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2′/3′ Regioselectivity of Enzyme-Free Copying of RNA Detected by NMR

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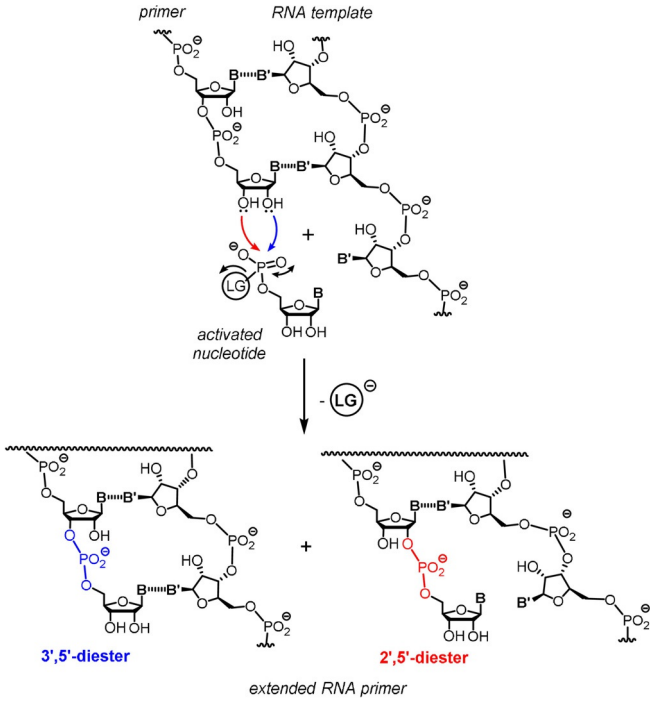
The RNA-templated extension of oligoribonucleotides by nucleotides produces either a 3′,5′ or a 2′,5′-phosphodiester. Nature controls the regioselectivity during RNA chain growth with polymerases, but enzyme-free versions of genetic copying have modest specificity. Thus far, enzymatic degradation of products, combined with chromatography or electrophoresis, has been the preferred mode of detecting 2′,5′-diesters produced in enzyme-free reactions. This approach hinges on the substrate specificity of nucleases, and is not suitable for in situ monitoring. Here we report how ^{1}H NMR spectroscopy can be used to detect the extension of self-templating RNA hairpins and that this reveals the regioisomeric nature of the newly formed phosphodiesters. We studied several modes of activating nucleotides, including imidazolides, a pyridinium phosphate, an active ester, and in situ activation with carbodiimide and organocatalyst. Conversion into the desired extension product ranged from 20 to 90%, depending on the leaving group. Integration of the resonances of H1′ protons of riboses and H5 protons of pyrimidines gave regioselectivities ranging from 40:60 to 85:15 (3′,5′ to 2′,5′ diester), but no simple correlation between 3′,5′ selectivity and yield. Our results show how monitoring with a high-resolution technique sheds a new light on a process that may have played an important role during the emergence of life.

Introduction

Because RNA can act as carrier of genetic information and as a catalyst, it is believed that RNA has played an important role in prebiotic evolution.[1,2] Best known in this context is the "RNA world" scenario,[3] which assumes that oligoribonucleotides preceded proteins and that spontaneously formed RNA sequences were replicated without the help of polymerases.[4–6] If so, the most likely reaction to underlie this form of replication was enzyme-free primer extension.[7] This template-directed reaction leads to new diester bonds between an existing oligonucleotide and an incoming ribonucleotide. For the reaction to occur with detectable rate and yield, the ribonucleotide has to be activated, using either pre-activation in a separate reaction[8,9] or in situ activation.[10] If it does occur, a mixture of regioisomeric products, possessing 2′-5′-linked diesters and 3′-5′-linked diesters, is formed (Scheme 1). This backbone heterogeneity is considered to be one of the major drawbacks of enzyme-free replication.[11]

The influence of the backbone linkage on the physical and chemical properties of RNA is significant. The 2′,5′-linkages affect the helical structure of natural RNA,[12] decrease the thermal stability of RNA duplexes,[13–15] and lower the hydrolytic stability up to 900-fold.[16] On the other hand, a lower duplex melting point can facilitate strand separation after enzyme-free genetic copying,[11] and 2′,3′-cyclic phosphates resulting from



Scheme 1. Enzyme-free primer extension leads to the formation of 3′,5′- and 2′,5′-diester linkages; B = nucleobase complementary to B′; LG = leaving group.

