Iterative Synthesis

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Iterative Synthesis of Nucleoside Oligophosphates with Phosphoramidites**

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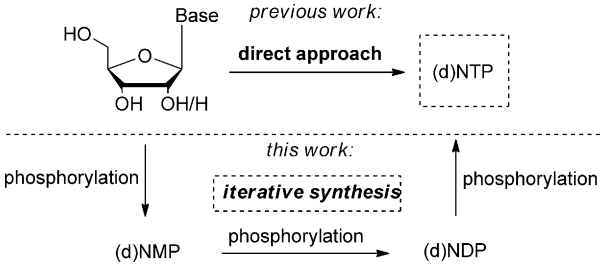
Abstract: *P-Amidites can be used in iterative couplings to selectively give mixed P^{III}–P^{V} anhydrides. These intermediates can be oxidized followed by a rapid removal of the two terminal fluorenylmethyl groups. An iterative synthesis (coupling, oxidation, deprotection) of nucleoside oligophosphates can be carried out in solution and on a solid support. The coupling rates and yields are high, the procedures convenient (non-dry reagents and solvents, ambient conditions, unprotected nucleotides), and the purification is very simple. The method works with all canonical nucleosides and holds promise for significant simplification of the usually cumbersome process of P-anhydride bond construction.*

In biology, many important molecules contain phosphoanhydride bonds.[1] Among the most prevalent targets in P-anhydride synthesis are the nucleoside triphosphates (NTP). The efficient generation of phosphoanhydride bonds and the ensuing purification of the target molecules still remains a substantial challenge.[2] The synthesis of nucleoside triphosphates is usually achieved by two different strategies (Scheme 1). In a fragment coupling approach, the triphosphate moiety is directly introduced on the nucleoside 5′ OH group. Alternatively, a diphosphate nucleophile can be condensed with an activated nucleoside monophosphate (NMP).[3,4] An unexploited strategy is the iterative coupling starting from the nucleoside and going through the different phosphorylation states (NMP via NDP to NTP). A substantial advantage of this approach is that all of the intermediates can be isolated that are also produced in the enzymatic synthesis of NTPs.[5] Additionally, an iterative approach would facilitate the synthesis of monodisperse polyphosphate chains of defined length.



Scheme 1. Direct nucleoside triphosphate (NTP) synthesis and a novel approach based on an iterative coupling strategy proceeding by the nucleoside monophosphate (NMP) and the nucleoside diphosphate (NDP).

An iterative strategy could either rely on P^{V} or P^{III} chemistry. Whereas condensation of activated P^{V} species with phosphates can be conducted without protecting groups, no such example exists for P^{III}-based couplings. On the other hand, P^{V} chemistry often suffers from long reaction times, large excess of reagents, low to moderate yields, and time-consuming purifications.[3] In comparison, P^{III} chemistry can be conducted using almost equimolar amounts of the reactants, and the high speed and efficiency of coupling is well-established in the context of oligonucleotide chemistry.[6] Thus, iterative couplings based on P^{III} chemistry could theoretically deliver the target NTPs in good yields, with easy purifications and efficient coupling rates.

To control the iterative process, two protecting groups at the terminal phosphate after coupling are needed. Ideally, the subsequent cleavage of these groups will occur rapidly, quantitatively, and without hydrolysis of the anhydride bonds to make the terminal phosphate accessible for another round of coupling. It is usually simple to cleave one protecting group at a terminal phosphate but the second is not as easily removed.[7] The fluorenylmethyl (Fm) group has been shown to enable double deprotection of phosphate triesters. However, the reported reaction times vary broadly (between 5 min to 18 h)[8] and the Fm group has not been studied in the context of P-anhydrides.

To improve P^{III}-based couplings significantly, it was necessary to avoid the use of protecting groups on the nucleoside. P-amidites (for example **1**; Scheme 2) were the reagents of choice in this study owing to their stability, versatility, and the possibility of activation by addition of acidic reagents. It is believed that activation proceeds through *N*-protonation of the amidite followed by displacement of the amine with the deprotonated activator. The released amine eventually acts as a base once the OH group of the target molecule is phosphitylated.[9] In the construction of P-anhydrides, a phosphoric acid could also be considered, as the acidic activator and displacement of the amine would directly yield the mixed P^{III}–P^{V} anhydride, potentially avoiding side reactions at free OH groups. The released amine would then neutralize the second proton on the initially provided phosphoric acid (not shown).

