of the terminal base pair of the primer/template duplex, governs the primer-extension reactions (Scheme 4).



Scheme 4.

If the cap stacks on the terminal nucleotide of the primer/template duplex **I**, it blocks the primer extension. This state must be in equilibrium with unstacked states **II** that in turn allow for the formation of the template/primer/monomer complex **III**. If state **I** is populated too much, the desired reaction will be slow, even if the likelihood of a reaction out of state **III** is greater than for the control reaction without cap. The stilbene cap of **5** may be better suited for accelerating primer extension than the trimethoxystilbene or the pyrenyl substituent of templates **4** as it stabilizes state **III**, but does not get "stuck" in state **I** too readily. The thymidine residue of the composite cap constitutes a more rigid linker than that found in the other appendages; this makes it less likely that the oxolinic acid residue can stack with the core duplex **I**.

The results of the assays performed with the three-strand system shown in Scheme 3 are interesting in light of the proposed three-state equilibrium. The downstream-binding or helper oligomer should provide a good cap, as it offers stacking surfaces on either side of the monomer, equivalent to those of a residue in the interior of a duplex. But even the three-strand system can oscillate between an open state that allows for the binding of a monomer and a closed state where the two helices stack on each other with the templating base positioned extrahelical. That the small molecular caps studied initially give rates and selectivities similar to those of the three-strand system shows how well the caps function. Further, the rate acceleration observed for the pyrenylmethylpyrrolidinol cap of **14** together with the fact that the more lipophilic stilbene was the winner among the caps on the short templates suggests that a lipophilic environment is favorable for the primer-extension reaction. So, a stably preorganized reaction site and a lipophilic environment for base pairing are apparently both important factors for rapid and selective primer extension. This conclusion could be of interest to those generating ribozymes with polymerases activity.[32]

The model proposed above does not explain why the stilbene of templates **5** has such a strong effect on the fidelity. How does this simple hydrocarbon cap prevent the incorporation of non-Watson–Crick-paired monomers? It lacks the shape complementarity that inhibits the formation of wobble base pairs and other non-Watson–Crick base pairs, and it does not have hydrogen-bond capabilities that allow it to recognize the right positioning of the universal hydrogen-bond acceptors of correctly formed base pairs. So, the presence of the stilbene moiety must enhance the intrinsic selectivity of Watson–Crick base pairing itself. In the hydrophobic environment created by a cap, hydrogen bonds should be strengthened compared with a state in which they are solvent exposed. Perhaps the stilbene cap offers a better hydrophobic shield for the canonical base pairs than for the mismatched base pairs. The attachment site and interactions with the templating base might position the cap to achieve this effect. The interactions with T should be less extensive than those with the larger A. Also, T itself is less likely to be permanently stacked on duplex. Together, this might explain why the T-templated reaction gains the least from the presence of the caps (Table 1).

The findings presented here are also of importance for the practical use of non-enzymatic primer-extension reactions. While the rate enhancements observed are modest compared with those achievable with enzymes, they turn reactions previously considered only in the context of prebiot-

ic evolution into reactions useful for applications that involve determining nucleotides in a template. The results with three-strand system (Scheme 3) show that with easy-to-synthesize oligonucleotides sequence-specific non-enzymatic primer-extension reactions can be accelerated enough to give half-life times for the primer well below 1 h. This makes these reactions attractive for applications such as genotyping single-nucleotide polymorphisms.[33] The selectivity achieved is sufficient to make clear base calls after incorporation of unlabeled monomers by mass spectrometry,[10] or to determine nucleotides in template DNA after incorporation of fluorophore-labeled monomers[34] followed by optical read-out. Either approach should be suitable for multiplexing, based on microarrays. Primer extension might also become important for template-directed assembly of new functional nucleic acids in a designable, sequence-specific fashion.[35] The active site created with downstream-binding oligonucleotides, such as **14**, should be more accommodating for chemical modifications, so that primer-extension reactions may be performed with modified monomers that are difficult to process by polymerases.[36] Experiments aimed at developing improved caps for the three-strand system are currently under way in our laboratories.

