JOC Featured Article

pubs.acs.org/joc

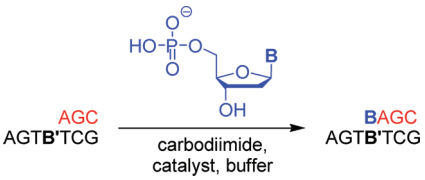
Chemical Primer Extension at Submillimolar Concentration of Deoxynucleotides

Manuel Röthlingshöfer[†] and Clemens Richert*[,‡]

[†]*Institute for Organic Chemistry, University of Karlsruhe/K.I.T., 76131 Karlsruhe, Germany, and* [‡]*Institute for Organic Chemistry, University of Stuttgart, 70569 Stuttgart, Germany*

lehrstuhl-2@oc.uni-stuttgart.de

Received February 10, 2010

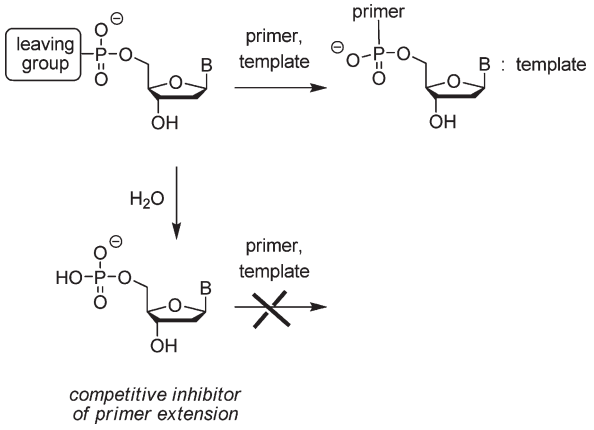


Template-directed primer extension usually requires a polymerase, nucleoside triphosphates, and magnesium ions as cofactors. Enzyme-free, chemical primer extensions are known for preactivated nucleotides at millimolar concentrations. Based on a screen of carbodiimides, heterocyclic catalysts, and reactions conditions, we now show that near-quantitative primer conversion can be achieved at submillimolar concentration of any of the four deoxynucleotides (dAMP, dCMP, dGMP and dTMP). The new protocol relies on in situ activation with EDC and 1-methylimidazole and a magnesium-free buffer that was tested successfully for different sequence motifs. The method greatly simplifies chemical primer extension assays, further reduces the cost of such assays, and demonstrates the potential of the in situ activation approach.

Introduction

Chemical primer extension is a DNA- or RNA-templated reaction that extends a primer by individual nucleotides, directed by Watson−Crick base pairing.[1] It encompasses template-directed incorporation of activated ribonucleotides on RNA[2−4] or activated deoxynucleotides on DNA.[5] Nature performs the corresponding reaction in the active site of polymerases, where the transphosphorylation step occurs in an environment shielded from water.[6] Like many enzyme-catalyzed reactions, energetically favorable hydrolysis of substrates and products can thus be avoided. But, chemical primer extension, a methodology that builds on work on potentially prebiotic modes of replication and transcription,[7] occurs on "naked" DNA. So, its reaction site is necessarily open to solvent. Nonaqueous solvents are not appropriate because they interfere with base pairing, and DNA as a polyanion does not readily dissolve in them. As a consequence, hydrolysis of the activated monomers competes with coupling,[8] curtailing yields to values below those for reactions in organic solvents without competing hydrolysis pathway (Scheme 1). For chemical primer extension reactions, "spent" monomers are free nucleotides that may

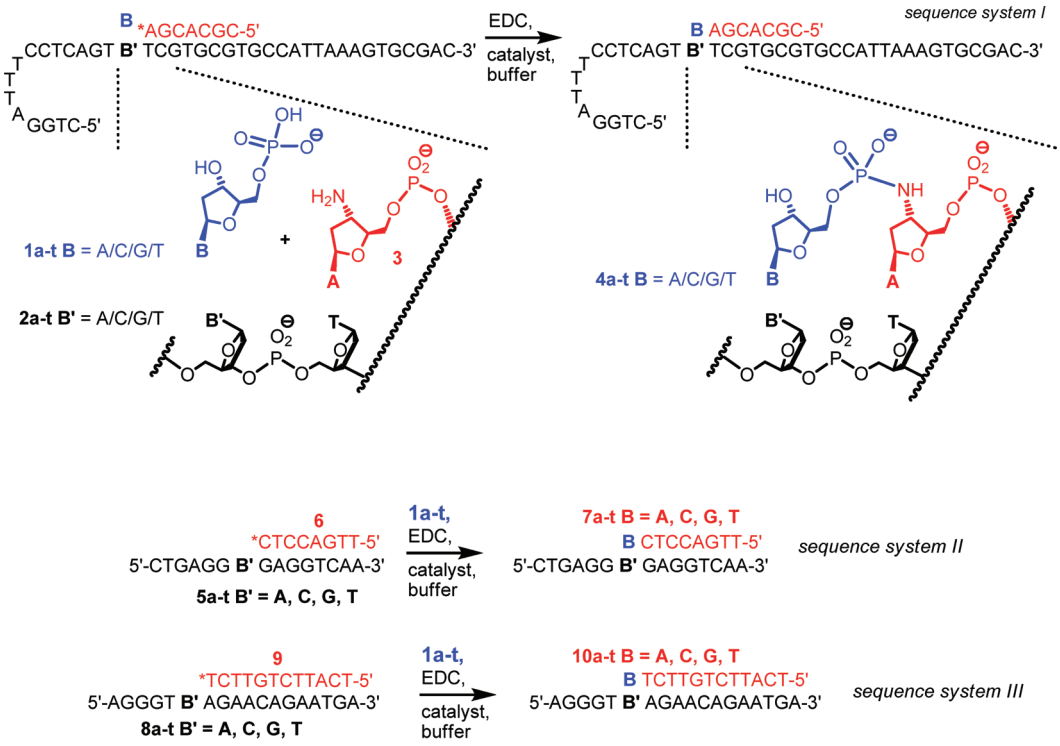
SCHEME 1. Possible Fates of Monomer



(1) Rojas Stütz, J. A.; Kervio, E.; Deck, C.; Richert, C. *Chem. Biodiversity* **2007**, *4*, 784.
(2) Prabahar, K. J.; Ferris, J. P. *J. Am. Chem. Soc.* **1997**, *119*, 4330.
(3) Chaput, J. S.; Sinha, S.; Switzer, C. *Chem. Commun.* **2002**, *15*, 1568.
(4) Kozlov, I. A.; Orgel, L. E. *Origins Life Evol. Biospheres* **1999**, *29*, 593.
(5) Schrum, J. P.; Ricardo, A.; Krishnamurthy, M.; Blain, J. C.; Szostak, J. W. *J. Am. Chem. Soc.* **2009**, *131*, 14560.
(6) Berdis, A. J. *Chem. Rev.* **2009**, *109*, 2862.
(7) (a) Orgel, L. E. *Crit. Rev. Biochem. Mol. Biol.* **2004**, *39*, 99. (b) Kozlov, I. A.; Orgel, L. E. *Mol. Biol.* **2000**, *34*, 781. (c) Monnard, P. A.; Szostak, J. W. *J. Inorg. Biochem.* **2008**, *102*, 1104.