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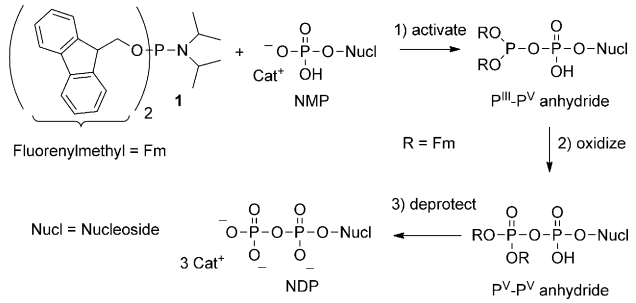


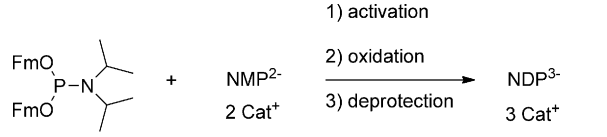
Scheme 2. Reagents, intermediates, and products occurring in the P-amidite-based homologation of nucleoside monophosphates to diphosphates.

To test the feasibility of this approach, commercially available NMPs in their diacid form were coupled with P-amidites and the reactions monitored by ^{31}P NMR spectroscopy. The reactions occurred smoothly and led to the selective generation of P-anhydride bonds within minutes. However, at about 50% conversion the reaction slowed down considerably, which can be attributed to the very different pKa values of diprotonated and monoprotonated phosphates. The transformation occurred in methanol as solvent without significant decomposition of the activated P-amidite and it tolerated water content up to 5% *v*/*v*. Addition of an external proton source such as pyridinium chloride drove these reactions to completion; however, just slight excess of the external activator led to decomposition of the generated mixed P-anhydrides and it was thus difficult to handle the reactions. As one goal of this study was the complete conversion of a given starting material to simplify purifications, another approach based on these findings was developed.

The NMPs were now provided in monoacidic form with a lipophilic counterion such as tetra-*N*-butyl ammonium (TBA) or trishexylammonium (THA; see Scheme 2), dissolved in either DMSO or DMF under ambient conditions. Neither the reagents nor the solvents were dried prior to use. A screening of activators revealed 1*H*-phenyltetrazole and 5-(ethylthio)-1*H*-tetrazole as appropriate reagents to activate P-amidite **1** without reactivity towards OH groups in for example, adenosine 5′-monophosphate (5′-AMP). The couplings occurred smoothly with as little excess as 1.1 equivalents of the P-amidite and selectively yielded the bis-Fm protected P^V–P^V anhydride after oxidation with *t*BuOOH or *m*CPBA (Scheme 2). No phosphitylation of the OH groups with excess reagent was observed. Moreover, primary OH groups as in 3′-AMP were not modified under these conditions (Supporting Information) as shown by ^{31}P NMR spectroscopy. Upon deprotection, only about 5% of 2′-3′ cyclic AMP formation were detected.

The reaction conditions were applied to different NMPs (see Table 1) and even in cytidine 5′-monophosphate (CMP) no appreciable side-reactions were detected (see Figure 1 and the Supporting Information). The reactions occurred in less than 30 min and the subsequent oxidation without isolation was complete within less than 5 min. 2′-Deoxynucleotides, such as thymidine 5′-monophosphate (TMP), were also excellent substrates under the conditions.

Table 1: Coupling of nucleoside monophosphates (NMP) to the corresponding nucleoside diphosphates (NDP).



Entry	NMP[a]	Conditions[b]	Time[c] [min]	Yield[d] [%]
1	5′-AMP	DMSO/1.4/5 *t*BuOOH/5	10/10/10	>95/75
2	5′-GMP	DMSO/1.2/10 *t*BuOOH/5	30/2/10	>95/75
3	5′-UMP	DMF/1.2/1.5 *m*CPBA/5	20/2/10	>95/93
4	5′-CMP	DMF/1.1/1.5 *m*CPBA/5	10/2/5	>95/77
5	3′-AMP	DMF/1.2/1.5 *m*CPBA/10	30/5/10	>95/87
6	5′-TMP	DMF/1.2/1.5 *m*CPBA/10	30/2/10	>95/80

[a] Lyophilized NMPs were used without previous drying. [b] All reactions were conducted in DMF or DMSO with phenyltetrazole or 5-(ethylthio)-1*H*-tetrazole as activator (1.1–2.5 equiv). The entries are given as: solvent/equiv P-amidite/equiv oxidant/deprotection with piperidine% v/v. [c] The entries are given as: coupling time in minutes/oxidation time in minutes/deprotection time in minutes. [d] The entries are given as: consumption of NMP based on ^{31}P NMR/yield isolated as piperidinium salt. Abbreviations: A: adenosine, G: guanosine, U: uridine, C: cytidine, MP: monophosphate, DP: diphosphate, Fm: fluorenylmethyl, *m*CPBA: *meta*-chloroperbenzoic acid, *t*BuOOH: *tert*-butyl hydroperoxide.