Conclusion

In conclusion, we have shown how small molecule appendages at the termini of templates can accelerate primer-extension reactions and increase their sequence fidelity. Terminal base pairs are otherwise difficult to replicate,[26a] and our results foster hopes that chemical replication from monomers can be realized. We propose a three-state model that helps to explain the unexpected activity of the stilbene carboxamide cap. This model also suggests a way of screening for caps that favor primer extension based on oligonucleotide duplexes. A good cap should increase the melting point of the full length duplex, but should have little effect on the melting point of a duplex that lacks the 3′-terminal residue of the target strand. If it shows these features, it should not block the reaction site and favor rapid and high yielding primer-extension steps.

Experimental Section

Abbreviations: chl, cholic acid residue; cpg, controlled pore glass; DIEA, *N*,*N*-diisopropylethylamine; dmf, *N*,*N*-dimethylformamidino group; DMF, *N*,*N*-dimethylformamide; HBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HEPES, 4-(2-hydroxyethyl)-piperazin-1-ethansulfonic acid; HOBt, 1-hydroxybenzotriazole; MALDI-TOF MS, matrix-assisted laser desorption ionization–time of flight mass spectrometry; N*, 5′-amino-2′,5′-dideoxynucleotide residue (N = A,C,G,T); OA, oxolinic acid residue; Pp, pyrenylmethylpyrrolidinol cap; Py, pyrenebutyric acid residue; St, stilbene cap; THAP, 2,4,6-trihydroxyacetophenone; TMS, trimethoxystilbene cap.

General methods: Reagents were obtained from Acros (Geel, Belgium), Aldrich/Fluka/Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany), unless otherwise noted and were used without further purification. HOBt and HBTU were from Advanced ChemTech (Louisville, KY). Phosphoramidites (dA^{Bz}, dC^{Bz}, T, dG^{dmf}) and reagents for DNA synthesis were from Proligo (Hamburg, Germany), except for dG^{dmf}-loaded controlled pore glass (cpg), which was from ABI (Warrington, UK). Phosphoramidites of modified nucleosides or caps used in this work, namely phosphoramidites of 5′-amino-2′,5′-dideoxynucleosides (N*),[9,37–40] stilbene phosphoramidites[20] and pyrenemetylpyrrolidonol phosphoramidite[9] were prepared as previously reported. The 5′-acylamidooligonucleotides (**2a**, **c**, **t**; **3a**, **c**, **t**), phosphoramidates (**11a–t**), primer (**9**) and 2-methylimidazolides (**10a–t**) were prepared as described previously.[11]

DNA synthesis: Oligonucleotides were prepared on a 1 μmol scale in polypropylene reaction chambers for DNA synthesis (Prime Synthesis, Aston, PA) on an 8909 Expedite DNA synthesizer from Perseptive Biosystems. β-Cyanoethyl phosphoramidites were employed as building blocks, following the recommendations of the manufacturer. Oligonucleotides were purified on Nucleosil 120-5 reversed phase C4 (modified oligonucleotides) and C18 (unmodified oligonucleotides) HPLC columns (both 250×4.6; Macherey–Nagel, Düren, Germany) with a gradient of MeCN (solvent B) in 0.1 M triethylammonium acetate (pH 7.0) (solvent A) at a flow rate of 1 mL min^{-1} and detection at 260 nm. Yields of oligonucleotides are based on the integration of the HPLC traces of crudes, without correction for the absorbance of the solvent front. Stock solutions of single-stranded oligonucleotide were prepared based on UV measurements, using calculated extinction coefficients for oligonucleotides generated by adding the extinctions of nucleotides and the caps.

