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Selective Phosphorylation of RNA- and DNA-Nucleosides under Prebiotically Plausible Conditions

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Nucleotides play a fundamental role in organisms, from adenosine triphosphate (ATP), the body's main source of energy, to cofactors of enzymatic reactions (e.g. coenzyme A), to nucleoside monophosphates as essential building blocks of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Although nucleotides play such an elemental role, there is no pathway to date for the selective formation of nucleoside 5′-monophosphates. Here, we demonstrate a selective reaction pathway for 5′ mono-phosphorylation for all canonical purine and pyrimidine bases under exceptionally mild prebiotic relevant conditions in water and without using a condensing agent. The pivotal reaction step involves activated imidazolidine-4-thione phosphates. The selective formation of non-cyclic mono-phosphorylated nucleosides represents a novel and unique route to nucleotides and opens exciting perspectives in the study of the origins of life.

Introduction

Phosphorylated compounds are ubiquitous in all living organisms, from the simplest plant protozoa to highly developed animals. They turn out to be one of the crucial key elements for life as we know it. In the human body, phosphorylated compounds are found in a wide variety of places and functions, e.g. as orthophosphates, a component of bones and teeth, as a central component of DNA and RNA, our cellular information storage, as phospholipids in cell membranes or as adenosine triphosphate (ATP), the body's primary energy source for use and storage.[1–4]

The formation of phosphorylated compounds, such as nucleotides, is a fundamental step towards the origins of life and has therefore been extensively studied.[5–11] One major problem in the use of phosphates as phosphorylating agents in absence of the biomolecular machinery is overcoming the "water problem", which describes the thermodynamically unfavorable release of a water molecule in an aqueous solvent.[12] This problem has so far been overcome by using non-aqueous solvents,[13] minimizing the solvent altogether,[14] applying high temperatures,[14] the addition of condensation agents or activated phosphates such as diamidophosphates (DAP)[15,16] or imidazole phosphate.[17]

The use of activated phosphates often allows mild reaction conditions for the phosphorylation of nucleosides to nucleotides, the reactions are usually carried out under water-poor conditions.

Most often, the reactions are performed as paste reactions, in order to minimize hydrolysis of the activated phosphates while simultaneously favoring the condensation reaction of the nucleoside and the phosphorylating agent.[15,17] Although yields can be increased in this way, selective phosphorylation of a single hydroxyl group is usually not possible. Krishnamurthy *et al.* demonstrated that using DAP, direct synthesis of cyclic 2′3′ nucleoside monophosphates (2′3′ cNMP) is possible, yielding only trace amounts of 5′-amidophosphates that eventually condensate to 5′ nucleoside monophosphates (5′ NMP) in an aqueous medium.[15] 2′3′ cNMPs not only play a role in the human body[18] but may also have provided a pathway for the formation of RNA on the early Earth.[19,20] It has been shown that the hairpin ribozyme or variants thereof are capable to catalyze the addition of 2′3′ cNMPs to RNA strands, and thus may play a fundamental role in the RNA world hypothesis.[19–23]

While these cyclic nucleotides represent a possible prebiotic pathway towards RNA oligomerization, they all require a previously formed ribozyme to elongate RNA. As the ribozyme itself is an RNA molecule, the origin of the RNA world must still have had a non-enzymatic emergence that gave rise to the first RNA/ribozyme.

Another route to RNA extension involves phosphate activation of 5′ NMPs with imidazole or imidazole-derivatives.[24–26] These reactions are usually carried out under mild reaction conditions in aqueous solution.[24,27] Consequently, uniform reaction conditions are a logical necessity, covering conditions ranging from phosphorylation of nucleosides to di- and oligomerization of nucleotides. Therefore, a phosphorylation method that selectively forms only 5′-NMP under mild conditions in solution is of great interest.

