Iterative Oligophosphate Synthesis

New Facile Approach to Phosphorylation

Phosphate anhydrides are ubiquitous modifications in nature. Their chemical synthesis is still a challenging task, most notably due to time-consuming purification procedures. The development of facile phosphorylation protocols devoid of chromatographic purification is thus of great interest. Herein, recent achievements towards this aim are highlighted, based on iterative P-anhydride homologations.

Introduction
The transfer of phosphate groups is an essential chemical reaction in all forms of life [1]. It is relevant to the biosynthesis of numerous phosphate-containing molecules including, for example, nucleic acids, signalling molecules and constituents of cell membranes. Phosphorylation represents one of the main mechanisms for the regulation of enzyme activity. The phosphate donors in such biological reactions contain P-anhydrides, often referred to as "high-energy phosphates".

To gain further insights into their biological role, access to these compounds is important. Whereas extraction and purification from biological sources is an appropriate way to obtain natural products, chemical synthesis can become necessary in cases of small in vivo concentrations or when purification is difficult. Synthetic procedures also enable the preparation of unnatural analogues, which can find applications as biochemical tools and therapeutics.
Several methods for the chemical synthesis of P-anhydrides have been developed in the last decades. They usually rely on the coupling of a phosphate, either with an activated $P^V$ or $P^{III}$ species, followed by oxidation of the coupling product. The most commonly used $P^V$ species, such as morpholidates and imidazolates, have been summarized [2,3,4]. In specific cases, these intermediates can react selectively with phosphates in the presence of other nucleophilic groups, enabling couplings without protecting groups. However, often these couplings are slow, require excess reagents and result in mediocre yields.

Reactions employing $P^{III}$ species are faster. The high reactivity of P-amidites, for example, facilitates much shorter coupling times with phosphates giving mixed anhydrides.
However, P-amidites are known to react within few minutes with many nucleophilic functions. This feature is applied in the phosphitylation of OH-groups in oligonucleotide synthesis. Thus, for the synthesis of P-anhydrides, protection of other nucleophilic groups seems to be required, adding unwanted protection/deprotection steps to the overall synthesis. Generally, phosphorylations are performed under dry conditions in order to minimize the possible hydrolysis of activated reactants. After the synthesis of P-anhydrides, chromatographic purification of the charged and unstable compounds is usually necessary. These demanding procedures often restrict P-anhydride synthesis to specialized laboratories. Overall, even if many phosphorylated compounds have been synthesized, different drawbacks remain for the established coupling procedures. The development of improved strategies to accelerate access to compounds containing P-anhydrides is fundamental for the advancement of the field. This initial investment will pay off with streamlined synthetic access to phosphorylated natural products and their analogues, enabling a more detailed analysis of their rich biological effects [5].

**Iterative Nucleotide Synthesis Based on P\textsuperscript{III} Chemistry**

Among the most important natural oligophosphates are nucleotides. Due to the vast number of their functions in vivo, they are important synthetic targets, both in the natural form but also with different modifications. Common methods for nucleotide synthesis have been summarized [3, 4]. Despite the variety of existing synthetic approaches, some basic drawbacks remain (vide supra), and consequently improved procedures are of great interest.