DOI: 10.1021/jo1002467 Published on Web 04/05/2010

SCHEME 2. Primer Extension Reactions Studied



bind to the template as tightly as the reactive monomers, thus acting as (competitive) inhibitors.[9] Enzyme-free copying reactions of DNA and RNA have long been known to stall after partial generation of complementary strands for all but the most favorable templates.[4,5,10,11]

Mononucleotides bind weakly to oligonucleotides in aqueous solution. This, together with background hydrolysis, leads to the need for high monomer concentrations and a large excess of monomer over template/primer in chemical primer extension.[12] To the best of our knowledge, no such reactions are known that lead to near-quantitative primer conversion at submillimolar monomer concentration, both for active esters[13] and active amides[14] as monomers. This includes assays with amino-terminal primers that are more reactive than their natural, hydroxy-terminated counterparts.[15] Both the traditional imidazolides and the oxyazabenzotriazolides introduced more recently require at least 3 mM monomer concentration for high-yielding assays.[13] More than 10 equiv of monomer are the rule, not the exception. This is unsatisfactory, particularly for expensive monomers, such as fluorophore-labeled OAt esters of deoxynucleotides with photolabile linkers.[16]

We reasoned that the limitations caused by competing hydrolysis may be overcome by providing an excess of an inexpensive condensation reagent as a source of freshly (re)activated monomer during the course of a primer extension. Template-directed chemical ligations of oligonucleotides with in situ activation, using the water-soluble carbodiimide EDC, were known from the literature,[17] including ligations via cyclic phosphates as active species.[18] In situ activation agents that have been used for ligations include cyanoimidazole and cyanogen bromide.[19] We decided to study carbodiimides, together with a catalyst, hoping to achieve full primer conversion at submillimolar nucleotide concentration.

In our study, we used unactivated 2′-deoxynucleoside 5′-*O*-monophosphates (dAMP, dGMP, dCMP, TMP, **1a**−**t**) as inexpensive monomers and a 3′-aminoterminal oligodeoxynucleotide (**3**) as primer in DNA-templated reactions that produce elongated primers (**4a**−**t**) with a phosphoramidate linkage (Scheme 2). Further, we decided to forego the use of a "helper oligonucleotide" that binds downstream of the templating base[13,20] and to focus on the most weakly binding deoxynucleotide (thymidine monophosphate) during the optimization study to ensure that the conditions established are as general as possible. Here we report such simplified chemical primer extension assays that avoid the preactivation step and

(8) Kanavarioti, A.; Bernasconi, C. F.; Alberas, D. J.; Baird, E. E. *J. Am. Chem. Soc.* **1993**, *115*, 8537.
(9) Vogel, S. R.; Richert, C. *Chem. Commun* **2007**, 1896.
(10) Wu, T.; Orgel, L. E. *J. Am. Chem. Soc.* **1992**, *114*, 317.
(11) Hey, M.; Hartel, C.; Göbel, W. *Helv. Chim. Acta* **2003**, *86*, 844.
(12) Röthlingshöfer, M.; Kervio, E.; Lommel, T.; Plutowski, U.; Hochgesand, A.; Richert, C. *Angew. Chem.* **2008**, *120*, 6154; *Angew. Chem., Int. Ed.* **2008**, *47*, 6065.
(13) Hagenbuch, P.; Kervio, E.; Hochgesand, A.; Plutowski, U.; Richert, C. *Angew. Chem.* **2005**, *117*, 6746; *Angew. Chem., Int. Ed.* **2005**, *44*, 6588.
(14) Inoue, T.; Orgel, L. E. *J. Am. Chem. Soc.* **1981**, *103*, 7666.
(15) Orgel, L. E.; Lohrmann, R. *Acc. Chem. Res.* **1974**, *7*, 368.
(16) Griesang, N.; Giessler, K.; Lommel, T.; Richert, C. *Angew. Chem.* **2006**, *118*, 6290; *Angew. Chem., Int. Ed.* **2006**, *45*, 6144.
(17) (a) Sievers, D.; v. Kiedrowski, G. *Nature* **1994**, *369*, 221. (b) Narayanan, S.; Dalpke, A. H.; Siegmund, K. H.; Heeg, K.; Richert, C. *J. Med. Chem.* **2003**, *46*, 5031. (c) Dolinnaya, N. G.; Tsytovich, A. V.; Sergeev, V. N.; Oretskaya, T. S.; Shabarova, Z. A. *Nucleic Acids Res.* **1991**, *19*, 3073. (d) Zielinksi, W. S.; Orgel, L. E. *Nature* **1987**, *327*, 346.
(18) Pitsch, S.; Krishnamurthy, R.; Bolli, M.; Wendeborn, S.; Holzner, A.; Minton, M.; Lesueur, C.; Schlönvogt, I.; Jaun, B.; Eschenmoser, A. *Helv. Chim. Acta* **1995**, *78*, 1621.
(19) (a) Luebke, K. J.; Dervan, P. B. *J. Am. Chem. Soc.* **1991**, *113*, 7447–7448. (b) Luebke, K. J.; Dervan, P. B. *Nucleic Acids Res.* **1992**, *20*, 3005–3009.
(20) Vogel, S. R.; Deck, C.; Richert, C. *Chem. Commun.* **2005**, 4922.

do not require a helper strand to achieve quantitative primer conversion at submillimolar monomer concentrations.