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hydrolysis of 2′,5′-linkages may react to 3′,5′-diesters regioselectively.[17] It has also been shown that RNA with 2′,5′-linkages can act as templates in primer extension.[18]

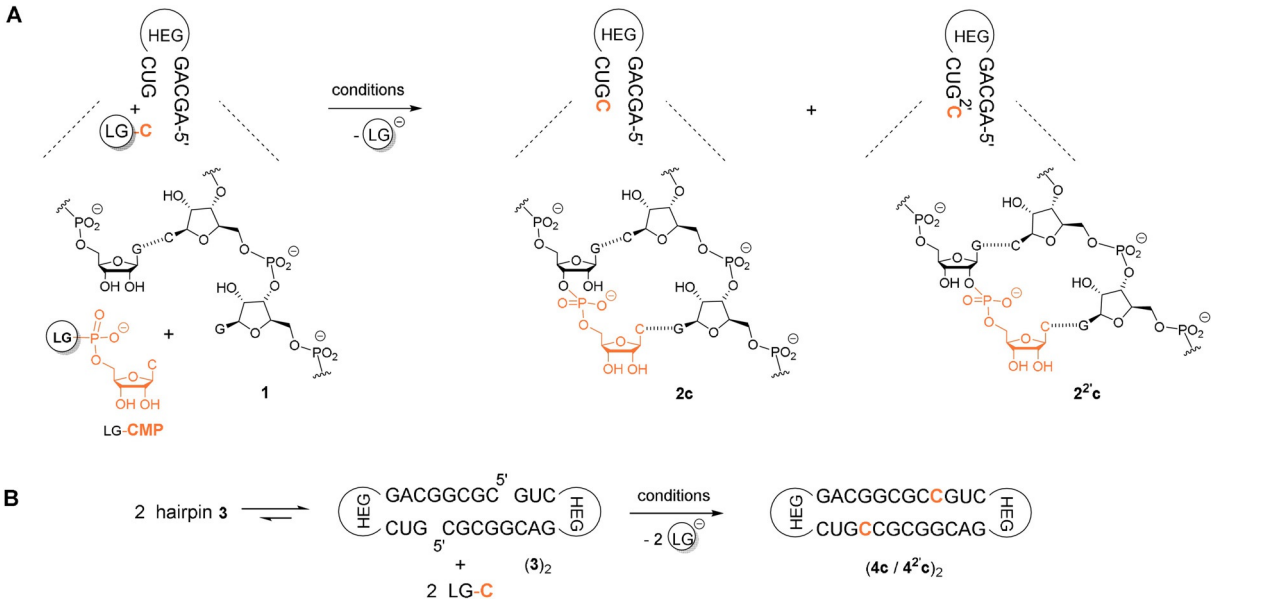
The regioselectivity of the templated formation of diester bonds has been studied for the oligomerization of 5′-phosphorimidazolides of ribonucleotides on homopolymer templates. Divalent cations, such as Zn^{2+} or Pb^{2+} were found to favor 3′,5′-linkages,[19–21] but heavy metals are not desirable in biological assays. Mixed sequence templates were studied later, often in the form of self-templating DNA hairpins with 3′-terminal ribonucleotides. The 2-methylimidazolide of guanosine 5′-monophosphate (2-MeIm-GMP) was reported to give mainly 3′,5′-linked products.[8] Investigating several imidazole-based leaving groups, the Szostak group found a dependence of the 3′,5′-selectivity, the rate, and the yield of enzyme-free primer extensions on the structure of the imidazole and the ability to form imidazolium bisphosphates with neighboring nucleotides.[22,23] Our own work on primer extension with RNA systems indicated that leaving groups and downstream-binding strands strongly affect rates and yields,[9,24,25] but it was unclear what the regioselectivity of phosphodiester formation was for reactions with leaving groups that cannot form imidazolium bisphosphates.[26] This included primer extension reactions with in situ activation that do not require a separate chemical activation step,[10] which are of particular interest in the context of prebiotic chemistry.[27]