We have previously reported the accessibility and application of imidazolidine-4-thiones (ITOs) as (photoredox) organocatalysts under early Earth conditions.[28] These ITOs are capable

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to mutate their own structure by dynamically adapting to their environment.[29] Preliminary investigations show that ITOs with a low steric hinderance, such as 2,5-dimethylimidazolidine-4-thione, can not only act as catalysts but also as an activator in the phosphorylation of nucleosides.[29]

Results and Discussion

Here, we report the regioselective phosphorylation properties of the prebiotically plausible 2,5-dimethylimidazolidine-4-thione under exceptionally mild reaction conditions in an aqueous reaction medium (Figure 1).

Amino phosphates such as imidazole phosphate have been shown to function very well as phosphorylating agents in paste reactions. However, when imidazole phosphate was used as a phosphorylating agent, no significant selectivity for one of the regioisomers was observed.[17] Presumably, for high selectivity over the 5′ hydroxyl group of nucleosides, not the higher reactivity of the primary hydroxyl groups must be considered, but also their sterically less hindered position. Therefore, various imidazolidine-4-thiones were synthesized from prebiotically accessible aldehydes and ketones such as acetaldehyde, acetone, and isobutyraldehyde.[30] Remarkably, it is the same pool of building blocks to build DNA nucleosides in an in situ formation of the deoxyribofuranosyl unit at the canonical nucleobases.[31,32] ITOs with a larger sidechain like isopropyl (ITO derived from isobutyraldehyde) were of great interest because of their side chains, which are sterically very demanding. Unfortunately, phosphorylation of 2,2,5-trimethylimidazolidine-4-thione, 2,5,5-trimethylimidzolidine-4-thione, 2,2,5,5-tetramethylimidazolidine-4-thione or 5-isopropyl-2-methylimidazolidine-4-thione was unsuccessful, probably due to steric effects of the phosphate group as well as the bulky side chains. This hypothesis was confirmed when phosphorylation of 2,5-dimethylimidazolidine-4-thione, which has the smallest side groups of all ITOs studied, was successful. This observation is consistent with our findings that the ITO's show different catalytic/activating properties depending on the substituents.[29]

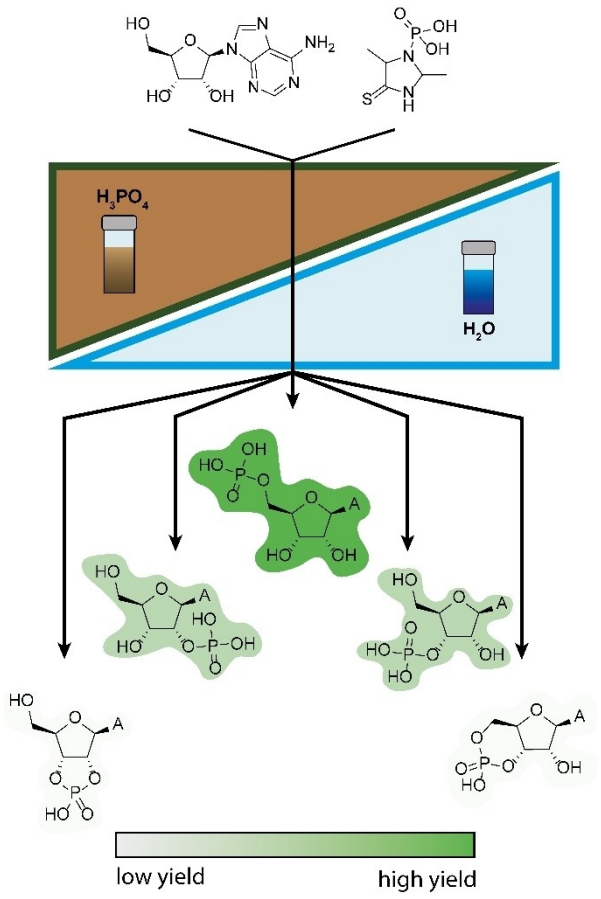

Figure 1. Selectivity of adenosine phosphate formation using phosphorylating agent ITO-P. Reactions were performed in an aqueous or a phosphate buffer solution, resulting in a high regioselective formation of 5′ adenosine monophosphate for both solvents. The yield ratio was determined by UHPLC QTOF-MS peak integration (applied mass filters: m/z 346.0541–346.0575; 328.0419–328.0485) analyzing reaction mixtures after 7 days.