Results

Scheme 2 shows the primer extension reactions studied. We chose thymidine-5′-monophosphate and template **2a** as our default system because incorporation of the weakly base pairing and weakly stacking thymine is the most difficult case among the four nucleotides. We reasoned that a solution found for this nucleotide would be readily transferable to the remaining three cases (A/C/G) that have more favorable binding properties.

We set up our initial assays with 200 equiv of EDC (100 mM) for the (re)activation of the monomer. A mixed sequence, containing any of the four nucleobases, was selected as template (**2a**−**t**), together with a corresponding short primer (**3**, Scheme 2). As mentioned above, the oligonucleotide complex does not feature a helper strand that would otherwise accelerate the primer extension reaction and improve its yield.[13]

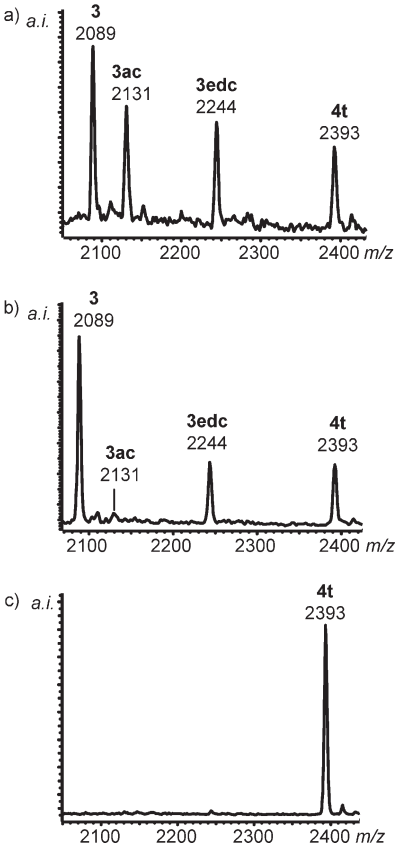


FIGURE 1. MALDI-TOF mass spectra of samples from primer extension reactions involving *sequence system I* and template **2a** at 0.5 mM dTMP (**1t**): (a) after 5 days at pH 7.9, 20 °C and 100 mM EDC without a catalyst; (b) under the same conditions, except that acetic acid was more rigorously excluded; (c) under optimized conditions (pH 7.0, 400 mM EDC, 100 mM 1-methylimidazole, 10 °C, without $MgCl_2$ after 7 days).

Our first experiment with in situ activation used 200 mM HEPBS buffer pH 7.9, 400 mM NaCl, 80 mM $MgCl_2$, i.e., standard buffer conditions for chemical primer extension,[21] and 0.5 mM of free, unactivated dTMP (**1t**) at 20 °C. As shown in Figure 1a, the extension reaction was slow under these conditions, with 38% remaining primer after 5 days. Two major side products were detected in MALDI-TOF mass spectra. One gave a mass 42 Da higher than that of the unreacted primer (**3ac**, Figure 2), suggesting acetylation with acetic acid. The acetic acid may have originated from residual triethylammonium acetate (TEAA) buffer used for HPLC purification of the oligonucleotides. The other side reaction led to a peak with a mass 155 Da higher than that of the primer. This was assigned to a guanidinium species (**3edc**) resulting from the addition of EDC to the terminal amine.[22]

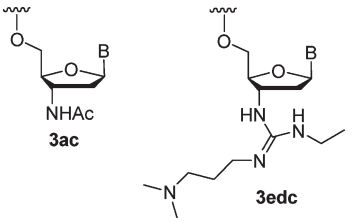


FIGURE 2. Side products observed in chemical primer extension reactions with in situ activation.

TABLE 1. Effect of Heterocycles on the Formation of the Desired Extended Primer Product and Side Products in Chemical Primer Extension Reactions with *Sequence System I* and Template 2a[a]

heterocycle	**4t** (%)	**3ac** (%)	**3edc** (%)
none (control)	9	< 1	18
1-hydroxy-7-azabenzotriazole[b]	16	77	7
1-hydroxybenzotriazole[b]	15	38	9
2-methylimidazole[c]	10	2	53
4,5-dicyanoimidazole[b]	65	6	10
2-nitroimidazole[b]	27	40	20
4-nitroimidazole[b]	13	4	18
benzimidazole[b]	< 1	< 1	11
1,2,4-triazole[b]	< 1	< 1	6
3-nitrotriazole[b]	76	10	10
tetrazole	13	85	2
5-ethylthiotetrazole[b]	2	41	< 1
pyridine	10	46	23
1-phenylimidazole[d]	11	36	17
1-vinylimidazole	21	18	14
1-hydroxyethylimidazole	41	< 1	10
1-ethylimidazole	30	2	12
1-methylimidazole	57	< 1	13
1-methyladenine[b]	22	1	16
3-methyladenine[b]	58	2	12
7-methyladenine[b]	5	2	10
9-methyladenine[b]	11	2	16

[a]Product distribution after 7 d reaction time. Conditions: 0.5 mM dTMP (**1t**), 100 mM EDC, 200 mM HEPBS pH 7.9, 400 mM NaCl, 80 mM $MgCl_2$, 20 °C; 100 mM heterocycle, except as described in the following footnotes. [b]0.5 μL of satd solution added (< 100 mM). [c]10 mM. [d]not miscible with water; 0.5 μL of a freshly vortexed mixture/emulsion (6%, v/v) was added.

We then focused on suppressing the side reactions. A simple reversed-phase cartridge purification step reduced the remaining amount of acetic acid to a level that made primer acetylation insignificant in the absence of catalysts (entry 1, Table 1). The reaction of the primer with EDC was suppressed by optimizing the reaction conditions. For this, the critical step was the identification of a catalyst that generates a more reactive version of the monomer and thus increases the rate of the desired reaction of the activated

(21) Rojas Stütz, J. A.; Richert, C. *J. Am. Chem. Soc.* **2001**, *123*, 12718.

(22) Williams, A.; Ibrahim, I. T. *Chem. Rev.* **1981**, *81*, 589.

monomer with the primer, with little accelerating effect on the side reactions. To achieve this, we tested a number of heterocycles and monitored the conversion by MALDI-TOF MS (Table 1).

