Phosphorylation

DOI: 10.1002/anie.201301092

Synthesis of Unsymmetric Diphospho-Inositol Polyphosphates**

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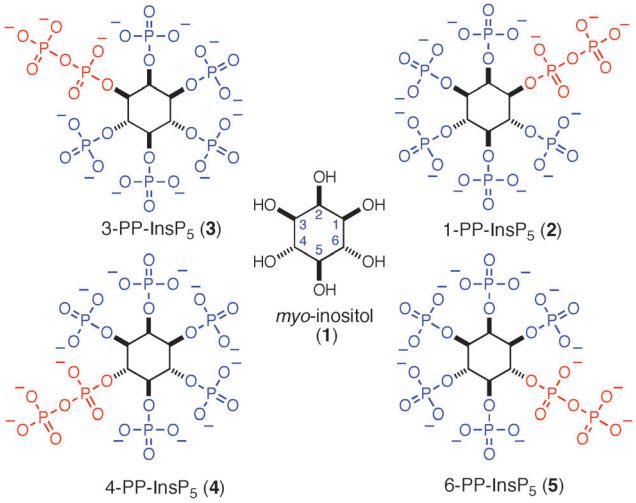
The phosphate esters of *myo*-inositol (**1**) have long been recognized as molecules of tremendous biological relevance in cellular signaling events.[1] Given the already high complexity of the inositol signaling cascade, a striking discovery was that inositol hexakisphosphate $InsP_6$ can be further phosphorylated by inositolhexakisphosphate kinases (IP6K) to pyrophosphorylated representatives such as 5-diphospho-inositol-1,2,3,4,6-pentakisphosphate 5-PP-$InsP_5$.[2] Different positional isomers of diphospho-inositol polyphosphates (X-PP-$InsP_5$, for example, **2**–**5**) have been discovered and were found to occur in all domains of biology ranging from archaea to mammals. Highly conserved enzymes tightly regulate their production and destruction.[3]



A concise synthetic approach allowing for the synthesis of all positional isomers of the X-PP-$InsP_5$ would enable the assignment of the specificity of the enzymes involved in X-PP-$InsP_5$ turnover. The possibility to generate derivatives as tools for chemical biology studies would open up new ways to understand their rich biological functions. X-PP-$InsP_5$ are involved in the regulation of such diverse processes as energy homeostasis, stress responses, viral release, cancer development, telomere length control, diabetes, and obesity.[3g,4] Furthermore, 5-PP-$InsP_5$ has been shown to act as a phosphate donor in vitro. This special process has been dubbed transphosphorylation, as in contrast to ATP-dependent phosphorylation, it occurs autocatalytically within specific serine-rich aminoacid sequences and the product of transphosphorylation is a serine pyrophosphate.[3d,4a,5]

X-PP-$InsP_5$ are difficult synthetic targets owing to their extreme hydrophilicity that is manifested in up to thirteen negative charges arranged around the rim of the inositol core structure. Recent investigations were focused on the synthesis of the symmetric *meso*-5-PP-$InsP_5$ and highlight the difficulties associated with obtaining reasonably pure material.[6] Although preparations of unsymmetric X-PP-$InsP_5$ have been reported,[1b,7] no unified approach exists to target all four unsymmetric X-PP-$InsP_5$.

The desymmetrization of *myo*-inositol (**1**) can be achieved in different ways.[8] A very efficient approach would be a desymmetrization by phosphorylation. Peptide-catalyzed enantioselective phosphorylations and phosphitylations have been described.[9] However, so far only phenol and benzyl protecting groups for the phosphite/phosphate esters are tolerated in this approach.

C_2-symmetric phosphoramidites have not yet been applied in the desymmetrization event to directly yield a phosphitylated intermediate. Conceptually, the chiral P-amidite should fulfil the following criteria: 1) easy accessibility, 2) C_2 symmetry to avoid chirality at phosphorous, 3) stability, and 4) mild removal as an orthogonal protecting group. With these prerequisites defined, a chiral auxiliary derived from the β-cyanoethyl protecting group[10] (β-CE, see Scheme 1, box) seemed to be a promising candidate. Analysis of the structure revealed a possible straightforward modification by introduction of a phenyl ring at C-α (**7**, Scheme 1). This novel chiral auxiliary/phosphate protecting group was derived from mandelic acid (e.r. 99.5:0.5) in a few synthetic steps but can also be prepared by enantioselective transfer hydrogenation.[11]

With the chiral P-amidite **7** in hand, different protected inositol-derived *meso* building blocks were needed that were suitable for the envisaged desymmetrization. For the construction of 1- and 3-PP-Ins-P_5 (**2**, **3**), a recently described regioselective *ortho*-ester cleavage was employed with a modified pattern of protecting groups.[12] Briefly, *myo*-inositol (**1**) was converted into its *ortho*-benzoate and subsequently *para*-methoxybenzylated (PMB) to *ortho*-ester **8**. Diisobutylaluminium hydride (DIBAL-H) was used for a regioselective reductive scission of the *ortho*-benzoate, thus releasing the 5-OH group. PMB-protection and selective acid-catalyzed rupture of the 1,3 benzylidene acetal without isolation of the intermediate yielded PMB-protected diol **9**.