In analogy to Krishnamurthy *et al.* phosphorylation reactions of RNA-nucleosides with DAP,[15] we initially used a saturated solution of adenosine (0.1 M) in ultrapure water (1 mL) and added ITO-P (5.0 equiv.) to the mixture. The reaction mixture was adjusted to pH 6 and was stirred at 50 °C for seven days. We verified the formation of adenosine monophosphate by UHPLC-QTOF MS using reference samples and high-resolution Orbitrap MS (Figure 2).

However, qualitative analysis of the sample by UHPLC-QTOF MS showed that not only 5′ AMP but also 3′ AMP and 2′ AMP were formed (Figure 2a).

The reaction conditions were optimized to find the best (selective) phosphorylation conditions. Furthermore, we compared these conditions to the reaction with DAP as well as the combination of DAP and imidazole in terms of yield and selectivity.

Quantitative analysis of the initial reaction screening showed that ITO-P yields more 5′ AMP than any of the other tested phosphorylation reagents under the examined condi-

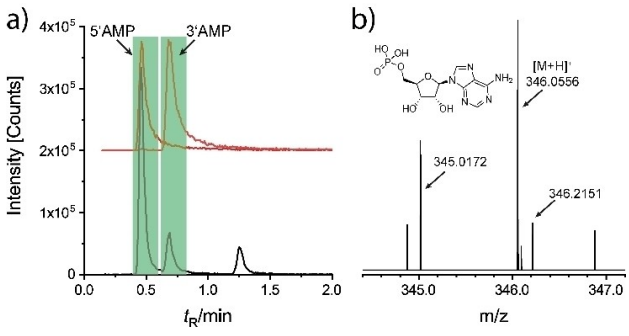

Figure 2. Formation of Adenosine monophosphates. a) Depiction of adenosine monophosphate by reaction of adenosine with ITO-P in water, as analyzed by UHPLC-QTOF MS (mass filter m/z 346.0541–346.0575, black curve) with 5′ AMP and 3′ AMP reference compounds (red curve). EIC and a retention time similar to the confirmed 5′ AMP and 3′ AMP peaks as well as similar reactivities of adenosines 2′ and 3′ hydroxyl groups suggest that the third observed peak (lower chromatogram in black) is 2′ AMP. b) High-resolution Orbitrap mass spectrum of adenosine monophosphate (0.1 mmol adenosine, 0.5 mmol ITO-P stirred in 1 mL H_2O for 7 days at 50 °C and pH 6).

tions (Figure 3a). As expected, DAP and especially DAP + Imidazole showed that they were the superior phosphorylating agents in terms of cyclic adenosine monophosphate formation (see Supplementary Figure S3). This was, expected given the higher steric hinderance of ITO-P and the fact that DAP has two amino leaving groups compared to the one imidazolidine-4-thione leaving group of ITO-P. 3′5′ cAMP could not be detected during reaction condition screening with any of the phosphorylating agents. This is coherent with results from Smith et al. who showed that temperatures above 60 °C are required to overcome the unfavorable formation of a trans six membered ring connected to a five membered ring.[33]

Considering that ITO-P is hydrolyzed more rapidly at higher temperatures, the screening range was extended to even milder reaction conditions. Applying phosphate buffer, to simulate a phosphate-rich environment on the early Earth,[34] the yield in 5′-AMP increased at room temperature for all phosphorylation reactions tested. This result represents a phosphorylation method under exceptionally mild reaction conditions. (Figure 3b).

Intriguingly, we observed that the 5′ AMP yields were higher for all reactions performed at pH 6 and 50 °C, without adjusting changes in the pH, compared to reactions executed at the same temperature but at pH 8. When the reaction was performed at room temperature the pH influence was more pronounced and varied with the particular reaction condition (Figure 3b).