The regioisomeric nature of newly formed diester linkages has thus far been determined by using selective enzymatic hydrolysis. Nucleases that cleave diesters with low specificity, such as snake venom phosphodiesterases, give different product patterns than RNase T2, which cleaves only 3′,5′-diesters readily.[11,28] The identification of remaining products obtained after digestion with a 3′,5′-specific diesterase either via liquid chromatography or gel electrophoresis reveals what percentage of 2′,5′-linkages were formed. Among the disadvantages of the enzyme-based methodologies is the limited regioselectivity and the sequence dependence of nucleases[29] that can affect the read-out. Further, non-biological components of assays, including activation agents and leaving groups may have adverse effects on enzyme activity, further complicating assays relying on nucleases. In addition, enzymatic digestion destroys oligonucleotides, complicating kinetic studies and making in situ monitoring impractical.

One way to avoid the disadvantages of digestion-based methods to determine regioselectivity is using spectroscopy. In particular, NMR spectroscopy provides a wealth of structural information and does not interfere with biomolecular reactions in aqueous buffer. Dissociation constants for complexes or primer-template duplexes or hairpins with nucleotides,[30–32] dinucleotides, or trinucleotides[33] have previously been measured by NMR. Further, a ^{31}P NMR-based methodology for in situ-monitoring of diester bond formation in oligomerization reactions has recently been described by us.[34] This encouraged us to ask whether the regioselectivity of primer extension can be measured without digestion or intervention. Here we report for the first time that this is indeed the case, using ^{1}H NMR signals. The results show that template-directed reactions are selective for the natural 3′,5′-diesters, with a modest dependence on the leaving group of the activated nucleotide.

Results and Discussion

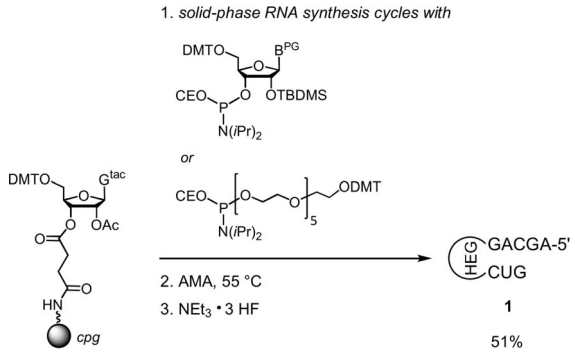
We selected the self-templating RNA hairpins shown in Scheme 2 to investigate the regioselectivity of phosphodiester formation in genetic copying reactions. Hairpins have previously been used in extension assays monitored by gel electrophoresis,[8] and in nucleotide binding studies.[30,25] Hairpin **1** has a short single-stranded overhang, consisting of two nucleotides,



Scheme 2. Sequences and primer extension reactions. A) Extension of hairpin **1**, and B) extension of hairpin **3** that forms a symmetrical duplex; LG = leaving group, HEG = hexaethylene glycol linker. See Figure 1 and Table 1 for leaving groups and conditions.

whereas hairpin **3** has a partially self-complementary 5′-overhang long enough to hybridize with a second hairpin to give a dumbbell-shaped dimer $(\mathbf{3})_2$. The intermolecular duplex formed by the 5′-terminal sequences provides stacking interactions to incoming nucleotides, and thus act like "helper" oligonucleotides that bind downstream of a primer terminus.[9] The templating base in either case was G, calling for cytidine monophosphate as nucleotide to be incorporated. A range of different activation modes, installing different leaving groups (LG) was to be tested.

