Primer Extension

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Chemical Primer Extension in Seconds**

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Dedicated to Professor Emanuel Vogel on the occasion of his 80th birthday

Chemical primer extension (CPE) generates nucleic acid strands elongated by the nucleotides encoded in a template strand in the absence of enzymes.[1] Enzymatic primer extension is a fundamental reaction that underlies both replication and transcription. It is also pivotal for biotechnology, as PCR and dideoxy sequencing are based on primer extension. Furthermore, the formation and template-directed extension of primers is probably a key process in the prebiotic phase of the evolution of life.[2] CPE can produce sequence information with unlabeled[3] or fluorophore-labeled mononucleotides.[4] Compared to polymerase-catalyzed primer extension, CPE is inexpensive as it avoids the cost of enzymes and triphosphate substrates, which makes it attractive for genotyping and sequencing applications. A reduction in cost is critical to meet the $1000 genome challenge.[5]

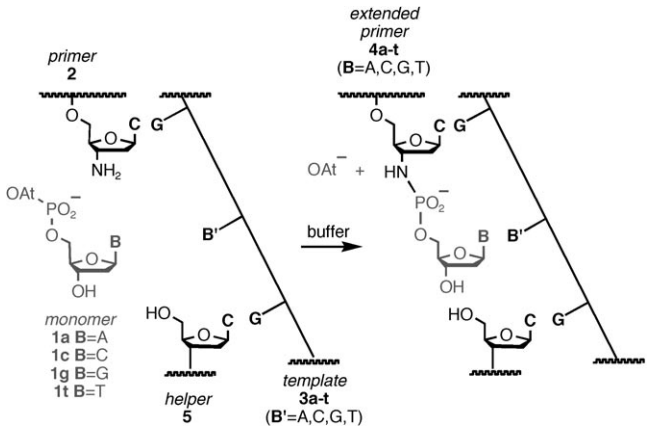
The chemical replication or copying reactions underlying CPE have been studied for decades in the context of prebiotic evolution.[6] A drawback of known forms of these reactions is that they are less efficient and much slower than their enzymatically catalyzed counterparts. Polymerase-catalyzed primer extension leads to replication of entire bacterial genomes within minutes, but chemical copying assays are run on the timescale of days to weeks.[7–9]

Attempts have been made to overcome the slow nature of primer generation[10] and CPE steps.[1] Oxyazabenzotriazolides were identified as reactive monomers that reduce the half-life time ($t_{1/2}$) of CPE involving amino-terminal DNA or RNA by more than an order of magnitude,[3,11] but all subsequent attempts to accelerate these reactions further appeared to hit a "solid wall" of unreactivity or lead to an unacceptable level of lability among the monomers. Control experiments meant to test the tolerance of the assay for pyridine (whose presence was needed to allow for direct addition of active esters of monomers generated in this solvent) led to an unexpected result. At high millimolar concentrations, pyridine massively accelerates spontaneous replication steps by what appears to be an organocatalytic process.

The range of reactions for which "organocatalysis" has been established has increased substantially. Although the phenomenon has been known for a long time,[12] the focus of contemporary research has been on enantioselective reactions catalyzed by substoichiometric amounts of chiral organic molecules.[13,14] Reactions as different mechanistically as aldol additions[15] and transfer hydrogenation[16] benefit from the presence of organocatalysts. However, transphosphorylation reactions are not among the synthetic transformations frequently catalyzed by organocatalysts. Instead, activation by metal ions, such as the magnesium ions found in the active site of polymerases or minerals, is more common. On the other hand, solid-phase DNA synthesis became rapid (and a successful commercial process) when phosphoramidites were combined with tetrazole as organocatalyst.[17,18] This catalyst is usually referred to as an "activator", as it is used above stoichiometric concentration.