Among the heterocycles chosen, some are known as additives for peptide coupling, like HOBt[23] and HOAt.[24] Others, like 2-methylimidazole,[14] triazole,[25] 1-methyladenine, and 3-methyladenine,[2] have previously been used as leaving groups in chemical primer extension reactions. Pyridine[12] and 1-hydroxyethylimidazole[26] have been employed as catalyst for these reactions. Yet other compounds tested, such as DCI, tetrazole, and 5-ethylthio-1*H*-tetrazole,[27] are used as catalysts in solid-phase DNA and RNA syntheses. Table 1 shows how strongly the putative catalysts affect the product distribution of the EDC-driven reaction. Most surprising was the finding that two compounds previously used in chemical primer extension with preactivated monomers (2-methylimidazole and pyridine) shift this distribution toward the EDC adduct. Others, like HOAt, a leaving group and additive for transacylation reactions, favored the acetylation of the aminoterminal primer.

TABLE 2. Effect of pH on the Product Distribution of Chemical Primer Extension Assays with *Sequence System I* and Template 2a[a]

catalyst	pH[b]	**4t** (%)	**3ac** (%)	**3edc** (%)
4,5-dicyanoimidazole[c]	7.0	62	15	9
	7.9	65	6	10
	8.9	6	4	11
3-nitrotriazole[c]	7.0	62	10	10
	7.9	79	8	8
	8.9	5	2	8
1-hydroxyethylimidazole[d]	7.0	81	3	10
	7.9	28	4	17
	8.9	2	6	13
1-ethylimidazole[d]	7.0	91	2	5
	7.9	27	3	14
	8.9	< 1	< 1	5
1-methylimidazole[d]	6.5	84	4	7
	7.0	91	< 1	5
	7.5	88	< 1	6
	7.9	40	3	20
	8.9	3	4	12

[a]Product distribution after 7 d reaction time. Conditions: 0.5 mM dTMP (**1t**), 100 mM EDC, 200 mM buffer, 400 mM NaCl, 80 mM $MgCl_2$, 20 °C. [b]pH of buffer added, HEPES (pH 6.5, pH 7.0 and pH 7.5), HEPBS (pH 7.9 and pH 8.9). [c]0.5 μL of satd solution added (< 100 mM). [d]100 mM.

The most promising candidates for catalysis were then tested at different pH values. Primer extension reactions of activated monomers with a 3′-aminoprimers are usually done under basic conditions to favor a sufficient fraction of unprotonated amine.[13] The activation reaction of the phosphate groups with EDC, on the other hand, can be expected to benefit from more acidic conditions.[28] The results compiled in Table 2 show that for the 3-nitrotriazole and dicyanoimidazole with their more acidic, electron-deficient heterocycle, pH 7.9 was indeed the best of the three pH values tested. For the imidazoles with a substituent at position 1, pH 7.0 gave higher yields. Since 1-methylimidazole and 1-ethylimidazole gave almost identical results, we used the less expensive 1-methylimidzaole for subsequent assays. The broad application of this catalyst favored this decision. For example, 1-methylimidazole has been successfully used as activator for the capping step in automated DNA synthesis,[29] and 1-methylimidazoliumphosphates of nucleotides and oligonucleotides have been used for the modification of enzymes,[30] as substrates for enzymes,[31] or for the synthesis of symmetrical pyrophosphates.[32] At pH 7.0, the half-life time of primer extension in *sequence system I* with template **2a**, dTMP (**1t**), and 1-methylimidazole was found to be 29 h.

We then tested buffers other than HEPES. Among these were those based on 2,6-lutidine[10] or imidazole hydrochloride,[33] known from other nonenzymatic template-directed assays. In our system, 2,6-lutidine gave a yield of 93%. This value is comparable to that achieved with HEPES, but the reaction was slower by a factor of 2. The imidazole buffer gave essentially no conversion. Next, exploratory experiments with water-soluble activators other than EDC were performed. These included 1-(3-(trimethylammonium)-propyl)-3-ethylcarbodiimide iodide (ETC),[34] *N*-cyclohexyl-*N*′-(β-[*N*-methylmorpholino]ethyl)carbodiimide *p*-toluenesulfonate (CME),[35] and 4-(4,6-dimethoxy-1,3,5-triazine-2-yl)-4-methylmorpholinium chloride (DMT-MM).[36] At 100 mM activator and 100 mM 1-methylimidazole, neither of the activation agents gave encouraging results, with low yields and a large extent of side product formation, both at pH 7.0 and pH 7.9. So, neither was pursued further. Instead, we studied the effect of the EDC concentration on the rate of primer extension reaction (Figure 3).

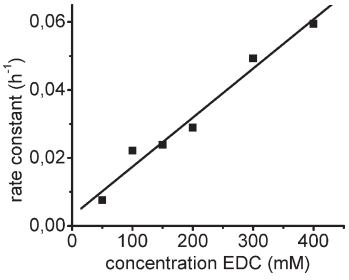


FIGURE 3. Effect of EDC concentration on rate of primer extension reaction with *sequence system I* and template **2a** (0.5 mM dTMP **1t**, 200 mM HEPES pH 7.0, 400 mM NaCl, 80 mM $MgCl_2$, 100 mM 1-methylimidazole, 20 °C).

(23) König, W.; Geiger, R. *Chem. Ber*. **1970**, *103*, 788.
(24) Carpino, L. A. *J. Am. Chem. Soc*. **1993**, *115*, 4397.
(25) Prabahar, K. J.; Ferris, J. P. *J. Am. Chem. Soc*. **1994**, *116*, 10914.
(26) Mansy, S. S.; Schrum, J. P.; Krishnamurthy, M.; Tobe, S.; Treco, D. A.; Szostak, J. W. *Nature* **2008**, *454*, 122.
(27) Sproat, B.; Colonna, F.; Mullah, B.; Tsou, D.; Andrus, A.; Hampel, A.; Vinayak, R. *Nucleosides Nucleotides* **1995**, *14*, 255.
(28) Ivanovskaya, M. G.; Gottikh, M. B.; Shabarova, Z. A. *Nucleosides Nucleotides* **1987**, *6*, 913.
(29) Brown, T.; Brown, D. J. S. In *Oligonucleotides and Analogues: A Practical Approach*; Eckstein, F., Ed.; Oxford University Press: Oxford, 1991.
(30) (a) Knorre, D. G.; Buneva, V. N.; Baram, G. I.; Godovikova, T. S.; Zarytova, V. F. *FEBS Lett*. **1986**, *194*, 64. (b) Godovikova, T. S.; Grachev, M. A.; Kutyavin, I. V.; Tsarev, I. G.; Zarytova, V. F. *Eur. J. Biochem*. **1987**, *166*, 611.
(31) Huang, K.; Arabshahi, A.; Wei, Y.; Frey, P. A. *Biochemistry* **2004**, *43*, 7637.
(32) Kanavarioti, A.; Lu, J.; Rosenbach, M. T.; Hurley, T. B. *Tetrahedron Lett*. **1991**, *32*, 6065.
(33) Rohatgi, R.; Bartel, D. P.; Szostak, J. W. *J. Am. Chem. Soc*. **1996**, *118*, 3340.
(34) Taha, S. M.; Ferguson-Miller, S. *Biochemistry* **1992**, *31*, 9090.
(35) (a) Sheehan, J. C.; Hlavka, J. J. *J. Org. Chem*. **1956**, *21*, 439. (b) Naylor, R.; Gilham, P. T. *Biochemistry* **1996**, *5*, 2772.
(36) (a) Kunishima, M.; Kawachi, C.; Hioki, K.; Terao, K.; Tani, S. *Tetrahedron* **2001**, *57*, 1551. (b) Li, X.; Gartner, Z. J.; Tse, B. N.; Liu, D. R. *J. Am. Chem. Soc*. **2004**, *126*, 5090. (c) Alongi, M.; Minetto, G.; Taddei, M. *Tetahedron Lett*. **2005**, *46*, 7069.