We then compared the formation of the monophosphorylated AMP products to examine the selectivity of the here presented phosphorylation reactions. 3′5′ cAMP was not detected.

It was shown that the formation of 5′ AMP was higher than the formation of 2′3′ cAMP under all tested conditions (except for the reactions performed at pH 8 and 1 eq. of ITO-P) (see Supplementary Figure S1). Not only was more 5′ AMP formed than 3′ AMP, but no cAMP could be detected by high resolution Orbitrap MS, if a phosphate buffer was used.

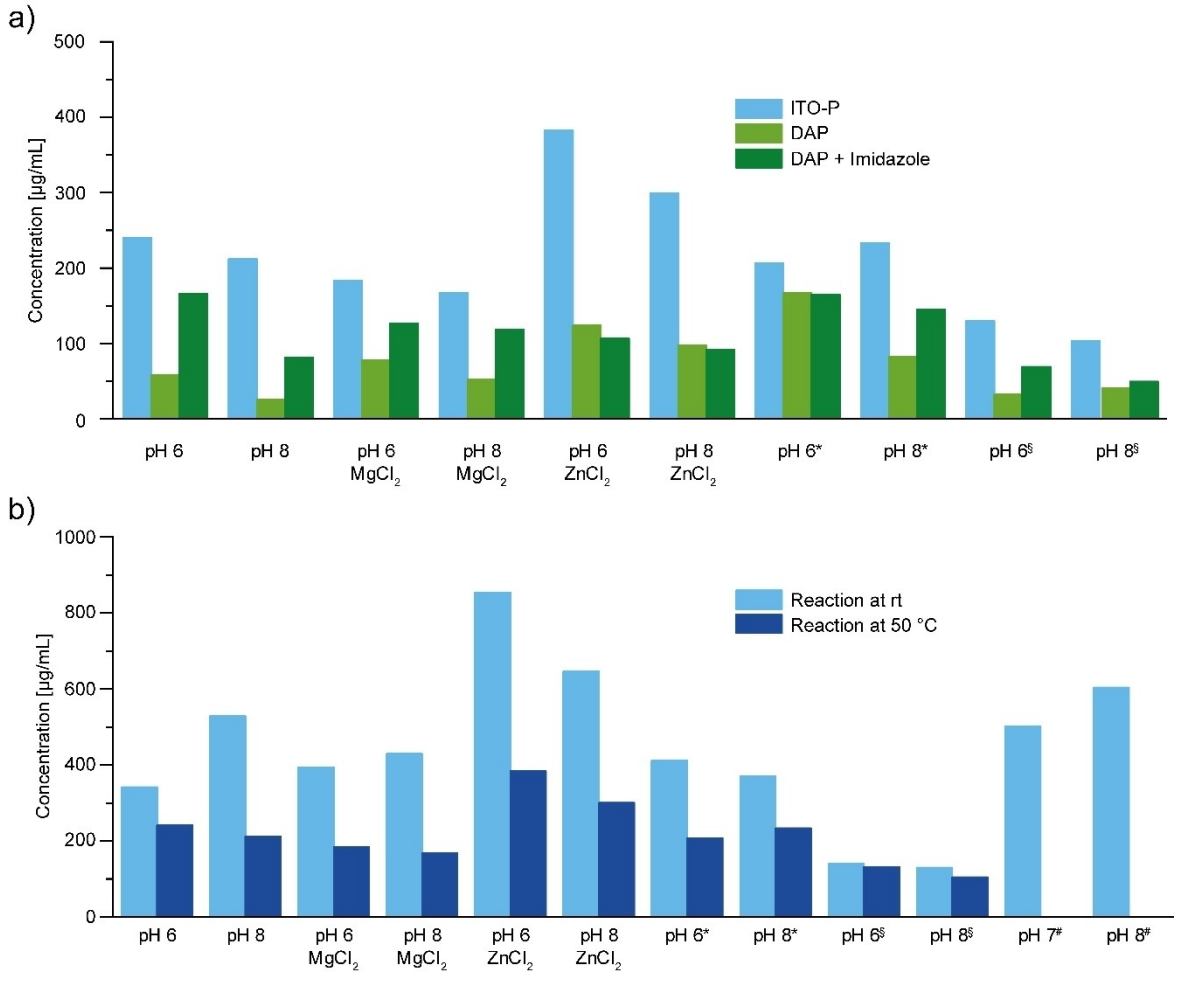

Figure 3. Screening of ITO-P mediated phosphorylation reactions of adenosine to 5′ AMP. a) Comparison of the yielded 5′ AMP with the phosphorylation agents ITO-P, DAP and DAP + imidazole for the screened reaction conditions. Reactions were carried out in 1 mL of ultrapure water at 50 °C and the described pH value. Reactions were performed with 5.0 equiv. of the phosphorylation agent (1.0 equiv. of imidazole if used), unless otherwise stated, and with or without additional 0.5 equiv. of additive. b) Extended screening for the optimal phosphorylation reactions of ITO-P. Reactions were run in 1 mL of ultrapure water or phosphate buffer at room temperature or 50 °C and the described pH value. Reactions were performed with 5.0 equiv. of ITO-P, and with or without additional 0.5 equiv. of additive. [*] pH was adjusted to pH 6/pH 