Our study on CPE employed oxyazabenzotriazolides of deoxynucleotides[3] (**1a**, **1c**, **1g**, **1t**) and 3′-amino-terminal primers (Scheme 1) to generate phosphoramidate linkages that are isoelectronic to phosphodiesters.[19] Some phosphoramidates are accepted by polymerases.[20] Oxyazabenzotriazolides produce phosphodiesters in RNA-based reactions.[11,21] Earlier attempts to employ known catalysts for ligation in this system, including proflavine,[22] had been essentially unsuccessful.[23]

For our screens, we transferred the assays involving **1a–t** to magnetic beads (Scheme 2). Excess monomers and buffer



Scheme 1. Chemical primer extension.

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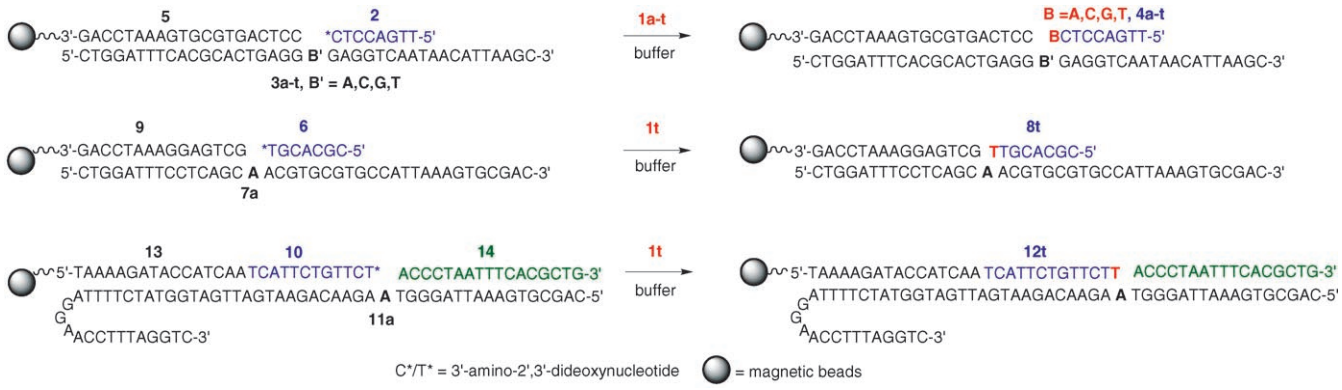

Scheme 2. Primer extension systems employed.

salts can be readily washed off from the bead-bound DNA, thus facilitating MALDI-TOF mass spectrometric analysis. By using the bead-based system, primer **2** was extended as directed by template **3**, to generate **4a–t** in the presence of biotin-bearing **5**, which served both as capture strand for the template and as helper oligonucleotide.[3] We monitored the reactions by briefly heating analytical samples and detecting the primer released into the supernatant by mass spectrometry under conditions that allowed for quantitative detection.[24] Under optimized conditions,[3] monomer **1c** reacted to give 92% primer conversion (Table 1). A significant acceleration was observed with a modest level of pyridine as "co-solvent". Neither 4-dimethylaminopyridine (DMAP), a well-established transacylation catalyst, nor tetrazole, the catalyst commonly used for solid-phase DNA synthesis, was able to produce the same acceleration as pyridine, which gave $t_{1/2} <$ 1 min and 95% primer conversion (Table 1; Figure 1). An optimization of the conditions (temperature, pyridine content, and monomer concentration) involving the "worst-case" monomer thymidine monophosphate, which forms weak base pairs and engages in weak stacking interactions, gave reaction conditions reminiscent of those of solid-phase DNA synthesis involving phosphoramidites[25] (0.1 M monomer concentration, 0.3 M activator, 23.5 °C; see Table 2).