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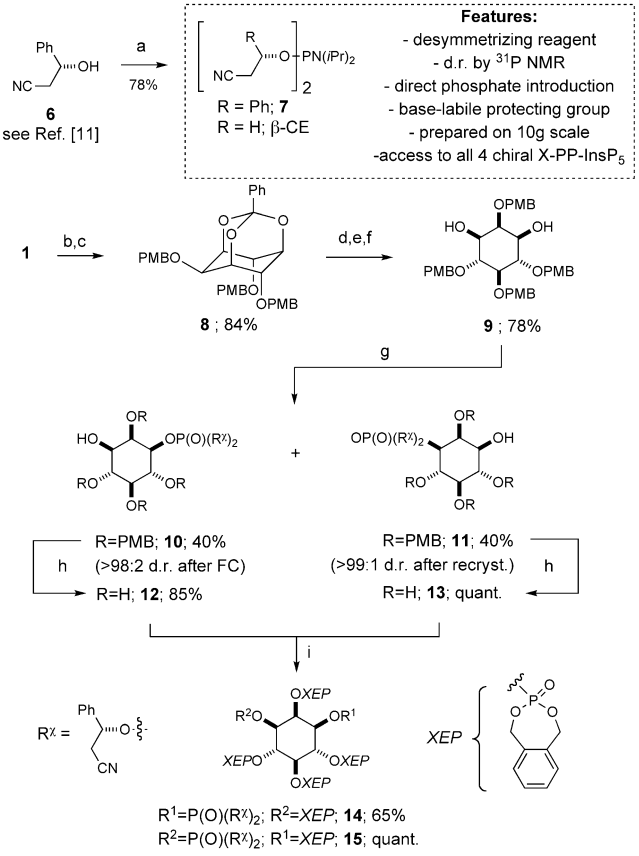
[**] The authors are grateful to Prof. J. Siegel for continuous support, and the NMR and MS facilities at UZH (O. Zerbe, L. Bigler). We thank A. Saiardi for kindly providing the plasmid encoding Ddp1. This work was supported by the Fonds der Chemischen Industrie (FCI, Liebig Stipend to H.J.J.) and the Swiss National Science Foundation (SNF, Ambizione Grant PZ00P2_136816 to H.J.J.).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201301092.



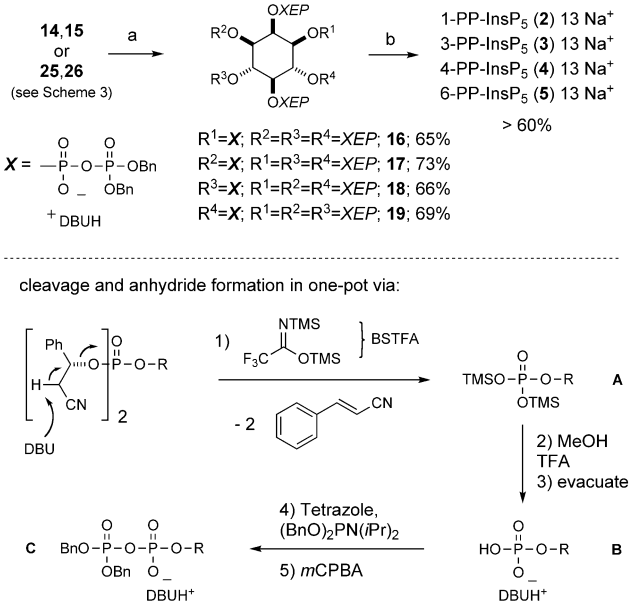
Scheme 1. Synthesis of unsymmetric hexaphosphates **14** and **15**. a) $Cl_2PN(iPr)_2$, NEt_3, 3 d; b) $PhC(OMe)_3$, CSA, DMSO, then recryst.; c) NaH, PMB-Cl, DMF, then recryst.; d) 2.7 equiv DIBAL-H, DCM, −78 °C; e) NaH, PMB-Cl, DMF; f) cat. *p*TsOH, H_2O/MeOH, 5 min reflux, then recryst.; g) 3 equiv **9**, 1 equiv **7**, 5-(*para*-F-phenyl)1*H*-tetrazole in MeCN, then 0 °C, *m*CPBA; 1:1 mixture of **10** and **11**, separated by recryst. and FC. Excess starting material recovered, yield based on **7**; h) 2.5% TFA in $CHCl_3$; i) 10 equiv *XEP*-amidite, 15 equiv DCI, 0 °C, MeCN, then 10 equiv *m*CPBA, 0 °C, then recryst. CSA = camphorsulfonic acid; DCI = 4,5-dicyanoimidazole; DCM = dichloromethane; DMF = dimethylformamide; DMSO = dimethylsulfoxide; DIBAL-H = diisobutylaluminium hydride; *m*CPBA = *meta*-chloro perbenzoic acid; PMB-Cl = *para*-methoxybenzyl chloride; *p*TsOH = *para*-toluene sulfonic acid; TFA = trifluoroacetic acid; *XEP*-amidite = *o*-xylylene *N*,*N*-diisopropylamino phosphoramidite.