8 once a day; [§] Reactions were performed with 1.0 equiv. instead of 5.0 equiv. of ITO-P; [#] Reactions were performed using a phosphate buffer of the described pH value instead of ultrapure water. For details see the Supporting Information.

We can show that no AMP or cyclic AMP is formed in experiments without phosphorylating agents in phosphate buffer. Thus, the phosphate group must have been transferred from the phosphorylating agent to the adenosine and does not derive from the buffer itself.

Comparison of our highest yielding 5′ AMP phosphorylation conditions shows that ITO-P provides not only an exceptionally high selectivity towards non-cyclic AMPs, but also a high selectivity towards 5′ AMP in general (Figure 4).

Following the screening of phosphorylation reaction conditions, the four best reaction conditions in terms of 5′ AMP yield and selectivity were selected for all canonical RNA and DNA nucleosides (Table 1).

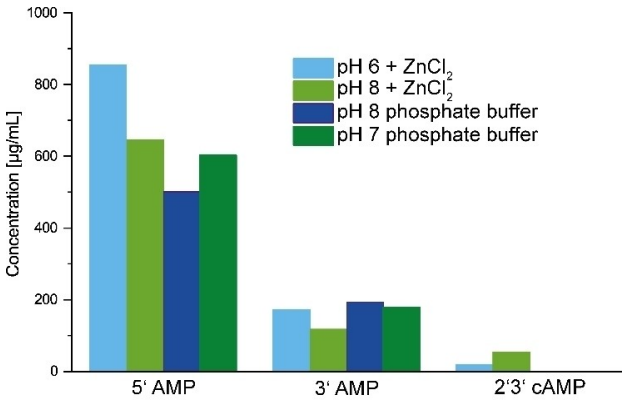

Figure 4. Yield distribution between 5′ AMP, 3′ AMP and 2′3′ cAMP for selected phosphorylation conditions. Selectivity towards 5′ AMP could be observed for all examined reaction conditions. No cAMP could be detected by Orbitrap MS or UHPLC-QTOF MS, if phosphate buffer was used instead of ultrapure water.

Table 1. RNA and DNA nucleosides phosphorylated by imidazolidine-4-thione phosphate under aqueous conditions.