We synthesized the self-templating hairpins by solid phase synthesis, as shown for hairpin **1** in Scheme 3, and we confirmed that they give sharp signals in ^{1}H NMR spectra. Then,



Scheme 3. Synthesis of RNA hairpin **1** via automated RNA solid phase synthesis. $B^{PG} = A^{Ac}$, C^{Ac}, G^{Ac}, or U; cpg = controlled pore glass, CE = cyanoethyl, HEG = hexaethylene glycol linker; AMA = ammonium hydroxide (28%)/aqueous methylamine (40%) 1:1 (*v/v*).

we prepared reference compounds of the expected regioisomeric products for extension with a cytidine residue at the 3′-terminus for either of the hairpins. The terminal C residue was either linked through a 2′,5′- or through a 3′,5′-diester bond, using phosphoramidite building blocks for the 5′-neighboring residue. Table S1 in the Supporting Information (SI) lists analytical data and yields of the hairpins. The 3′-TBDMS-protected 2′-phosphoramidite of guanosine, first employed in solid-phase RNA synthesis in 1990,[35] is commercially available. The presence of the 2′,5′-diester in the extended hairpins is indicated by a "$^{2'}$**c**" in the name, as in **2**$^{2'}$**c**. With the regioisomers in hand, their ^{1}H NMR spectra were assigned using two-dimensional spectra. Selected spectra are displayed in Figures S9–12 in the SI. The assignments of product peaks in assay solution were later also confirmed by spiking with the synthetic compounds (SI Figures S13 and S14).

Figure 1 shows the activated forms of cytidine 5′-monophosphate employed in the extension assays. For the active amides/active ester prepared in separate reactions, the protocols followed literature precedents,[22,36,9,37] as detailed in the Supporting Information. The leaving groups introduced in situ are formed when the initial isourea activation product from the ribonucleotide and EDC react with the organocatalyst in the condensation buffer. We have previously shown that primer extension gives no detectable product in the absence of a suitable organocatalyst.[10] In some instances, the rapid reaction of the organocatalyst with the isourea leads to high-yielding but slower reactions, a phenomenon recently termed "organocapture" by us.[38]

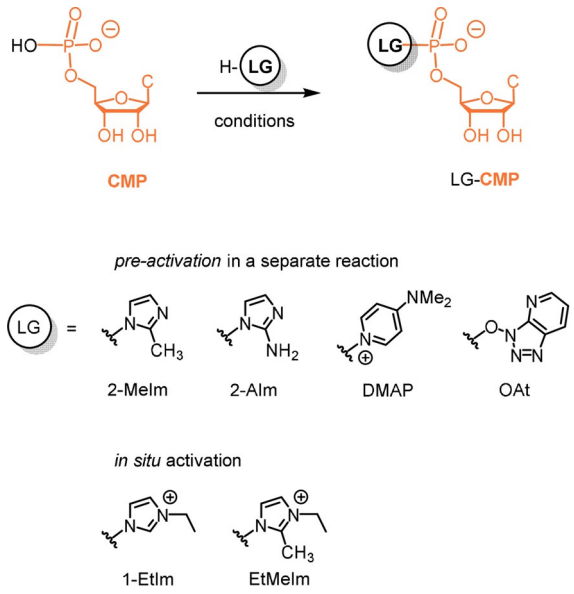


Figure 1. Activation reaction, structures of leaving groups employed, and the mode of activation used. The activation of pre-activated nucleotides involved redox condensation with dipyridyldisulfide/PPh_3 (2-MeIm, 2-AIm),[39,22] condensation with uronium salt HATU (OAt),[9] or condensation with carbodiimide in aqueous medium, as described in the SI (DMAP). Leaving groups employed in assays with in situ activation are the active intermediates of organocatalysis or organocapture that form after the initial step of activating the ribonucleotide with EDC.[40,38]