Even thymidine (**1t**) underwent template-directed addition to the growing primer with a half-life time of 0.3 min (Figure 1). For the more readily incorporated monomers that engage in strong base pairing and/or strong stacking interactions, near-quantitative conversion was observed after 30 s, the earliest time point accessible with our methodology (Figure 2). As crude active esters of monomers were

Table 1: Effect of additives on CPE with **1c**, **2**, **3g**, and **5**.[a]

Additive	$t_{1/2}$ [min]	Conversion [%][b]
– (control)	3.9	92
pyridine	0.8	95
DMAP	–	0
pyridine *N*-oxide	6.4	94
chinoline	4.3	90
2-methoxypyridine	3.6	99
dicyanoimidazole[c]	1.9	99
tetrazole	1.7	99

[a] Conditions: 25 pmol **2**, **3g**, and **5**, 3.6 mM **1c**, 200 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(4-butanesulfonic acid) (HEPBS), pH 8.9, 400 mM NaCl, 80 mM $MgCl_2$, 100 mM additive. [b] Extrapolated from fit. [c] 20 vol% of saturated solution (< 100 mM).

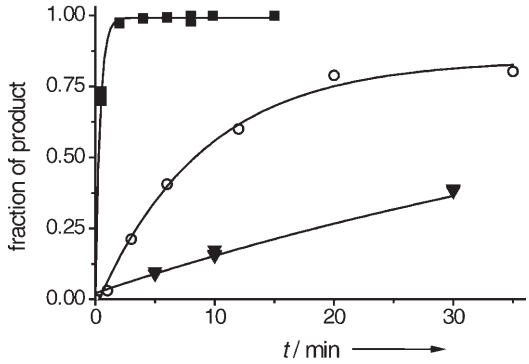

Figure 1. Kinetics of extension of **2** templated by **3a**/**5** with 3.6 mM **1t** alone (▼), 3.6 mM **1t** and 100 mM pyridine (○), or 100 mM **1t** and 300 mM pyridine (■).

Table 2: Effect of reaction conditions on primer extension.

Substrates[a]	[**1**] [mM]	Pyridine [mM]	*T* [°C]	$t_{1/2}$ [min]	Conversion [%][b]
3a/2/5/1t	3.6	100	10	6.9	91
3a/2/5/1t	3.6	100	20	6.2	84
3a/2/5/1t	3.6	100	23.5	5.4	80
3a/2/5/1t	3.6	100	30	8.4	47
3a/2/5/1t	5.0	100	23.5	3.4	95
3a/2/5/1t	10	100	23.5	2.3	95
3a/2/5/1t	20	100	23.5	2.1	99
3a/2/5/1t	50	300	23.5	1.5	95
3a/2/5/1t	100	300	23.5	0.3	99
3a/2/5/1t[c]	100	300	23.5	0.3	99
3t/2/5/1a	100	300	23.5	< 0.15	97
3c/2/5/1g	100	300	23.5	< 0.15	99[d]
3g/2/5/1c	100	300	23.5	< 0.15	99[d]
7a/6/9/1t	100	300	23.5	0.4	98
11a/10/13/14/1t[e]	100	300	23.5	0.8	99

[a] Conditions: 25 pmol oligomers, 200 mM HEPBS, pH 8.9, 400 mM NaCl, 80 mM $MgCl_2$. [b] Determined from fit. [c] **1t** purified by HPLC analysis. [d] 99% conversion after 30 s. [e] 3.6 equivalents of **14** used.

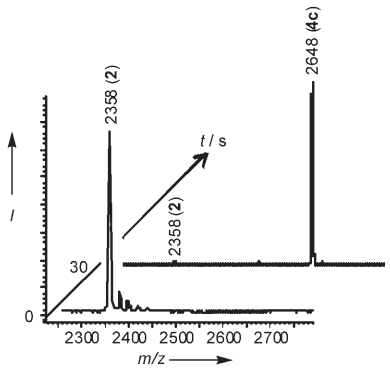

Figure 2. MALDI-TOF mass spectra from primer extension with **2**, **3g**, **5**, and **1c** (100 mM) at 300 mM pyridine, at 0 s and after 30 s.

employed without purification (in analogy to amino acid activation in peptide synthesis), control assays were performed with HPLC-purified **1t**. These gave the same $t_{1/2}$ values as the assays with the crude material, within experimental error.

