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Preparation of Synthetically Challenging Nucleotides Using Cyanoethyl P-Imidazolides and Microwaves

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ABSTRACT

We describe a general method for the elongation of nucleoside oligophosphate chains by means of cyanoethyl (CE) phosphorimidazolides. Though the method requires a phosphorylation and subsequent deprotection reaction, both steps could be achieved in one pot without isolation/purification of the initial phosphorylation product. We have also found that pyrophosphate bond formation by this method is significantly accelerated by microwave irradiation.

Nucleoside oligophosphates such as NDPs, NTPs, and NP_4s, and their phosphate-modified analogues are widely used as molecular probes, enzymatic inhibitors, signaling pathway regulators, etc., in investigations of cellular processes. The potential of even minor chemical modifications to alter biological properties of nucleotides is reflected in numerous discoveries in the fields of biology, biotechnology, biophysics, and medicine, made by means of NDP, NTP, and their analogues. For example, various P-modified analogues of NTP have been used to study the mechanisms and substrate specificities of DNA and RNA polymerases and to obtain appropriately modified nucleic acids.[1] Phosphorothioate, (methylenebis)phosphonate, and imidodiphosphate analogues of GDP and GTP have been employed to study signaling pathways mediated by G-protein-coupled receptors.[2] Nucleotides containing various phosphate modifications have been identified as promising drug candidates for targeting P2X and P2Y receptors.[3] Recently, some nucleoside 5′-phosphorothioate analogues have been identified as biocompatible antioxidants that dissolve β-amyloid–metal ion aggregates, with potential for Alzheimer disease treatment.[4]

Because of the increasingly important applications of nucleoside oligophosphates and their analogues, efficient and relatively straightforward methods for their synthesis are required. Important contributions in this field include methods developed by numerous researchers,[5] especially in the context of the synthesis of NTPs (and less commonly, NDPs) modified at the α-phosphate with nonbridging oxygen-to-sulfur, -borane, or -selenium substitution. These methods, the majority of which are based on

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trivalent phosphorus chemistry and involve formation of trimetaphosphate-like intermediates, have gained much appreciation since they usually enable assembly of the desired oligophosphate bridge via one-pot reaction and directly form a properly protected nucleoside in satisfactory yield.

In this study, we focused on the synthesis of nucleoside oligophosphates that have yet to be reported, are commercially unavailable, or are synthesized in relatively low yields using one-pot approaches. Among them are nucleoside

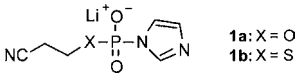

Figure 1. Phosphorylating reagents **1a** and **1b**.

tetraphosphates and their derivatives, as well as nucleotides that bear an α,β-bridging or β-nonbridging modification in the triphosphate bridge. We employed imidazolide derivatives of cyanoethyl phosphate or thiophosphate (**1a** and **1b**, respectively; Figure 1) as electrophilic reagents that can be coupled to various phosphate-modified nucleotides under mild conditions in reactions mediated by divalent metal ions.[6] In contrast to many of methods mentioned above, this approach requires multistep phosphate chain build-up, which is generally less efficient than one-pot synthesis. However, this method enables precise construction of the oligophosphate bridge with strictly defined positions of the required modifications.[7] Moreover, several compounds such as nucleoside triphosphates modified at the α,β-bridging position (ppXpN) are not accessible through trimetaphosphate-like intermediates. Thus, they are usually synthesized through enzymatic phosphorylation of the corresponding diphosphate analogue.[8] The use of phosphorylating reagents **1a** and **1b** constitutes an attractive chemical alternative to enzymatic procedures that are often constrained by enzyme specificity.

In general, the present synthetic procedure relies on the reaction of a properly P-modified nucleoside 5′-*O*-mono-, di-, or triphosphate, including boranophosphates and 2-selenodiphosphates,[9] with reagent **1a** or **1b** in DMF in the presence of a divalent metal chloride, followed by β-elimination of the cyanoethyl (CE) protecting group (Scheme S1, Supporting Information). To prove the utility of **1a** and **1b** as phosphorylating reagents, we synthesized 23 ribonucleotides (**2**−**24**) bearing phosphate chains of different lengths with various modifications and nucleobases of different polarity and chemical stability (Table 1).