In the one-dimensional ^{1}H NMR spectra of extension products, well resolved resonances were identified for integration. Figures 2A, B show expansions of spectra of **4c** and **4**$^{2'}$**c**. These were the largest compounds to be detected in our study. The spectra were acquired in D_2O containing 25 mM HEPES and 6 mM $MgCl_2$ at pH 7.8, that is, conditions that are typical for single time-point analysis of assays performed on the usual small scale (20 µL) and diluted for NMR analysis. Figure 2C shows the same spectral region for a representative reaction mixture from an assay that ran for 5 d in condensation buffer containing 1-ethylimidazole as organocatalyst, diluted with D_2O to a final volume of 180 µL immediately before the spectrum was recorded.[10] The relative intensity of the signals for the 3′,5′-diester and its 2′,5′-regioisomer shows that the former is formed preferentially.

Table 1 gives an overview of the results obtained from assays using NMR analysis. Reaction conditions were those found in the literature for the respective activation mode. For 1-ethyl-2-methylimdiazole, which was employed as organocatalyst at 250 mM concentration, the condensation buffer previously reported for 1-ethylimidazole was used.[10] It was tested as organocatalyst because the methyl group at the 2-position was known to enhance regioselectivity for imidazolides.[41] An assay with 5 mM (rather than 80 mM) $MgCl_2$, otherwise performed identically to the one of the first entry of Table 1, gave just 19% conversion after 7 d, confirming that magnesium ions

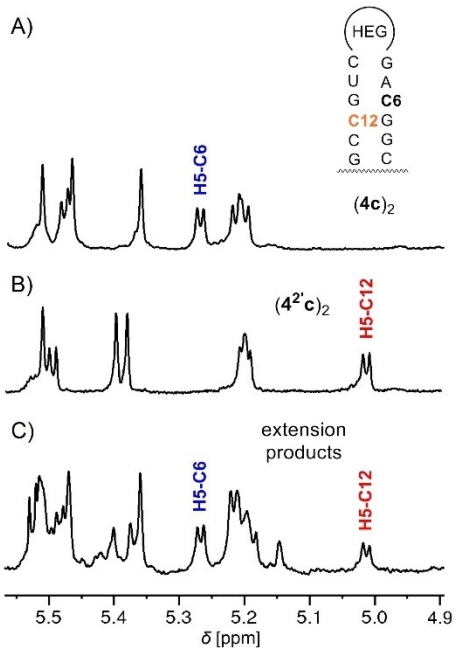


Figure 2. Excerpts of ^{1}H NMR spectra (700 MHz) of regioisomeric hairpins in pure form and as obtained from an extension assay mixture. A) Spectrum of 3′,5′-hairpin **4c** recorded in D_2O containing 25 mM HEPES, 6 mM. $MgCl_2$ at pH 7.8, and B) spectrum of hairpin **4²'c** with a single terminal 2′,5′-linkage under the same conditions. C) Spectrum from extension assay with hairpin **3** and in situ activation and 1-EtIm after 5 d, showing peaks for either of the regioisomeric products; assay conditions: 10 mM CMP, 0.8 M EDC, 0.15 M 1-EtIm, 0.5 M HEPES, 0.08 M $MgCl_2$, pH 7.5, 0 °C, spectrum acquired after nine-fold dilution with D_2O. The numbers of the nucleotide residues in question are shown in the upper right-hand corner of part A.

are critical for extension. The regioselectivity values of Table 1 were measured when monitoring indicated little further conversion, that is, toward the end of the reaction. So, the time points listed in the sixth column of Table 1 give an indication of the reactivity and hydrolysis rate of monomers,[25] with fast-reacting monomers like OAt-CMP studied in short assays, whereas slow acting monomers like 2-MeIm-CMP being allowed longer reaction times in the assay with hairpin **1**. A spectrum from each of the assays is shown in Figures S15–S24 of the SI.