MALDI-TOF mass spectrometry: MALDI-TOF mass spectra were acquired on a Bruker REFLEX IV spectrometer featuring a nitrogen laser ($\lambda = 337$ nm) and software XACQ 4.0.4 and XTOF 5.1.0. Spectra were acquired in negative, linear mode at a total extraction voltage of 20, 18.6 kV delayed extraction (on IS2), 9.6 kV lens and 1.55 kV detector voltage. Each spectrum consisted of the sum of the signal from 150 to 300 shots at a repetition rate of 1–3 Hz. The MALDI matrix mixtures for oligonucleotides was THAP (0.3 M in EtOH) and diammonium tartrate (0.1 M in water) (2:1, v/v). Calculated masses are average masses, m/z found are those for the unresolved pseudomolecular ion peaks ($[M-H]^-$). The accuracy of mass determination with the external calibration used is approximately ±0.1%.

General protocol A—Cleavage of oligonucleotides from solid support: The cpg loaded with the oligonucleotide was briefly dried (0.1 Torr), and treated with ammonium hydroxide (sat. aqueous NH_3, 150 μL per 5 mg cpg) in a polypropylene vessel. After 16 h at RT, excess ammonia was removed with a gentle stream of compressed air for 1 h. The supernatant was aspired, and the solid support was washed with water (2×200 μL). The combined aqueous solutions were filtered (0.2 μm pore size) and used directly for HPLC purification.

General protocol B—Coupling of modified phosphoramidites on solid support: The cpg loaded with the unmodified portion of the oligonucleotide (5 mg, approx. 0.2 μmol DNA) was dried at 0.1 Torr for 3 h in a polypropylene vial. A solution of the modified phosphoramidite (0.16 to 1 M in MeCN, 150 μL) and 1*H*-tetrazole (0.5 M in MeCN, 150 μL) were added under argon and the mixture was shaken for 30 min. The supernatant was aspired and the solid support was washed with MeCN (2×1 mL). After drying at 0.1 Torr for 1 h the coupling reaction was repeated once before standard oxidation.

General protocol C—Acetylation on solid support: The following protocol is for the final step of the assembly of Ac-G*ACGTGCG (**2g**) and is representative. The acetylation step of the 5′-amino terminated oligonucleotide, prepared with the phosphoramidite of 5′-amino-2′,5′-dideoxyguanosine[40] (GP B) was performed on the support with the "capping" reaction mixture for DNA synthesis. A mixture of acetic anhydride/2,6-lutidine/THF ("cap A", 0.5 mL 1:1:8) and DMAP/pyridine ("cap B", 0.5 mL, 6.5% w/v) (1:1 v/v) was added to the amino terminal oligonucleotide chain on cpg, and the mixture was shaken for 10 min. The supernatant was aspired and the support was washed with acetonitrile (2× 2.5 mL), followed by drying at 0.1 Torr. Cleavage from solid support followed GP A.

General protocol D—Amide coupling on solid support: A mixture of the corresponding carboxylic acid (100 μmol), HOBt (13.5 mg, 100 μmol) and HBTU (34.1 mg, 90 μmol) was dried at 0.1 Torr, dissolved in DMF (600 μL) and treated with DIEA (40 μL, 230 μmol). After 10 min, the resulting solution was injected into a polypropylene reaction chamber for DNA synthesis containing the cpg-bound 5′-amino terminated DNA (5 mg, approx. 0.2 μmol DNA), prepared by GP B, with the aid of two syringes. After 45 min, the solid support was rinsed with DMF (2 mL), washed with MeCN (3×2 mL) and dried in vacuo. 5′-Acylamidooligonucleotides were cleaved from solid support following GP A.

Ac-G*ACGTGCG (2g): Prepared by GP C; MALDI-TOF MS: calcd for $[M-H]^-$: 2491.7, found 2492.4; HPLC: C18, gradient: 0% B for 5 min, to 17% B in 40 min, elution after 40 min; yield: 24%.

chl-G*ACGTGCG (3g): Prepared by GP D; MALDI-TOF MS: calcd for $[M-H]^-$: 2840.2, found 2839.3; HPLC: C4, gradient: 0% B for 5 min, to 34% B in 40 min, elution after 34 min; yield: 24%.