Although the proposed approach is relatively straightforward and universal, several procedures required optimization with regard to the coupling and deprotection conditions and reaction workup. Interestingly, we also discovered that both key steps, pyrophosphate bond formation and removal of the terminal CE group, were accelerated by microwave irradiation. The optimized conditions for the synthesized compounds are shown in Table 1.

We began by investigating the syntheses of GDP and its α-thio and α-borano analogues, GDPαS (**2**) and GDPαBH$_3$ (**3**), respectively. In order to find optimal conditions for the reaction of **1a** with GMP or its analogues (GMPS or GMPBH$_3$), the influence of different metal chlorides ($ZnCl_2$, $MgCl_2$, or $MnCl_2$) on coupling rate and efficiency was examined. In the case of GDP-CE formation, $ZnCl_2$ was most effective; after 30 min, the HPLC conversion of GMP to GDP-CE reached 99%, even when only 1.2 equiv of **1a** was used. In the case of GMPS, the reaction rate was highest in the presence of $MnCl_2$; however, the overall yield did not depend on the metal chloride used (the HPLC profile of an exemplary coupling reaction is shown in Figure S1, Supporting Information). In contrast, efficient coupling of GMPBH$_3$ with **1a** to yield GDPαBH$_3$-CE was observed only in the presence of $MgCl_2$.

Next, we sought conditions for effective CE group removal, preferably without the need for isolation and purification of the synthesized CE-protected nucleotides. The CE group is commonly used as a phosphate protecting group in oligonucleotide synthesis, but it has also been applied to the synthesis of nucleoside oligophosphates.[10,11] CE removal is easily performed via β-elimination under basic conditions, e.g., by ammonolysis, especially in the case of uncharged phosphate moieties. The removal of CE from negatively charged phosphates is more difficult. It is usually performed in 0.1 M NaOH at elevated temperature;[12] hence, we began by applying these conditions to the deprotection of GDP-CE and its analogues. After diluting the reaction mixture with aqueous NaOH and heating it at 50 °C, GDP-CE was converted to GDP almost quantitatively within 1 h. However, under the same conditions, both GDPαS-CE and GDPαBH$_3$-CE underwent hydrolysis almost exclusively to yield the corresponding monophosphates, GMPS and GMPBH$_3$, respectively.

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(9) For the preparation of starting materials that are not commercially available, see the Supporting Information.

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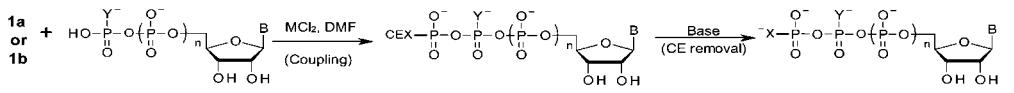
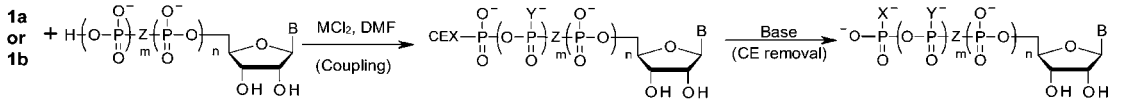
Table 1. Summary of the Phosphate-Modified Nucleotides Synthesized Using Reagents **1a** and **1b**