The data of Table 1 show several things: 1) The smaller, more open hairpin **1** gives lower regioselectivity than the dumbbell system throughout. This confirms that the additional stacking interactions provided by the downstream duplex region helps to steer the reaction toward the phosphodiester found in today's RNA that is copied via enzymatic primer extension. 2) There is a modest difference in regioselectivity between the different activation modes, but no drastic change in product distribution. 3) The well-established 2-methylimidazolide gives the lowest yields at the time points sampled, but is a close second in regioselectivity, after the largely isosteric 2-aminoimidazolide as activated nucleotide that gave up to 85% of the 3′,5′-regiosomer for **3** and up to 66% of this regioisomer for **1**. Neither of the activation modes gives a large enough regioselectivity to make the 2′,5′-isomers irrelevant as a threat to "genome integrity" during enzyme-free copying.

The NMR-based data does not show the strong correlation between the rate of the extension reaction and its regioselectivity that was recently reported for a (more focused) set of imidazolides.[22] Our data from the broader set of active species also does not hint at a dominant role of bridged dimers in the extension reaction. For example, the OAt ester, with its different geometry and basicity rivals the imidazolides in regioselectivity (penultimate entry in Table 1). Further, the 1-ethylimidazolium nucleotide that forms upon in situ activation with 1-EtIm that cannot form phosphate-bridged dimers at all, was one of only two active species to give 90% extension of **3** in just five days, confirming that it is as reactive as the imidazolides that can. Most importantly, though, the highest regioselectivity was found for dumbbell duplex $(\mathbf{3})_2$ with single-nucleotide binding sites and unphosphorylated 5′ termini that does not allow for the binding or formation of imidazolium bisphosphate dimers in the gap, again indicating that there is no requirement for such species. Finally, because the pyridinium phosphate DMAP-CMP, a species described by Ferris et al. as strongly favoring 3′,5′-linked oligoadenylates,[42] gives a modest level of 3′,5′ selectivity (63:37) with well templating hairpin **3**, it appears that the directing effects of this leaving group and the template are not additive.

While the primary goal of our study was to obtain unambiguous data on the regioselectivity of primer extension, we also

Table 1. Results of primer extension assays.

Hairpin[a]	LG	Monomer concentration [mM][b]	Buffer[c]	pH	*T* [°C]	Analysis time point [days]	Ratio 3′,5′ to 2′,5′	Conversion [%][d]
1	1-EtIm	10	A	7.5	0	7	40:60	82
1	EtMeIm	25	A	7.5	0	13	46:54	39
1	2-AIm	20	B	7.75	25	6	66:34	90
1	2-MeIm	30	B	7.75	25	11	65:35	20
1	OAt	30	B	8.9	25	4	55:45	39
3	1-EtIm	10	A	7.5	0	5	62:38	90
3	2-AIm	20	B	7.75	25	5	85:15	90
3	2-MeIm	20	B	7.75	25	5	84:16	51
3	OAt	30	B	8.9	25	4	79:21	85
3	DMAP	30	B	7.75	25	4	63:37	75

[a] Starting concentration 2 mM hairpin. [b] Either LG-C or starting concentration of CMP for in situ activation (1-EtIm or EtMeIm). [c] A = 0.8 M EDC, 0.15 M 1-EtIm or 0.25 M EtMeIm, 0.5 M HEPES, 0.08 M $MgCl_2$; B = 0.2 M HEPES, 0.05 M $MgCl_2$. [d] Determined by MALDI-TOF-MS at the time point given.

asked whether NMR data can provide kinetic insights. Figure 3A shows kinetics of the extension of hairpin **1** using in situ activation with 1-ethylimidazole as organocatalyst together with data for three pre-activated monomers. The corresponding kinetics of the extension with EtMeIm as organocatalyst are shown in SI Figure S25. The imidazolides give slow kinetics. The OAt ester reacts much faster, albeit with the lower conversion noted in the single time point analysis (Table 1).