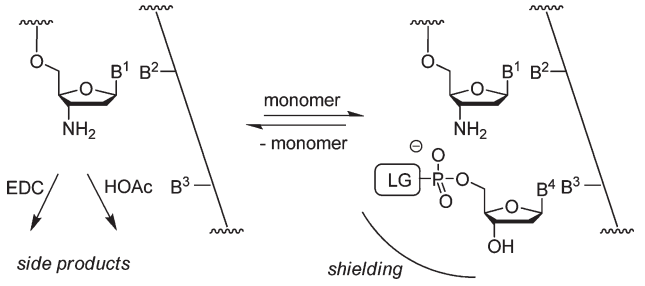
The rate of primer reaction was found to increase with increasing EDC concentration over the entire concentration range tested (Figure 3). This suggests that the activation reaction itself is a rate-limiting process. On the other hand, the overall rate was found to be largely independent of the 1-methylimidazole concentration between 5 and 100 mM catalyst, while much lower (1 mM) and higher (200 mM) 1-methylimidazolde concentrations led to slower primer extension (Table S1, Supporting Information). High levels of the carbodiimide also led to more formation of EDC adduct **3edc** as side product (Table S2, Supporting Information). This prompted us to study the effect of temperature on the rate and yield of the chemical primer extension reaction (Table 3).

TABLE 3. Effect of Temperature on the Rate of Primer Conversion and Product Distribution of Chemical Primer Extension with *Sequence System I* and Template 2a[a]

temp (°C)	[EDC] (mM)	$t_{1/2}$[b] (h)	**4t** (%)	**3ac** (%)	**3edc** (%)
25	100	22	78	1	21
20		36	95	< 1	3
15		38	98	< 1	< 1
20	400	12	93	< 1	7
15		14	95	1	4
10		16	> 99	< 1	< 1

[a]Product distribution after 7 d reaction time. Conditions: 0.5 mM dTMP (**1t**), 200 mM HEPES pH 7.0, 400 mM NaCl, 80 mM $MgCl_2$, 100 mM 1-methylimidazole. [b]Calculated from fit to kinetic data.

SCHEME 3. Shielding Effect of Bound Monomer on the Amino Terminus of the Primer[a]



[a]Lowering the temperature shifts the binding equilibrium to the bound state, leading to a stronger shielding effect.

We reasoned that at lower temperature, the binding equilibrium involving monomer and template/primer would be shifted to the bound side. The bound monomer would then shield the amino group at the terminus of the primer from attack by both EDC and activated acetate (Scheme 3). Gratifyingly, a decrease in temperature was indeed found to significantly reduce the extent of formation of either of the side products. At the same time, a colder assay solution was found to have only a modest effect on the rate of the primer extension, most probably because a stronger template effect accelerates the extension, compensating for the lower intrinsic reactivity of the monomer−oligonucleotide complex.

Finally, we studied the effect of buffer and salt concentration on the assay. Surprisingly, reducing the concentration of $MgCl_2$ in the buffer increased the rate of primer extension (Table 4). We observed 98% extended primer and a half-life time of 11 h at 10 °C and 400 mM EDC. This is in sharp contrast to polymerase-catalyzed primer extension reactions, where two essential magnesium ions coordinate to the triphosphate in the active site.[37] Further, Mg^{2+} is known to increase duplex stability[38,39] and should therefore increase the template effect. But the Mg^{2+} ions may also bind to the phosphate group of the monomer[40] and block its reactivity toward EDC. Increasing the HEPES concentration from 200 to 300 mM gave no significant change in either yield or rate of the desired extension reaction. Lowering the NaCl concentration from 400 to 200 mM slightly reduced the rate of the extension.

TABLE 4. Effect of $MgCl_2$ concentration on Half-Life Time and Yield of Chemical Primer Extension Assay with *Sequence System I* and Template 2a[a]

temp (°C)	[EDC] (mM)	$MgCl_2$ (mM)	yield **4t**[b] (%)	$t_{1/2}$[c] (h)
20	100	80	95	36
		40	96	28
		10	98	22
10	400	80	> 99	16
		10	93	13
		0	98	11

[a]Conditions: 0.5 mM dTMP (**1t**), 200 mM HEPES pH 7.0, 400 mM NaCl, 100 mM 1-methylimidazole, 10 °C. [b]As detected in MALDI-TOF spectra after 7 days. [c]Calculated from fit to kinetic data.

Having established conditions that lead to near-quantitative incorporation of thymidine monophosphate, we then tested the remaining three monomers dAMP, dCMG, and dGMP (**1a**, **1c**, **1g**) under the optimized conditions. We did observe full primer conversion for the monophosphates of dA, dC, and dG (Figure 4). There was a significant difference in the rate of reaction for the different monomers (Figure 5). Compared to previous studies with amino-terminal primer,[13] the order of reactivity was somewhat unexpected. The fact that deoxycytidine monophosphate was incorporated more slowly than thymidine monophosphate cannot readily be explained by differences in base pairing strength, as dCMP (**1c**) should form three hydrogen bonds where dTMP (**1t**) forms only two when binding to the templating base. Cytosine should experience slightly weaker stacking interactions, though, as it lacks the methyl group at the 5-position of thymine.