A) Nucleotides modified at non-bridging position(s) of the oligophosphate chain



Entry	Product No	Abbrev.	Y, X, B, n	Starting[a] materials	μw[a]	Time/ HPLC yield	CE removal /time[a]	Prep. yield	Prev. synt.[b]
1		GDP[f]	O, O, G, 0	GMP + **1a**	–	0.5 h/99%	A/1 h	n.d.	-
2	**2**	GDPαS[f]	S, O, G, 0	GMPS + **1a**	–	3 d/83%	B/5 h	37%	10%[17]
3	**3**	GDPαBH$_3$	BH$_3$, O, G, 0	GMPBH$_3$ + **1a**	–	24 h/81%	B/5 h	62%	26%[5d]
4	**4**	ADPαS	S, O, A, 0	AMPS + **1a**	–	3 d/95%	B/5 h	61%	30%[5d]
5	**4**	ADPαS	S, O, A, 0	AMPS + **1a**	+	0.5 h/88%	C, μw/20 min	63%	
6	**5**	ADPαBH$_3$	BH$_3$, O, A, 0	AMPBH$_3$ + **1a**	–	24 h/84%	B/3 h	65%	28%[5d]
7	**5**	ADPαBH$_3$	BH$_3$, O, A, 0	AMPBH$_3$ + **1a**	+	0.5 h/76%	C, μw/20 min	58%	
8	**6**	UDPαS[f]	S, O, U, 0	UMPS + **1a**	–	3 d/80%	B/5 h	65%	30%[18]
9	**7**	m^{7}GDPαS	S, O, m^{7}G, 0	m^{7}GMPS + **1a**	–	3 d /90%	B/6 h	39%	N
10	**7**	m^{7}GDPαS	S, O, m^{7}G, 0	m^{7}GMPS + **1a**	+	20 min/92%	C, μw/20 min	60%	
11	**8**	2′,3′-*c*PS-UDPαS	S, O, U*[d], 0	2′,3′-*c*PS-UMPS + **1a**	–	5 d /82%	B/5 h	59%	N
12	**9**	ADPαSβS[f]	S, S, A, 0	AMPS + **1b**	–	5 d /85%	B/5 h	20%	72%[11]
13	**10**	GDPαSβS	S, S, G, 0	GMPS + **1b**	–	5 d/90%	B/5 h	36%	32%[5b]
14	**10**	GDPαSβS	S, S, G, 0	GMPS + **1b**	+	15 min/85%	C, μw/30 min	30%	
15	**11**	ATPβS	S, O, A, 1	ADPβS + **1a**	–	2h/92%	B/12 h	62%	12%[5a]
16	**12**	GTPβS	S, O, G, 1	GDPβS + **1a**	–	24h/96%	B/5 h	73%	45%[17]
17	**13**	ATPβBH$_3$	BH$_3$, O, A, 1	[ADPβBH$_3$][c] + **1a**	–	5h/60%	B/2 h	47%	N
18	**13**	ATPβBH$_3$	BH$_3$, O, A, 1	[ADPβBH$_3$][c] + **1a**	+	5 min/40%	C, μw/5 min	20%	
19	**14**	GTPβBH$_3$	BH$_3$, O, G, 1	[GDPβBH$_3$][c] + **1a**	–	5 h/48%	B/2 h	18%	N
20	**15**	ATPβSe	Se, O, A, 1	ADPβSe + **1a**	+	0.5 h/60%	C, μw/5 min	43%	N
21	**16**	GTPβSe	Se, O, G, 1	GDPβSe + **1a**	–	24 h/52%	B/2h	15%	N