Nucleoside	Method	5′ NMP Yield [µmol/mL]	cNMP found
Adenosine	A	2.46	yes
Adenosine	C	1.74	no
Guanosine	B	1.61	yes
Guanosine	C	2.93	no
Cytidine	B	2.79	yes
Cytidine	C	2.17	no
Uridine	A	3.10	yes
Uridine	D	7.04	no
deoxyAdenosine	B	0.10	yes[a]
deoxyAdenosine	D	0.05	yes[a]
deoxyGuanosine	B	0.51	no
deoxyGuanosine	C	0.37	no
deoxyCytidine	A	4.19	no
deoxyCytidine	C	4.78	no
Thymidine	B	2.79	no
Thymidine	D	2.95	no

Method A: 1 mL ultrapure water, 0.05 mmol $ZnCl_2$, 0.1 mmol nucleoside and 0.5 mmol ITO-P were stirred at pH 6 for 7 days at room temperature. Method B: 1 mL ultrapure water, 0.05 mmol $ZnCl_2$, 0.1 mmol nucleoside and 0.5 mmol ITO-P were stirred at pH 8 for 7 days at room temperature. Method C: 1 mL pH 7 phosphate buffer, 0.1 mmol nucleoside and 0.5 mmol ITO-P were stirred for 7 days at room temperature. Method D: 1 mL pH 8 phosphate buffer, 0.1 mmol nucleoside and 0.5 mmol ITO-P were stirred for 7 days at room temperature.
[a] was only observed under the following conditions: injection volume: 10 µL, nozzle voltage: 2 kV, capillary voltage: 3 kV.

As hypothesized, 5′ NMP was formed in all phosphorylation reactions. Moreover, cNMP was not detected in any of the ribonucleoside phosphorylation reactions when a phosphate buffer was used as solvent.

Surprisingly, 3′ NMP of dA, dG and dC was not formed in the phosphorylation of deoxynucleosides under various conditions, demonstrating the high regioselectivity of ITO-P.

Conclusion

We have presented here an important application for prebiotically plausible ITO organocatalysts, extending the scope of reactions. It has been shown that these catalysts are not only capable of functionalizing their own building blocks[28,29] as well as potentially other reactions, but can also act as phosphorous activators in prebiotically relevant phosphorylation reactions. The presented ITO-P is able to phosphorylate all canonically plausible nucleosides under different reaction conditions. Moreover, the phosphorylation reactions can be carried out under exceptionally mild reaction conditions in aqueous mixtures. Furthermore, the high regioselectivity of ITO-P was demonstrated not only between 5′ AMP and 2′3′ cAMP but also for the formation of 5 AMP in general.

In addition to the phosphorylated canonical nucleosides presented in this work, the phosphorylation is also conceivable for other prebiotically relevant molecules