Experiments with any of the four base pairs at a $MgCl_2$ concentration of 80 mM and in the absence of this salt showed that Mg^{2+} ions have different effects on the rate of reaction for the different monomers. The incorporation of dTMP (**1t**) opposite **2a** was significantly faster without $MgCl_2$, while primer extension by the other three nucleotides, templated by **2g**, **2t**, or **2c** showed less of an effect (Table S3, Supporting Information). These differences may be caused by different affinities of the nucleobases for the divalent cation and effects of the resulting complexes on base pairing.

Control experiments with preactivated monomers (oxyazabenzotriazolides)[13] under buffer conditions optimized for these building blocks (200 mM HEPBS pH 8.9, 400 mM NaCl, 80 mM $MgCl_2$) at the same monomer concentration (0.5 mM) showed much lower yields (52% for incorporation of dAMP, 33% for dCMP, 70% for

(37) Doublie, S.; Tabor, S.; Long, A. M.; Richardson, C. C.; Ellenberger, T. *Nature* **1998**, *391*, 251.
(38) Dove, W. F.; Davidson, N. *J. Mol. Biol.* **1962**, *5*, 467.
(39) Owczarzy, R.; Moreira, B.,G.; You, Y.; Behlke, M. A.; Walder, J. A. *Biochemistry* **2008**, *47*, 5336.
(40) Bloomfield, V. A.; Crothers, D. M.; Tinoco, I. *Nucleic Acids Structures, Properties, and Functions*; University Science Books, Sausalito, 2000.

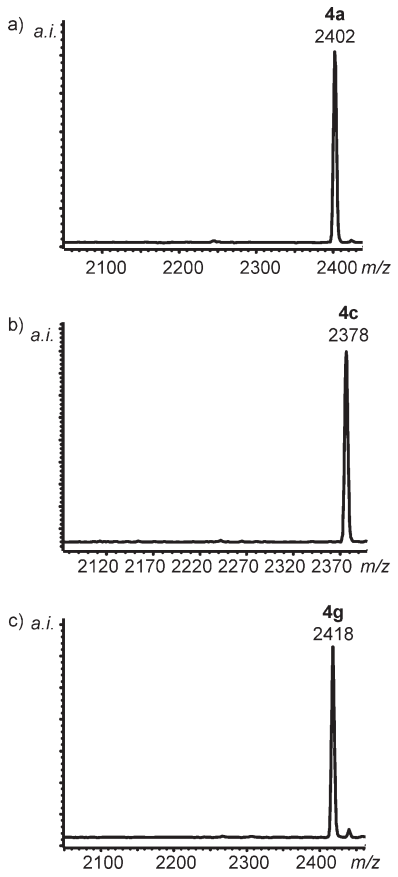


FIGURE 4. MALDI-TOF mass spectra of samples from primer extension reactions drawn after 7 days reaction time under conditions optimized for the incorporation of dTMP **1t** (200 mM HEPES pH 7.0, 400 mM NaCl, 0.5 mM monomer, 400 mM EDC, 100 mM 1-methylimidazole, 10 °C): (a) incorporation of dAMP (**1a**), templated by **2t**, (b) incorporation of dCMP (**1c**), templated by **2g**, (c) incorporation of dGMP (**1g**), templated by **2c**.

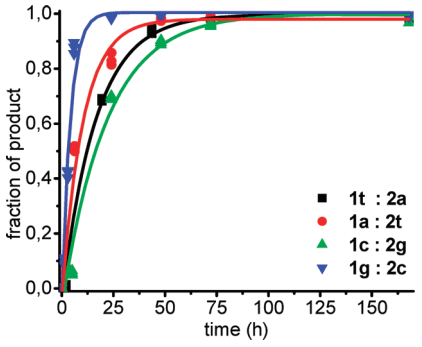


FIGURE 5. Kinetics of primer extension reactions for any of the four base pairs with *sequence system I* under optimized conditions for incorporation of dTMP **1t** (200 mM HEPES pH 7.0, 400 mM NaCl, 0.5 mM monomer, 400 mM EDC, 100 mM 1-methylimidazole, 10 °C).

dGMP and 11% for dTMP), confirming that the EDC-mediated extensions described here are not only attractive for the simplicity of the protocol (no preactivation) but are also indeed superior in terms of yield.

Experiments with mixtures of all four monomers were then performed to demonstrate sequence selectivity. This included both a templating base forming a weak base-pair with the complementary nucleotide (template **2t**) and a strongly base-pairing templating base (template **2c**). With an equimolar mixture of all for dNMPs (**1a**−**t**, 0.5 mM each), excellent sequence selectivity was observed, with a product distribution of >97:1:1:1 (extension of primer by matched monomer vs extension by mismatched monomers). Figure S1 shows MALDI spectra acquired after 24 h reaction time, when >93% of either primer had been converted. No misincorporation of either dCMP or dTMP is discernible (see Supporting Information).

TABLE 5. Effect of Monomer Concentration on Product Distribution of Chemical Primer Extension Assay with *Sequence System I*[a]

dNMP	[**1a**−**t**] (mM)	yield **4a**−**t**[b] (%)	**3ac**[b] (%)	**3edc**[b] (%)
1a	0.5	>99	<1	<1
	0.1	92	<1	7
	0.01	8	3	36
1c	0.5	98	<1	1
	0.1	69	10	13
	0.01	7	13	27
1g	0.5	>99	<1	<1
	0.1	98	<1	<1
	0.01	34	8	27
1t	0.5	98	<1	1
	0.1	79	4	12
	0.01	3	11	24