The terminally Fm-protected intermediates were deprotected by addition of 5% *v*/*v* piperidine with or without prior crystallization. These deprotections occurred rapidly in both DMF and DMSO (5 min), highlighting the solvent dependence of the rate of cleavage of the Fm group on phosphate anhydrides. The nucleoside diphosphates usually precipitated from the reaction mixture upon complete deprotection in almost pure form, containing neither coupling nor oxidizing reagent. The major byproduct of these reactions was phosphate generated from excess P-amidite after oxidation and deprotection, which can be removed easily.

The coupling efficiency based on consumption of NMP was in all cases more than 95% An example of the course of the coupling from CMP (as 1.3 TBA salt) to cytidine 5′-diphosphate (CDP) monitored by ^{31}P NMR spectroscopy is given in Figure 1. The unpurified CDP generated in this way contained less than 10% impurity derived from excess starting P-amidite and precipitation of the protected intermediate (diethyl ether/hexanes) before deprotection removed impurities completely (see Supporting Information). The yield of isolated pure CDP was 77% without any need for chromatography and the total reaction time for all of the combined transformations did not exceed 30 min.

The same method was applied to couple P-amidite **1** to different unprotected nucleoside diphosphates, yielding the terminally blocked triphosphates (Figure 2). Again, the reactions were found to be chemoselective, with an average of 1.6 equivalents of P-amidite **1** being necessary. Under these conditions, the couplings occurred almost quantitatively within minutes, as judged by ^{31}P NMR spectroscopy followed by slow hydrolysis of the newly formed bond, which complicated the synthesis as compared to the NDP preparation. Precipitation after oxidation yielded the protected NTPs