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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[1] S. von Euw, Y. Wang, G. Laurent, C. Drouet, F. Babonneau, N. Nassif, T. Azaïs, *Sci. Rep.* **2019**, *9*, 8456.
[2] J. D. Watson, F. H. C. Crick, *Nature* **1953**, *171*, 737–738.
[3] T. Harayama, H. Riezman, *Nat. Rev. Mol. Cell Biol.* **2018**, *19*, 281–296.
[4] J. R. Knowles, *Annu. Rev. Biochem.* **1980**, *49*, 877–919.
[5] A. M. Schoffstall, E. M. Laing, *Ori. Life Evol. Biosph.* **1985**, *15*, 141–150.
[6] A. M. Schoffstall, *Orig. Life* **1976**, *7*, 399–412.
[7] G. Costanzo, R. Saladino, C. Crestini, F. Ciciriello, E. Di Mauro, *J. Biol. Chem.* **2007**, *282*, 16729–16735.
[8] T. Georgelin, M. Jaber, T. Onfroy, A.-A. Hargrove, F. Costa-Torro, J.-F. Lambert, *J. Phys. Chem. C* **2013**, *117*, 12579–12590.
[9] H.-J. Kim, Y. Furukawa, T. Kakegawa, A. Bita, R. Scorei, S. A. Benner, *Angew. Chem. Int. Ed.* **2016**, *55*, 15816–15820; *Angew. Chem.* **2016**, *128*, 16048–16052.
[10] M. Akouche, M. Jaber, M.-C. Maurel, J.-F. Lambert, T. Georgelin, *Angew. Chem. Int. Ed.* **2017**, *56*, 7920–7923; *Angew. Chem.* **2017**, *129*, 8028–8031.
[11] S. C. Kim, L. Zhou, W. Zhang, D. K. O'flaherty, V. Rondo-Brovetto, J. W. Szostak, *J. Am. Chem. Soc.* **2020**, *142*, 2317–2326.
[12] M. A. Pasek, *Chem. Rev.* **2020**, *120*, 4690–4706.
[13] B. Burcar, M. Pasek, M. Gull, B. J. Cafferty, F. Velasco, N. V. Hud, C. Menor-Salván, *Angew. Chem. Int. Ed.* **2016**, *55*, 13249–13253; *Angew. Chem.* **2016**, *128*, 13443–13447.
[14] C. Ponnamperuma, R. Mack, *Science* **1965**, *148*, 1221–1223.
[15] C. Gibard, S. Bhowmik, M. Karki, E.-K. Kim, R. Krishnamurthy, *Nat. Chem.* **2018**, *10*, 212–217.
[16] M. Yadav, R. Krishnamurthy, *Org. Lett.* **2019**, *21*, 7400–7404.
[17] O. R. Maguire, I. B. A. Smokers, W. T. S. Huck, *Nat. Commun.* **2021**, *12*, 5517.
[18] J. D. Verrier, T. C. Jackson, R. Bansal, P. M. Kochanek, A. M. Puccio, D. O. Okonkwo, E. K. Jackson, *J. Neurochem.* **2012**, *122*, 115–125.
[19] E. Y. Song, E. I. Jiménez, H. Lin, K. Le Vay, R. Krishnamurthy, H. Mutschler, *Angew. Chem. Int. Ed.* **2021**, *60*, 2952–2957; *Angew. Chem.* **2021**, *133*, 2988–2993.
[20] E. I. Jiménez, C. Gibard, R. Krishnamurthy, *Angew. Chem. Int. Ed.* **2021**, *60*, 10775–10783.
[21] H. Mutschler, P. Holliger, *J. Am. Chem. Soc.* **2014**, *136*, 5193–5196.
[22] R. Hieronymus, S. P. Godehard, D. Balke, S. Müller, *Chem. Commun.* **2016**, *52*, 4365–4368.
[23] W. Gilbert, *Nature* **1986**, *319*, 618.
[24] L. Li, N. Prywes, C. P. Tam, D. K. O'Flaherty, V. S. Lelyveld, E. C. Izgu, A. Pal, J. W. Szostak, *J. Am. Chem. Soc.* **2017**, *139*, 1810–1813.
[25] M. Jauker, H. Griesser, C. Richert, *Angew. Chem. Int. Ed.* **2015**, *54*, 14559–14563; *Angew. Chem.* **2015**, *127*, 14767–14771.
[26] S. Motsch, D. Pfeffer, C. Richert, *ChemBioChem* **2020**, *21*, 2013–2018.
[27] W. Zhang, A. Pal, A. Ricardo, J. W. Szostak, *J. Am. Chem. Soc.* **2019**, *141*, 12159–12166.
[28] A. C. Closs, E. Fuks, M. Bechtel, O. Trapp, *Chem. Eur. J.* **2020**, *26*, 10702–10706.
[29] A. C. Closs, M. Bechtel, O. Trapp, *Angew. Chem. Int. Ed.* **2022**, *61*, e202112563.
[30] J. C. Aponte, D. Whitaker, M. W. Powner, J. E. Elsila, J. P. Dworkin, *ACS Earth Space Chem.* **2019**, *3*, 463–472.
[31] J. S. Teichert, F. M. Kruse, O. Trapp, *Angew. Chem. Int. Ed.* **2019**, *58*, 9944–9947; *Angew. Chem.* **2019**, *131*, 10049–10052.
[32] F. M. Kruse, J. S. Teichert, O. Trapp, *Chem. Eur. J.* **2020**, *26*, 14776–14790.
[33] M. Smith, G. I. Drummond, H. G. Khorana, *J. Am. Chem. Soc.* **1961**, *83*, 698–706.
[34] J. D. Toner, D. C. Catling, *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 883–888.

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