Py-G*ACGTGCG (4g): Prepared by GP D; MALDI-TOF MS: calcd for $[M-H]^-$: 2720.0, found 2720.7; HPLC: C4, gradient: 0% B for 5 min, to 34% B in 40 min, elution after 42 min; yield: 18%.

Py-A*ACGTGCG (4a): Prepared by GP D; MALDI-TOF MS: calcd for $[M-H]^-$: 2704.0, found 2706.0; HPLC: C4, gradient: 0% B for 5 min, to 34% B in 40 min, elution after 45 min; yield: 57%.

Py-T*ACGTGCG (4t): Prepared by GP D; MALDI-TOF MS: calcd for $[M-H]^-$: 2695.0, found 2695.4; HPLC: C4, gradient: 0% B for 5 min, to 34% B in 40 min, elution after 38 min; yield: 32%.

Py-C*ACGTGCG (4c): Prepared by GP D; MALDI-TOF MS: calcd for $[M-H]^-$: 2680.0, found 2682.1; HPLC: C4, gradient: 0% B for 5 min, to 34% B in 40 min, elution after 38 min; yield: 66%.

St-GACGTGCG (5g): Prepared by GP B, A; MALDI-TOF MS: calcd for $[M-H]^-$: 2793.0, found 2794.2; HPLC: C4, gradient: 0% B for 5 min, to 28% B in 40 min, elution after 35 min; yield: 78%.

St-AACGTGCG (5a): Prepared by GP B, A; MALDI-TOF MS: calcd for $[M-H]^-$: 2777.0, found 2778.9; HPLC: C4, gradient: 0% B for 5 min, to 26% B in 40 min, elution after 36 min; yield: 90%.

St-TACGTGCG (5t): Prepared by GP B, A; MALDI-TOF MS: calcd for $[M-H]^-$: 2768.0, found 2766.3; HPLC: C4, gradient: 0% B for 5 min, to 24% B in 40 min, elution after 42 min; yield: 92%.

St-CACGTGCG (5c): Prepared by GP B, A; MALDI-TOF MS: calcd for $[M-H]^-$: 2753.0, found 2751.5; HPLC: C4, gradient: 0% B for 5 min, to 23% B in 40 min, elution after 31 min; yield: 72%.

TMS-GACGTGCG (6g): Prepared by GP B, A; MALDI-TOF MS: calcd for $[M-H]^-$: 2883.0, found 2883.7; HPLC: C4, gradient: 0% B for 5 min, to 24% B in 45 min, elution after 39 min; yield: 64%.

TMS-AACGTGCG (6a): Prepared by GP B, A; MALDI-TOF MS: calcd for $[M-H]^-$: 2867.0, found 2866.7; HPLC: C4, gradient: 0% B for 5 min, to 28% B in 30 min, elution after 26 min; yield: 76%.

TMS-TACGTGCG (6t): Prepared by GP B, A; MALDI-TOF MS: calcd for $[M-H]^-$: 2858.0, found 2859.7; HPLC: C4, gradient: 0% B for 5 min, to 24% B in 45 min, elution after 43 min; yield: 80%.

TMS-CACGTGCG (6c): Prepared by GP B, A; MALDI-TOF MS: calcd for $[M-H]^-$: 2843.0, found 2844.9; HPLC: C4, gradient: 0% B for 5 min, to 24% B in 45 min, elution after 35 min; yield: 85%.

OA-T*-GACGTGCG (7g): Prepared by GP D; MALDI-TOF MS: calcd for $[M-H]^-$: 2996.1, found 2993.7; HPLC: C4, gradient: 0% B for 5 min, to 27% B in 40 min, elution after 33 min; yield: 70%.