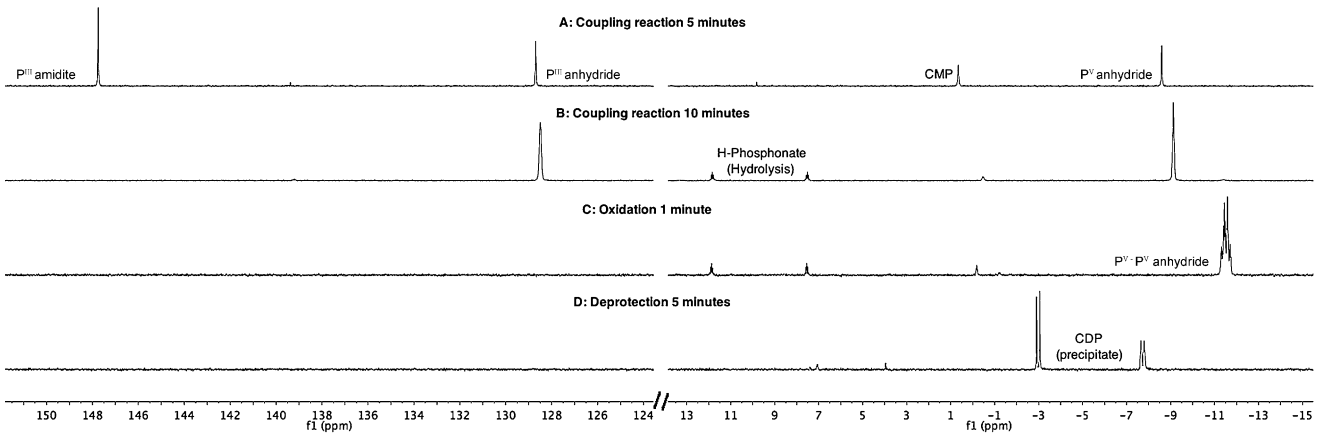


Figure 1. ^{31}P NMR (^{1}H coupled) study monitoring the formation of CDP from CMP in [D_7]DMF under ambient conditions (for assignment of the intermediates, see Scheme 2). A) Bis-Fm P-amidite **1** (1.1 equiv) and CMP (1.0 equiv, 1.3 TBA salt) were mixed in [D_7]DMF and 2.5 equiv 5-(ethylthio)-1*H*-tetrazole were added. Consumption of CMP and P-amidite is accompanied by the rise of a coupled signal of P^{III} and P^{V} mixed anhydride. B) The coupling reaction is complete after 10 min. Excess P-amidite **1** is hydrolyzed to H-phosphonate. C) Oxidation with 1.5 equiv *m*CPBA is complete within 1 min, generating a nonsymmetric P^{V}–P^{V} anhydride. D) Addition of 5% piperidine v/v leads to deprotection of the terminal Fm protected phosphate within 5 min. CDP precipitated, and it was collected and dissolved in MeOD for NMR analysis.

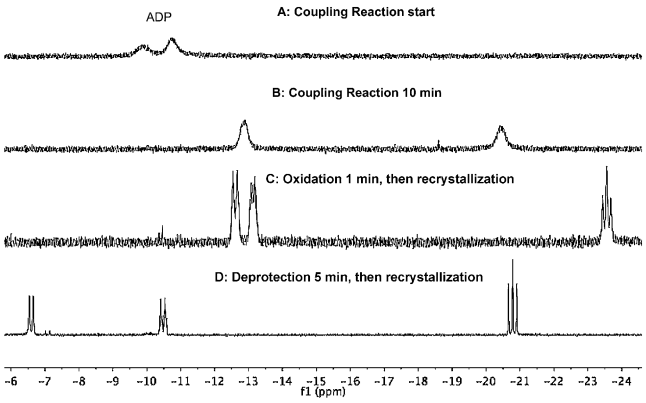
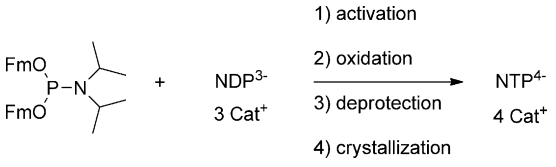


Figure 2. Partial ^{31}P NMR (^{1}H coupled) spectra demonstrating the formation of ATP from ADP in [D_7]DMF under ambient conditions (for details, see the Supporting Information). A) Bis-Fm P-amidite **1** (1.5 equiv) and ADP (1.0 equiv, 1.2 TBA salt) were mixed in [D_7]DMF and 2.0 equiv 5-(ethylthio)-1*H*-tetrazole were added. B) The coupling reaction is complete after 10 min. Consumption of ADP was monitored, and a new set of signals arises ($\delta = -21$ ppm corresponds to β-phosphate in the P^{V}–P^{V}–P^{III} anhydride, $\delta = -12$ ppm corresponds to α-phosphate). C) Oxidation with 1.9 equiv *m*CPBA is complete within 1 min, generating a nonsymmetric P^{V}–P^{V}–P^{V} anhydride. The product was crystallized to remove impurities and dissolved in [D_6]DMSO. D) Addition of 5% piperidine in [D_6]DMSO leads to deprotection of the terminal Fm-protected phosphate within 5 min. ATP precipitated upon addition of diethyl ether, and it was collected and dissolved in MeOD for NMR analysis.

Table 2: Coupling of NDPs to the corresponding NTPs.



Entry	NDP[a]	Conditions[b]	Time[c] [min]	Yield[d] [%]
1	5′-GDP	DMF/1.6/2 *m*CPBA/5	5/2/10	>95/75
2	5′-ADP	DMF/1.7/2 *m*CPBA/5	10/2/5	>95/76
3	5′-UDP	DMF/1.6/2 *m*CPBA/5	20/2/5	>95/68
4	5′-CDP	DMF/1.6/2 *m*CPBA/5	20/1/5	>95/79

[a] Lyophilized NDPs were used without previous drying. [b] All of the reactions were conducted in DMF or DMSO with 2 equiv phenyltetrazole or 5-(ethylthio)-1*H*-tetrazole as activator. The entries are given as: solvent/equiv P-amidite/equiv oxidant/deprotection with piperidine % v/v. [c] The entries are given as: coupling time in minutes/oxidation time in minutes/deprotection time in minutes. [d] The entries are given as: consumption of NDP based on ^{31}P NMR/yield isolated as piperidinium salt (with 4–12% NDP). Abbreviations: A: adenosine, G: guanosine, U: uridine, C: cytidine, DP: diphosphate; TP: triphosphate. Fm: fluorenylmethyl; *m*CPBA: *meta*-chloroperbenzoic acid.

that contained about 4–12% NDP (but no other impurities), arising from some hydrolysis during oxidation (Table 2).