Two additional sequence motifs were tested, both of which featured adenine, that is, the poorest-templating base (Scheme 2). The first of the motifs, which consisted of **7a**, **6**, and **9**, gave $t_{1/2} = 0.4$ min. The other, which involved long template **11a**, primer **10**, capture oligonucleotide **13**, and helper **14**, reacted with $t_{1/2} = 0.8$ min (Table 2).

CPE fueled by 2-methylimidazolides[7] was also accelerated in the presence of pyridine. For incorporation of deoxycytidine monophosphate opposite guanine, a fourfold increase in rate was observed at 150 mM pyridine ($t_{1/2} = 1$ h).

Exploratory experiments with a T-boosted mixture of all four oxyazabenzotriazolide-activated deoxynucleotides (OAtdNs) nucleotides[3] were run to demonstrate sequence selectivity. These showed sequence-selective nucleotide incorporation for both the weakly base-pairing (template **3a**) and strongly base-pairing cases (**3g**; Figure 3). Small peaks, on a level of those of salt adducts, were discernible for presumed extension products with misincorporated nucleotides. These are roughly equally distributed among the three possible nucleobases, which points to untemplated synthesis as a possible low-level side reaction. If side products were to be extended in subsequent extensions (which is far from certain), a significant background could build up after multiple extensions.

Our methodology is not limited to single-nucleotide extension. Figure 4 shows a MALDI-TOF mass spectrum of a doubly extended primer. Both extension reactions were performed in the presence of 300 mM pyridine. The first employed **15**, photolably 3′-protected with a nitrophenylpropyloxycarbonyl (NPPOC) group.[26] Monomer **15** reacted only 1.8-fold less rapidly than **1t** under the same conditions. Deprotection with UV light involved 10×30 s of irradiation. The half-life time for the second extension step was 62 s under unoptimized conditions. Either extension reaction occurred with near-quantitative yield (see the Supporting Information). Full primer conversion can also be achieved with NPPOC-protected **15** in the absence of helpers (unpublished results). Multiple extensions may be performed with short, displaceable helpers, as demonstrated for RNA.[11,21]

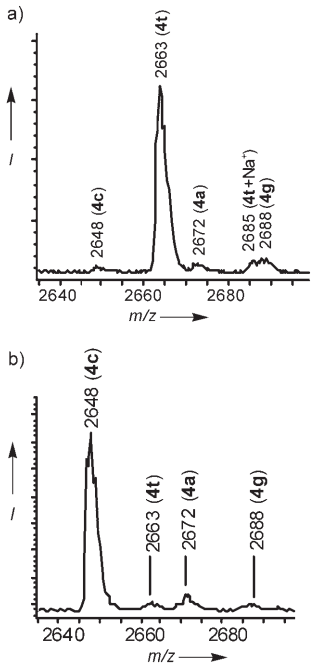

Figure 3. MALDI-TOF mass spectra from primer extension with a mixture of **1t**, **1a**, **1c**, and **1g** and template a) **3a** or b) **3g** in the presence of **5** at 300 mM pyridine after a reaction time of 15 min.