OA-T*-AACGTGCG (7a): Prepared by GP D; MALDI-TOF MS: calcd for $[M-H]^-$: 2980.1, found 2980.0; HPLC: C4, gradient: 0% B for 5 min, to 27% B in 40 min, elution after 32 min; yield: 70%.

OA-T*-TACGTGCG (7t): Prepared by GP D; MALDI-TOF MS: calcd for $[M-H]^-$: 2971.1, found 2970.0; HPLC: C4, gradient: 0% B for 5 min, to 26% B in 40 min, elution after 32 min; yield: 71%.

OA-T*-CACGTGCG (7c): Prepared by GP D; MALDI-TOF MS: calcd for $[M-H]^-$: 2956.1, found 2953.0; HPLC: C4, gradient: 0% B for 5 min, to 25% B in 40 min, elution after 32 min; yield: 69%.

Pp-GATTCCAG (14): Prepared by GP B, A; MALDI-TOF MS: calcd for $[M-H]^-$: 2772.0, found 2772.3, 2557.9 $[M-PyCH_2-H]^-$; HPLC: C4, gradient: 0% B for 5 min, to 25% B in 45 min, elution after 38 min; yield: 78%.

Primer-extension assay: The procedure employed was similar to the one reported earlier.[11] Briefly, aqueous solutions of primer **9** (1.8 nmol) and the corresponding template (**1a–t** to **8a–t**, 1.8 nmol) or template plus downstream-binding strand (**12a,g** and **13** or **14**, 1.8 nmol each) were added to a polypropylene vial (volumes of individual aliquots were between 0.5 and 2.5 μL). Then, aliquots of stock solutions of NaCl (2 μmol) and $MgCl_2$ (0.4 μmol) were added, and the resulting solution was lyophilized to dryness. A freshly prepared solution of the four 2-methylimidazolides (**10a–t**, 20 mM each) in a buffer of HEPES (0.2 M, pH 7.9, 5 μL) was added to the residue and the mixture vortexed for 15 s. The reaction was allowed to proceed while the vial was immersed in a water bath at 20 °C. Aliquots (0.3 μL) were withdrawn at stated intervals, diluted 100-fold with a deionized water treated with beads of ammonium form of Dowex 50W X4, 50–100 mesh cation-exchange resin, the slurry was vortexed for 15 s, and stored under liquid nitrogen before being used for MALDI-TOF MS analysis. For mass spectra, samples (0.5 μL) of the supernatant were mixed with the matrix/comatrix mixture (THAP, 0.3 M in ethanol and diammonium tartrate; 0.1 M in water; 2:1, v/v, 0.5 μL) containing T_{12} as an internal standard. Spectra were acquired until at least 5000 ion counts were accumulated for the most prominent peak of spectra. For each reaction, 10–12 time points were chosen and three mass spectra acquired per time point. Peak heights over extrapolated background, relative to the internal standard were measured in each mass spectrum.

Kinetic analysis: The kinetic analysis was similar to that described earlier.[11] Pseudo first-order rate constants were obtained by fitting a monoexponential to the experimental data. Rate constants for individual incorporation of the four competing monomers were derived by fitting two parameters: an exponential factor representing the rate constant and a preexponential factor giving the fraction of product being formed. Second-order rate constants were calculated via dividing the pseudo first-order rate constant for formation of the extended primers by the concentration of the activated monomer. The rate constants for the control reactions given in Table 1 are representative second-order rate constants (in $[h^{-1}M^{-1}]$). The complex of oligonucleotides was treated as one and the respective monomer as the other reactant. Relative rate constants reported are based exclusively on experiments performed in parallel, and absolute rate constants of the control reactions tested might vary slightly (approx. ±10%) from those reported earlier.[11] Formulae used for the kinetic analysis are given in the Supplementary Information. Fits were obtained by using the program "Slide Write Plus" for Windows, version 3.0.

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