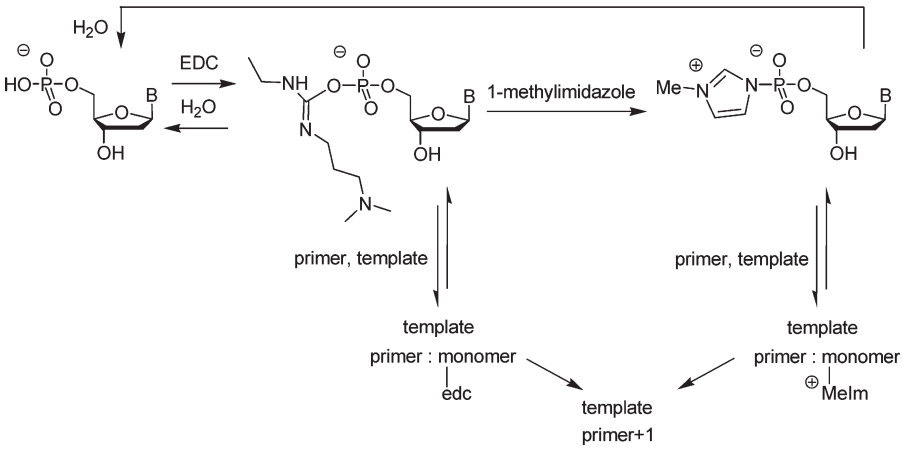
[a]Conditions: 200 mM HEPES pH 7.0, 400 mM NaCl, 400 mM EDC, 100 mM 1-methylimidazole, 10 °C. [b]As detected in MALDI-TOF spectra after 7 days (dNMPs **1a**−**t** = 0.5 mM) or 14 days (dNMPs **1a**−**t** = 0.1 mM or 0.01 mM).

To probe for the limits of our current method, we then decreased the monomer concentration further, to values of 0.1 and 0.01 mM (Table 5). The latter value means that only 1 equiv of mononucleotide is present in the assay solution. We extended the reaction time to 14 days to compensate for expected decreases in rate. At 0.1 mM monomer concentration, we observed at least 69% conversion for all four monomers. For dGMP (**1g**), the monomer with the greatest base pairing potential, full primer conversion was found. At the very lowest monomer concentration tested (0.01 mM) only dGMP gave more than 30% conversion, while the other three monomers (A/C/T) gave less than 10% extended primer. Here, deoxycytidine performed better than thymidine, in line with expected base pairing strength. So, the monomers showing the fastest reaction (dAMP **1a** and dGMP **1g**, Figure 5) also show the highest yield at 0.1 mM monomer concentration.

Finally, to demonstrate that our protocol is suitable for a range of sequence contexts, we tested the methodology with other oligonucleotides (*sequence system II and III*, compare Scheme 2), again including templates for the incorporation of any of the four monomers. We observed almost full conversion in every case, with significant differences in the rate of the reactions (Table 6). Overall, *sequence system I* (**2a**−**t**) showed the fastest reactions. This system features an adenine at the 3′-terminal position of the primer (**3**), the nucleobase offering the best stacking interactions.[41] Among the templating bases, adenine leads to the slowest reactions, though, and incorporation of dGMP (**1g**) on template **5c** was the fastest reaction tested, with a half-life time of just under 2 h.

(41) (a) Bommarito, S.; Peyret, N.; SantaLucia, J., Jr. *Nucleic Acid Res.* **2000**, *28*, 1929. (b) Isaksson, J.; Chattopadhyaya, J. *Biochemistry* **2005**, *44*, 5390.

SCHEME 4. Mechanism Proposed for EDC-Mediated Primer Extension Reactions[a]



[a]Binding of the unactivated monomer and hydrolysis of the template-bound monomer species are omitted for clarity.

TABLE 6. Kinetic Data of Chemical Primer Extension Assays Involving Different Sequence Motifs[a]

template	dNMP	yield[b] (%)	$t_{1/2}$[c] (h)
2a	**1t**	> 99	11
2c	**1g**	> 99	2.8
2g	**1c**	> 99	15
2t	**1a**	> 99	7.1
5a	**1t**	97	30
5c	**1g**	> 99	1.9
5g	**1c**	99	8.9
5t	**1a**	> 99	12.7
8a	**1t**	92	25
8c	**1g**	> 99	3.6
8g	**1c**	> 99	3.1
8t	**1a**	96	31

[a]Conditions: 200 mM HEPES pH 7.0, 400 mM NaCl, monomer 0.5 mM, 400 mM EDC, 100 mM 1-methylimidazole, 10 °C. [b]As detected in MALDI-TOF spectra after 7 days. [c]Calculated from fit to kinetic data.

Discussion

These results show that in situ activation of nucleotides can be successfully used for high-yielding chemical primer extension reactions. In the absence of a catalyst, these reactions are rather slow and low yielding. When combined with a heterocyclic catalyst, extension occurs at low monomer concentrations that otherwise lead to incomplete conversion of primer. The use of the catalyst is critical because accelerating reactions in conventional fashion, through heating, is problematic, as this would further weaken what is already a modest template effect, resulting from a single base pair in aqueous medium. How significant the strengthening of the template effect at low temperatures is, may be gleaned from the rate of primer conversion below 20 °C (Table 3).

Our mechanistic proposal (Scheme 4) assumes that the phosphate group of the monomer reacts first with the carbodiimide to form an active species. This step that may either involve free nucleotide or the template-bound fraction of the monomer, appears to be rate limiting, as demonstrated by the dependence of the reaction rate on the EDC concentration (Figure 3). This activated phosphate may then either be hydrolyzed back to the free nucleotide, or it may undergo the desired primer extension reaction, with or without the intermediacy of a species formed with the catalyst. Using the kinetic approach reported previously,[21] apparent second-order rate constants (k_{2app}) of between 44 and 860 M^{-1} h^{-1} are obtained for the reactions between the different primer/template complexes and the nucleotides listed in Table 6. These values are misleading, though, as activation with EDC, rather than primer extension itself, appears to be rate-limiting (vide supra).

Substituted imidazolides appear to be particularly well suited as catalysts, as evidenced by the results of our screen (Table 1) and the large number of studies with (methyl)imidazolides as preactivated mononucleotides.[5,10,11,21,42] Apparently, the combination of minimal steric demand, favorable electronic properties, and the ability to undergo proton transfer at or near neutral pH makes these heterocycles well suited for chemical primer extension. There is one important difference between the 2-methylimidazolides commonly used as preactivated monomers and the imidazolium ions that can be expected to form from 1-methylimidazole as catalyst (Scheme 4). The former are neutral moieties in unprotonated form, while the latter necessarily exist as cationic species, once they have reacted with the nucleotide. Depending on the pH, the neutral imidazolide may survive for extended periods of time in aqueous solution, whereas the imidazolium species will most likely be hydrolyzed rapidly, if they fail to react with the primer terminus. For the in situ activation procedure, with its slow, EDC-driven activation, the more reactive imidazolium species appears to be better suited than its uncharged, protonable counterpart.