Recently, we found that under appropriate conditions, P-amidites react at much higher rates with phosphates as compared to other nucleophiles. In the presence of phosphates, water, and alcohols, a P-amidite can be coupled to the phosphate with exquisite selectivity, resulting in a mixed P\textsuperscript{III}-P\textsuperscript{V} anhydride. Only little excess of the P-amidite reagent 1 (fig. 1) is necessary to ensure complete consumption of the starting material. After the phosphate has been consumed, excess P-amidite preferably hydrolyses to an unreactive H-phosphonate. Since hydrolysis is much faster than phosphitylation of OH groups, no protecting groups on the nucleoside are required, and the reaction can be run under ambient conditions without drying of any reagents and reactants. To demonstrate that this finding opens new possibilities for selective phosphorylation, a procedure for the homologation of nucleotides by iterative addition of one phosphate group per cycle was developed [6].
This phosphorylation method consists of a three-step sequence (fig. 1). In a first step, a nucleotide salt 2 with a lipophilic counterion, such as tetrabutylammonium (TBA), dissolved in wet DMF, is treated with a slight excess of P-amidite 1 and a tetrazole activator (5-phenyl-1H-tetrazole, 5-ethylthio-1H-tetrazole or 5-methylthio-1H-tetrazole) to initiate the coupling. The nucleotide is fully converted to the mixed $P^{	ext{III}}$-$P^{	ext{V}}$ anhydride 3 within 30 minutes or less, whereas excess 1 is hydrolyzed by water. Subsequent oxidation of 3 by adding a slight excess of mCPBA to the mixture yields the $P^{	ext{V}}$-$P^{	ext{V}}$-anhydride 4. Finally, the fluorenylmethyl (Fm) groups on the terminal phosphate are rapidly cleaved with 5-10 vol % piperidine. Upon addition of diethyl ether, the product 5 precipitates as a piperidinium salt, whereas reactants and side products remain in solution.

**High Purity Confirmed by NMR**

In comparison with other syntheses of nucleoside oligophosphates, this procedure has certain advantages. The coupling step is not only fast, but also highly selective. No phosphitylation of OH groups or amines was observed, and nucleotides can be directly homologated in their unprotected form. Moreover, the reaction is insensitive to water and thus can be run in open flasks with wet solvents and reactants. The oxidation with mCPBA is quantitative and complete within few minutes. Subsequently, the addition of piperidine not only allows for a fast and quantitative cleavage of both Fm groups, but also the precipitation of the homologated nucleotide as a piperidinium salt in good yields and high purity (see fig. 2).

The coupling works extremely well for the conversion of nucleoside monophosphates (NMPs) to nucleoside diphosphates (NDPs). All canonical NMPs were homologated to the corresponding diphosphate in less than 1 hour of combined reaction time for the three steps, including purification. The three steps can usually be run in a one-pot operation. In all cases, the precipitated products did not require further purification other than precipitation and the isolated yields ranged from 75% to 93% (fig. 3).

Reiteration of the reaction sequence after ion exchange enabled homologation to the triphosphates (NTP). Due to decreased stability of the $P^{	ext{III}}$-$P^{	ext{V}}$-$P^{	ext{V}}$ anhydride intermediates, slightly higher amounts of reagent 1 were necessary (1.6-1.7 eq.). After deprotection, the triphosphates were precipitated in 68-79 % yield (fig. 3). They contained 3-12 % of NDP as sole impurity due to a decay of the intermediate. For many biological applications, this purity is sufficient; otherwise strong-anion-exchange (SAX) chromatography can be applied for the removal of trace impurities.
Besides further homologation of ATP to obtain adenosine tetraphosphate (AP4) in 50% isolated yield, it was also shown that these couplings work on controlled pore glass (CPG) bound nucleosides. The homologation of CPG-bound 2‘-deoxyguanosine to dGMP, dGDP and dGTP was similarly fast and selective and demonstrates the possibility to build up oligophosphate chains of defined length on solid support.

Conclusion
Nature synthesizes P-anhydrides by iterative phosphorylation. Consequently, our PIII based iterative homologation can be considered a biomimetic approach. Additionally, it offers the possibility to introduce modifications at different defined positions in the growing phosphate chain, due to the occurrence of a PIII intermediate. The development of automated oligophosphate synthesis on solid-bound substrates, such as 5‘-cap-structures found in oligonucleotides, is an exciting potential application of these homologations with significant impact also in industrial research.

The possibility of chemoselective P-anhydride couplings using PIII chemistry is a discovery that holds promise for the synthesis of a broad range of natural products and chemical tools for biological studies. The developed protocol is a powerful method to extend oligophosphate chains by one phosphate unit in each coupling; it is highly selective, reliable, works in open flasks and under ambient conditions without tedious purification procedures. As such, it has the potential to open P-anhydride synthesis to less specialized labs as a routine technique.

References

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