B) Nucleotides modified at the bridging position of the oligophosphate chain



Entry	Product No	Abbrev.	Z, X, B, m, n	Starting[a] materials	μw[a]	Time/ HPLC yield[a]	CE removal /time	Prep. yield	Prev. synt.[b]
22	**17**	ppCH$_2$pA[f]	CH$_2$, O, A, 1, 1	pCH$_2$pA + **1a**	–	1.5 h/92%	B/4 h	77%	NR
23	**18**	ppCH$_2$pG	CH$_2$, O, G, 1, 1	pCH$_2$pG + **1a**	–	1.5 h/95%	B/4 h	78%	30%[21]
24	**18**	ppCH$_2$pG	CH$_2$, O, G, 1, 1	pCH$_2$pG + **1a**	+	0.5 h/95%	C, μw /0.3 h	80%	
25	**19**	ppNHpA[f]	NH, O, A, 1, 1	pNHpA + **1a**	–	2h/88%	B/4 h	76%	77%(e)[8]
26	**20**	ppNHpG	NH, O, G, 1, 1	pNHpG + **1a**	–	1.5 h/93%	B/4 h	72%	NR
27	**21**	p$_4$A[f]	O, O, A, 1, 2	ATP + **1a**	–	3 h/97%	B/3 h	82%	NR
28	**22**	ppCH$_2$pp(m^{7}G)	CH$_2$, O, m^{7}G, 1, 2	pCH$_2$pp(m^{7}G) + **1a**	–	3.5 h/69%	C/2 h	56%	NR
29	**23**	pppCH$_2$p(m^{7}G)	CH$_2$, O, m^{7}G, 2, 1	ppCH$_2$p(m^{7}G) + **1a**	–	4 h/71%	C/2 h	51%	NR
30	**24**	p$_S$pCH$_2$pG	CH$_2$, S, G, 1, 1	pCH$_2$pG + **1b**	+	0.5 h/98%	C, μw/0.5 h	70%	N

[a] Coupling conditions: 8 equiv of $MgCl_2$, DMF. CE removal: (A) 0.1 M NaOH, 50 °C; (B) 10–15% DBU; DMF; open vessel or reduced pressure or DTT; (C) 10–15% DBU; DMF, DTT; μw: 40 °C, closed vessel, dynamic power max. 10 W. [b] The reference list is for the purpose of a general overview and may not be comprehensive. For details, please see the references cited. [c] This nucleotide, because of its instability, has been generated in situ via reaction of an appropriate nucleoside 5′-phosphorimidazolide with boranophosphate triethylammonium salt. [d] U* denotes uracil nucleoside that bears additionally a cyclic 2′,3′-thiophosphate. [e] Chemoenzymatic synthesis; NR, the compound is known, but the yield was not reported; N, new compound. [f] These nucleotides have also been synthesized via μw-assisted procedure, but without isolation (for HPLC yields, see Table S1, Supporting Information).

To overcome this problem, we sought a milder method of CE removal. We tested several conditions, including ammonolysis, treatment with TEA, DIPEA, EDA and DBU. Only DBU treatment under elevated temperature enabled efficient removal of CE,[13] yet only in the cases when $MgCl_2$ was used as a coupling mediator. The two other metal chlorides deactivated DBU, probably because of their stronger Lewis acidity compared to $MgCl_2$. Thus, for the preparation of all other nucleotides, use of $MgCl_2$ was preferable since it enabled subsequent one-pot deprotection with DBU, even though it was not the most efficient mediator of pyrophosphate bond formation in all cases. The reactions were monitored by RP-HPLC–ESI-MS.[14] In the one-pot reactions with $MgCl_2$, complete removal of protecting groups from GDPαS-CE and GDPαBH$_3$-CE required heating at 50 °C for 2–4 h. These optimized conditions (without any further amendment) were successfully used for the synthesis of compounds **2**–**8** (Table 1). It is worth emphasizing that the reaction conditions proved to be mild enough to carry out the synthesis of a nucleotide

(13) The same conditions are used, e.g., to remove EtSH in the synthesis of NTP and NDP via the oxathiaphospholane approach (ref 5e).

(14) It was difficult to follow the deprotection by RP-HPLC only, because of the existence of numerous DBU ion pairs that complicated the HPLC profiles.

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bearing 7-methylguanosine (**7**), which is known to be labile in alkaline solutions.[15]

Next, we tested **1b** as a thiophosphorylating reagent (Table 1, synthesis of **9** and **10**). The pyrophosphate bond formation rates and efficiencies in the reactions of **1b** were generally comparable to those of **1a**. However, a known problem associated with CE removal is the possibility of readdition of acrylonitrile to the nucleotide.[16] Therefore, β-elimination of the CE group should be performed in a manner that prevents readdition of acrylonitrile after neutralization. In our syntheses, performing the reaction in an open flask properly protected from moisture was sufficient to achieve efficient CE removal from the oxygen center. However, CE removal from sulfur was much more problematic; it proceeded slower and with significant levels of side-reactions. To facilitate sulfur deprotection, we added 1,4-dithiothreitol (DTT) to the reaction mixture. DTT acted as an efficient acrylonitrile scavenger (Figure S1C, Supporting Information), resulting not only in diminished amounts of side-products, but also shortened deprotection time.