Since EDC is the only reactant that cannot be regenerated upon hydrolysis, an excess of the carbodiimide is required to ensure full conversion of the primer. This measure suffices, down to fairly low monomer concentration, where hydrolysis is the predominant reaction pathway. High EDC concentrations are tolerated very well by DNA.[43] In our experiments, no EDC adducts, other than the one forming with the amino-terminal primer, were detected in our mass spectra, even after extended periods of time. Still, the search for improved versions of EDC is most likely key to more rapid primer extension with free nucleotides at low monomer

(42) Heuberger, B. D.; Switzer, C. *Org. Lett.* **2006**, *8*, 5809.
(43) See, e.g.: Li, X.; Liu, D. R. *Angew. Chem.* **2004**, *116*, 4956. Snyder, T. M.; Tse, B. N.; Liu, D. R. *J. Am. Chem. Soc.* **2008**, *130*, 1392. Gorin, D. J.; Kamlet, A. S.; Liu, D. R. *J. Am. Chem. Soc.* **2009**, *131*, 9189.

concentration. Assays with just one equivalent of monomer (entries 3, 6, 9, and 12 in Table 5) may be best suited for identifying improved in situ activating agents, as these allow for unambiguous determination of the relative rates of competing pathways, via product analysis.

It is also interesting to discuss the current results in more general terms. Nature chiefly employs two strategies to combat the problem of hydrolysis of building blocks. One of these strategies is to utilize metastable building blocks whose background hydrolysis is very slow outside the active site of enzymes. Their active form is then efficient shielding in the active site. The other strategy is to ensure a constant flux in all living organisms, where achieving thermodynamic equilibrium equals death, and frequent uptake of nutrients ensures that losses through hydrolysis will be compensated. Reactivation of monomers is a strategy that mimics this approach to a certain extent. The current study shows than an excess of approximately 10 equiv is required for guanine as nucleobase and 50 equiv for thymine. These values provide benchmarks for processes driven by nucleotides in aqueous solution and thus useful numbers for the estimation of the need of activating "energy" required for autonomous systems and possibly systems driven by other sources of energy than carbodiimides. Such calculations may be particularly interesting for experiments that mimic simple evolutionary processes thriving on activation agents that will dwindle over the course of lengthy extension assays, where multiple extension steps occur.

Independent of such considerations, the current results show that a set of simple, inexpensive, and commercial chemicals (deoxynucleotides, EDC, and 1-methylimidazole) are sufficient to induce high-yielding primer extension, even for the deoxynucleotides experiencing the weakest base pairing and stacking interactions (dT and dC). This is encouraging, as it shows that a simple condensing agent can keep reactions alive that would usually die down as a result of progressing hydrolysis. The methodology presented here may also prove useful for ligation assays involving two oligonucleotides or dumbbell motifs.[17] Further, it will be interesting to see whether the current methodology is suitable for incorporating pyrene nucleotides opposite abasic sited in DNA templates, a reaction known for the corresponding nucleoside triphosphates, directed by polymerases.[44]

Conclusions

In conclusion, here we show how chemical primer extension can be performed at submillimolar monomer concentration. A combination of in situ activation with high EDC concentrations and 1-methylimidazole as catalyst gives full incorporation of all four nucleotides for reacting on different template motifs. Our results show the importance of optimized reaction conditions (temperature, pH, salt content) for avoiding side reactions and thus for achieving acceptable rates for the desired extension reaction. Some of the effects observed, e.g., on the acetylation as side reaction, would have been as difficult to predict at the outset as the successful conversion of the primer under a lean monomer regime.

Experimental Section

Synthesis of 3′-Aminoterminal Primers. Primers **3**, **6**, and **9** were synthesized via standard automated DNA synthesis on controlled pore glass with immobilized 3′-amino-2′,3′-dideoxynucleoside as described previously.[45] Primer **CGCACGA*** (**3**) MALDI-TOF-MS calcd for $[M - H]^-$ 2088.4, found 2088.5. **TTGCCTC*** (**6**) MALDI-TOF-MS calcd for $[M - H]^-$ 2358.6, found 2359.1. **TCATTCTGTTCT*** (**9**) MALDI-TOF-MS calcd for $[M - H]^-$ 3575.4, found 3575.4

Primer Extension (General Protocol). The following protocol is for assays performed under optimized conditions and is representative. Assays were carried out in aqueous solution at 10 °C (2.5 μL total volume). To 1 μL of a stock solution of the buffer [HEPES (pH 7.0) (500 mM), NaCl (1 M)] were added aliquots of oligonucleotide solutions (**2a**−**t** and **3**, 0.25 μL each, 100 μM in water) and 1-methylimidazole (0.5 μL, 500 mM). The reaction was started by the addition of 0.5 μL of a freshly prepared aqueous solution of monomer (dNMPs **1a**, **1c**, **1g**, or **1t**, 2.5 mM) and EDC hydrochloride (2 M). The final concentrations were as follows: HEPES (200 mM), NaCl (400 mM), oligonucleotides (10 μM), monomer (0.5 mM), EDC·HCl (400 mM), 1-methylimidazole (100 mM). Samples (0.4 μL) were taken and diluted with water (10 μL). The resulting solution was treated with a few grains of Dowex 50 WX8-200 cation-exchange beads (ammonium form) for at least 10 min. A sample (1 μL) of the supernatant was then analyzed by MALDI-TOF MS. Assays with mixtures of monomers were performed by adding 0.5 μL of a freshly prepared solution of the monomers (**1a**−**t**, 2.5 mM each) and EDC hydrochloride (2 M) to give a final concentration of each monomer (**1a**−**t**) of 0.5 mM and 400 mM EDC·HCl.

Acknowledgment. We thank Eric Kervio, Christopher Deck, and Tanja Lommel for helpful discussions. This work was supported by DFG (Grant No. RI 1063/8-1 to C.R.).

Supporting Information Available: Tables S1−S3, Figure S1, general information, additional experimental details, and MALDI-TOF spectra of 3′-aminoterminal primers (**3**, **6**, and **9**). This material is available free of charge via the Internet at http://pubs.acs.org.

(44) Matray, T. J.; Kool, E. T. *Nature* **1999**, *399*, 704.

(45) Eisenhuth, R.; Richert, C. *J. Org. Chem.* **2009**, *74*, 26.