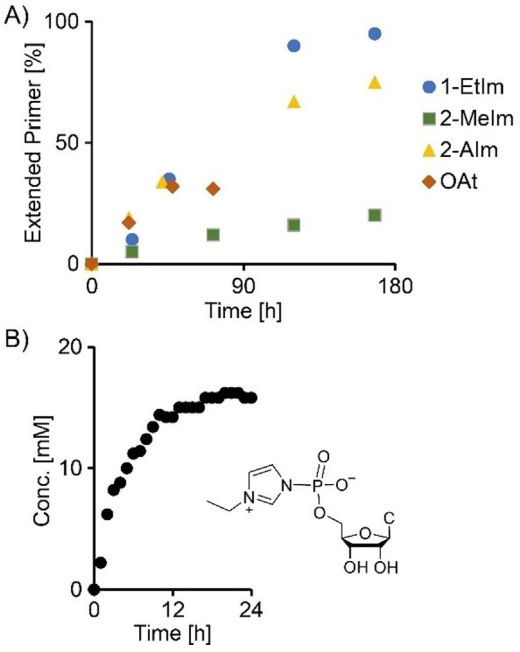


Figure 3. Kinetics of reactions in condensation buffer. A) Kinetics of extension of hairpin **1** with different activation modes and leaving groups, as detected by MALDI MS. Conditions are the same as those reported for the respective monomers in Table 1, except that the hairpin concentration was 1 mM for 1-EtIm and 2-MeIm and 1.5 mM for 2-AIm-CMP, and that the monomer concentration was 25 mM (2-AIm), 20 mM (2-MeIm), or 40 mM (OAt). B) Kinetics of build-up of the imidazolium phosphate 1-EtIm-CMP during the early phase of an assay with in situ activation, using 0.8 M EDC, 0.15 M 1-EtIm, 0.08 M $MgCl_2$, 0.5 M HEPES, 0.24 mM 1, D_2O, pH 7.5, 0 °C, as detected by its ^{31}P NMR signal at −11.3 ppm. Note the different scale of the *x*-axes.

The extension reaction with in situ activation starts slowly, but then surpasses the other reactions. So, there is a lag phase at the beginning of the assay with in situ activation. We suspected that this lag phase is due to the kinetics of formation of the organocatalytic imidazolium phosphate 1-EtIm-CMP. Figure 3B shows that this is indeed the most likely explanation. During the first 24 h of this extension assay, there is a build-up of the kinetically relevant intermediate, and the extension is slow. Once the concentration of EtIm-CMP has reached a steady state, a more rapid and high-yielding extension occurs.

Conclusions

The first application of NMR spectroscopy for monitoring primer extension revealed that the effect of organic leaving groups on regioselectivity is modest, but that the template effect is quite strong. Untemplated oligomerizations of ribonucleotides usually produce 2′,5′-diesters as the dominant product in magnesium-containing buffer.[34] In most cases studied here, the natural 3′,5′-diester is formed preferentially, even though a high concentration of magnesium ions (80 mM) was used to obtain high yields. For the dumbbell $(\mathbf{3})_2$ with its duplex flanking the extension sites, the 3′,5′-diester is the major product for all activation modes studied. Further, the regioselectivity with in situ activation and 1-ethylimidazole as organocatalyst is not far behind that with imidazolides and can be shifted further toward the natural diester with the sterically more demanding organocatalyst 1-ethyl-2-methylimidazole. This confirms the hypothesis that simple heterocycles could have played roles not unlike those of enzymes in prebiotic chemistry.[38] The in situ activation has the advantage of requiring no synthetic preparation of activated nucleotides[27] and may have operated in reactions with different primary activation agents.[38]

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: genetic copying · prebiotic chemistry · primer extension · regioselectivity · RNA

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