Next, we turned our attention to the syntheses of nucleoside triphosphates modified at the β-nonbridging position (**11**−**16**), which are known to be particularly difficult. In the case of these compounds, pyrophosphate bond formation proceeded rather smoothly, but oligophosphate cleavage was observed because of reaction with acrylonitrile generated upon treatment with DBU. This was again overcome by addition of DTT.

Phosphorylation of compounds bearing either bridging (CH_2 or NH, **17**−**20**, **22**−**24**) or no modifications (**21**) using **1a** was more rapid, straightforward, and efficient than that of compounds with nonbridging modifications. Moreover, these compounds were generally less susceptible to pyrophosphate bond cleavage, and hence, CE removal could be achieved either in the presence of DBU or aqueous NaOH, provided that the nucleobase was sufficiently stable in aqueous alkali (Table 1, entries 22−27). Applying **1b** as a thiophosphorylating reagent and DBU/DTT for CE removal, we also synthesized a nucleoside triphosphate modified with a methylene group at the α,β-position and with sulfur at the γ-position (**24**).

It should be noted that some of the nucleotides used as starting materials in this study are chemically labile compounds. The nucleoside 5′-monoboranophosphates ($NMBH_3$) can be easily synthesized from corresponding H-phosphonates,[20] but they gradually undergo hydrolysis upon chromatographic purification in aqueous conditions. Nonetheless, we found that the reaction with **1a** could be performed very efficiently on unpurified $NMBH_3$. The nucleoside β-boranodiphosphates were even more labile, and we failed to isolate them, but were able to generate them in situ immediately before the reaction with **1a**.[19] NDPβSe obtained via our previously reported procedure[19,20] were prone to Se−Se dimerization and hence should be handled under an inert atmosphere.

Finally, we investigated whether time-consuming coupling reactions (e.g., of **7** and **10**) could be accelerated by microwave irradiation. Interestingly, we discovered that microwave irradiation significantly accelerated coupling between nucleotides and **1a** or **1b**. Complete conversion of the starting materials to CE-nucleotides could be achieved in 5−30 min, rather than the hours or days required in traditional synthesis.[19a] This was observed for all reactions tested (Table 1; entries 5, 7, 10, 14, 18, 20, 24, and 30; Figure S4, Supporting Information, and 7 other examples in Table S1, Supporting Information). Furthermore, formation of additional side-products was not observed under these conditions. Importantly, conventional heating to 50 °C did not have the same effect but instead led to extensive side reactions. Encouraged by these findings, we also tested the use of microwave heating for the deprotection step. Since the reactions were performed in closed vessels, DTT was added along with DBU to trap acrylonitrile released upon CE removal. Again, we found that the reaction was significantly accelerated (in the presence of DBU/DTT in DMF, 40 °C, 10 W) from 2−4 h to 5−30 min. Hence, microwave-assisted synthesis was a valuable alternative, particularly in the case of lengthy procedures. Moreover, to the best of our knowledge, these experiments are the first examples of microwave-facilitated pyrophosphate bond formation, which opens the possibility for further studies on this phenomenon.

In conclusion, we have described a general, one-pot synthetic approach for the elongation of nucleotide oligophosphate bridges by a single phosphate or thiophosphate subunit. Employing this approach, we synthesized various synthetically challenging nucleoside oligophosphates in satisfactory yields (20−82%). It is worth mentioning that this is the first report of some of the synthesized nucleotides, e.g., NTP$\beta$$BH_3$ and NTPβSe. We also demonstrated the high potential of microwave-assisted synthesis for the acceleration of metal-ion mediated pyrophosphate bond formation.

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Supporting Information Available. Synthetic procedures, Scheme S1, Figures S1−S4, Table S1, characterization data and NMR spectra for products **2**−**24**. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.