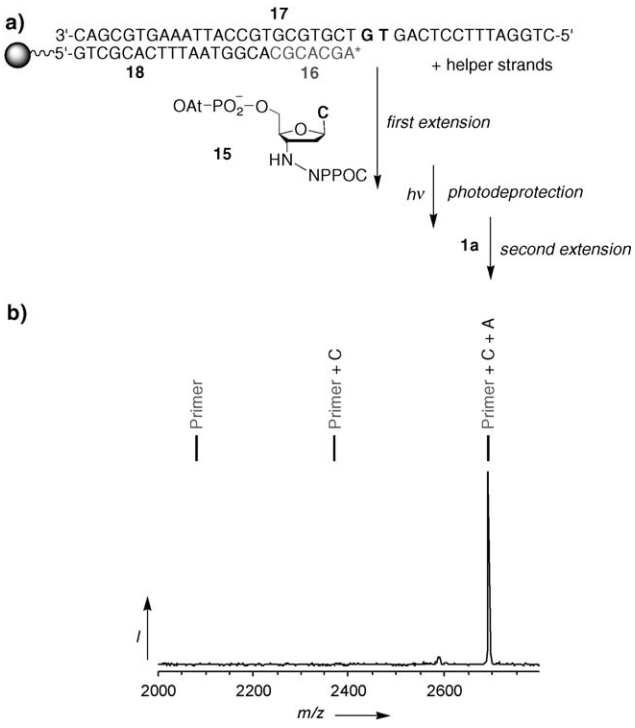

Figure 4. Double extension of primer. a) Reaction system featuring primer **16**, template **17**, capture oligonucleotide **18**, and photolably protected monomer **15**, or monomer **1a**. b) MALDI-TOF mass spectrum of the doubly extended primer after reactions with **15** (20 mM), *hν*, and **1a** (100 mM). See the Supporting Information for details.

It is likely that pyridine exerts its rate-accelerating effect through the formation of a covalent intermediate (Scheme S3, Supporting Information). Pyridinium phosphates have been occasionally proposed as reactive intermediates in reactions leading to phosphoramidates.[27,28]

Our results demonstrate that CPE reactions can be accelerated to a degree that makes them as fast as extension cycles on DNA synthesizers. CPE as presented here also shares key features with automated DNA synthesis: it involves in situ generation of the active species through an activator, produces near-quantitative yields, and occurs on a solid support. Unlike DNA synthesis, CPE is directed by a template, which allows, in principle, for processes like "chemical PCR". An array of templates exposed to the same reaction solution may undergo simultaneous extension, each directed by the sequence to which the primer is bound. Release of the products requires no more than heating of the support.

Primer extension is important for several fields, but fundamental studies thus far have focused on polymerase-based systems,[29] whereas enzyme-free replication reactions are considered mostly in the context of the origin of life.[30] Given the reaction rates in the current system, enzyme-free primer extension may become attractive for both. In the latter field, the catalytic effect of pyridine demonstrated herein could lead to new scenarios for classical replication reactions involving phosphoramidates.[30]

Experimental Section

Assays were carried out in a suspension (2.5 μL) containing biotin-binding magnetic beads (loading approximately 10 pmol μL^{-1}). A freshly suspended slurry of beads loaded with capture oligonucleotide **5**, **9**, **13**, or **18** (2.5 μL) was transferred to a polypropylene vessel. The storage buffer solution was removed with a magnet under the vessel. After addition of the stock solution (1 μL) of the reaction buffer (HEPBS (500 mM), NaCl (1M), $MgCl_2$ (200 mM), and the stated amount of pyridine at pH 8.9), aliquots of the oligonucleotide solutions (**2** and **3a**–**t**, or **6** and **7a**, or **10**, **11a**, and **14**, 0.25 μL, 100 μM strands in water) were added. Addition of the solution (1 μL) of the activated monomer gave the final concentrations (oligonucleotides (10 μM), HEPBS (200 mM), NaCl (400 mM), and $MgCl_2$ (80 mM)) and started the assay. Samples (0.4 μL) were taken, and the beads washed three times with aqueous NH_4OAc (1 μL, 1M). After addition of water (1.5 μL), the sample was heated to 70 °C for 3 min. The hot supernatant was immediately aspired and treated with Dowex 50 WX8-200 cation-exchange beads (ammonium form). A sample of the supernatant was analyzed by MALDI-TOF mass spectrometry. Assays with mixtures of monomers were performed with a stock solution (1 μL) of a mixture of the activated nucleotides **1a**, **1c**, **1g** (25 mM), and **1t** (125 mM) to give a final concentration of activated nucleotides of 10 mM (**1a**, **1c**, **1g**) and 50 mM (**1t**).

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