The deprotection was conducted in DMSO, and the unprotected NTPs precipitated from solution upon addition of Et_2O. The purity of this material was high and only diminished by the presence of small amounts of NDP (4–12%), which can be easily removed by reverse-phase or strong anion exchange chromatography (see Figure 2, line D, for an example of the quality of crude material). Underlining the general applicability of this coupling procedure, it was also possible to homologate ATP to the pure tetraphosphate (AP_4) in 50% yield of isolated product.[10]

The observed selectivity is the result of an attenuation of the nucleophilicity of alcohols as compared to phosphate and water in DMSO or DMF. While monitoring the reactions, it became apparent that first the phosphate would react almost exclusively, followed by hydrolysis of excess P-amidite. If conducted under dry conditions with excess P-amidite, significant reactions at OH groups were detected. Thus, water intercepts excess of the amidite and protects the hydroxy functions from phosphitylation during the synthesis.

Besides the fact that P-amidites can be used in protecting group free coupling chemistry, an interesting application of iterative nucleoside oligophosphate synthesis would be the introduction of the phosphoanhydrides with the (oligo)nucleotide bound on a solid support.[4f,11] In these cases, the method would become applicable also in the synthesis of oligophosphate end-capped RNA or DNA strands, which have recently emerged as important compounds in biology, for example, in the innate immune response.[12] An iterative P^{III} amidite coupling strategy would allow using commercially available synthesizers that have been optimized towards the application of these reagents.

As proof-of-concept, controlled pore glass (CP-glass)-bound protected 2′-deoxyguanosine (dG) was 5′-detritylated, and subsequently phosphitylated with P-amidite **1**, oxidized and deprotected, yielding CP-glass-bound dG 5′-monophosphate. Iteration led to dG 5′-diphosphate and dG 5′-triphosphate in a straightforward fashion (Figure 3). All of the

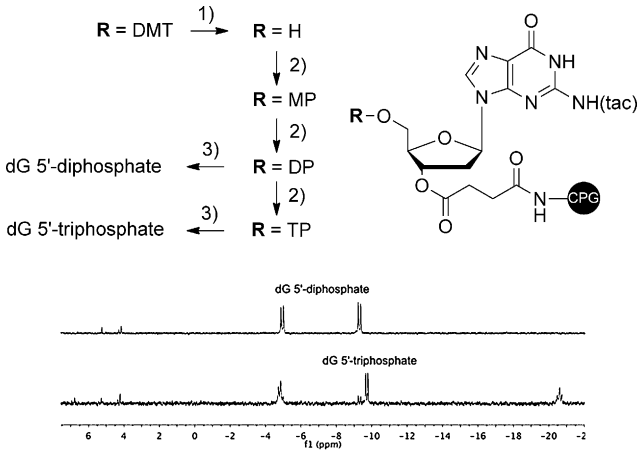


Figure 3. Iterative solid-phase-supported synthesis of dG 5′-oligophosphates. 1) TFA/DCM; 2) a. Amidite **1**, MeCN, 1*H*-tetrazole; b. *m*CPBA; c. rinse with DMF, 5% piperidine; d. wash with MeCN; 3) NH_4OH. ^{31}P NMR (^1H decoupled) spectra demonstrating the formation of dGDP and dGTP (containing ca. 20% dGDP) in D_2O after cleavage from the solid support. Abbreviations: CPG: controlled pore glass, DMT: dimethoxytrityl, MP: monophosphate, DP: diphosphate, TP: triphosphate, tac: *tert*-butylphenoxyacetyl.

involved P-anhydride forming reaction steps occurred within 10 to 20 min under ambient conditions. Cleavage from CP-glass gave rise to reasonably pure dG 5′-oligophosphates.

In summary, this study proves that P-amidites can be used in protecting-group-free iterative couplings to selectively give mixed P^{III}–P^V anhydrides. These intermediates can be oxidized followed by a rapid removal of the two terminal fluorenylmethyl groups. The concept allows conducting an iterative synthesis of nucleoside oligophosphates in solution and on solid support. The coupling rates and yields are high, the procedures convenient (non-dry reagents and solvents, ambient conditions), and the purification is very simple. This method holds promise for significant simplification of the usually cumbersome process of P-anhydride bond construction.

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