Meso-compound **9** with free 1- and 3-OH groups was now set for desymmetrization by phosphitylation. As an activator, 5-(*para*-F-phenyl)1*H*-tetrazole was used and the P^{III} intermediates were oxidized in situ. This procedure resulted in a 1:1 mixture of both the 1- and 3- phosphorylated products **10** and **11** according to ^{31}P NMR analysis. No attempts were undertaken to bias the selectivity, as both products were needed in the following steps.

Diastereomer **11** was separated from the mixture by crystallization resulting in a diastereomeric ratio of > 99:1 as judged by proton decoupled ^{31}P NMR spectroscopy. The other diastereomer **10** was purified to a ratio 98:2 by normal flash column chromatography (FC). The regioselectivity was assigned by cleavage of the protecting groups giving the enantiomeric inositol-1-phosphate or inositol-3-phosphate for which the optical rotations are known (see Supporting Information).[8d]

Cleavage of the PMB groups resulted in the diastereomeric protected inositol-phosphates **12** and **13** that were subjected to exhaustive phosphitylation with an *o*-xylylene-derived phosphoramidite (*XEP*-amidite)[6c] followed by in situ oxidation yielding hexaphosphates **14** and **15**.

The next challenge was the mild removal of the chiral auxiliary and the generation of the phosphoanhydride bond. Although mono-deprotection was readily achieved by addition of one equivalent of DBU under elimination of cinnamonitrile (thus proving equivalence to the β-CE protecting group; see Scheme 2), the second group was not as easily removed. With the assumption that the generated negative charge at phosphorous after cleavage of the first



Scheme 2. One-pot deprotection/P-anhydride formation sequence followed by global reductive deprotection yielding the natural products (**2**–**5**) as sodium salts. a) 1. DBU, BSTFA in MeCN (**A**), then 2. TFA in MeOH, then 3. remove solvents in vacuo (**B**), then 4. dibenzyl diisopropylamino phosphoramidite, 1*H*-tetrazole in MeCN, then 5. *m*CPBA (**C**), recryst., then RP-FC (C_{18}); b) 80 bar H_2, PtO_2 or Pd (black) in *t*BuOH/H_2O 4:1 to 1:1, $NaHCO_3$, 3 h, then recryst. DBU = 1,8-Diazabicyclo[5.4.0]undec-7-ene; BSTFA = *N*,*O*-bis(trimethylsilyl) trifluoroacetamide, RP-FC = reversed phase flash chromatography.

protecting group is blocking the cleavage of the second protecting group due to repulsive coulombic interaction, a transient masking of the negative charge with a trimethylsilyl (TMS) group should enable cleavage of the second chiral auxiliary.[13]

N,*O*-Bis(trimethylsilyl) trifluoroacetamide (BSTFA) proved to be a suitable, mild TMS donor in this transformation and produced the bis-TMS-protected phosphate (**A**, Scheme 2) within a few minutes in the presence of excess DBU. The bis-TMS phosphate was then directly methanolyzed to the monophosphate. Suppression of an immediate

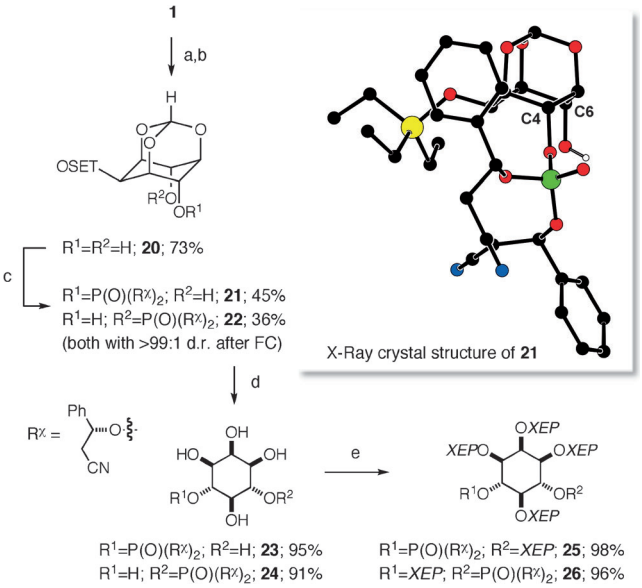
attack of the strongly nucleophilic dibasic phosphate to the adjacent phosphate triesters was achieved by addition of trifluoroacetic acid (TFA) during methanolysis (**B**, Scheme 2) resulting in the less nucleophilic monobasic phosphate. This intermediate was then directly converted into the mixed P-anhydride with bis-benzyl-*N*,*N*-diisopropylamino phosphoramidite in the presence of 1*H*-tetrazole, subsequently oxidized, and the product was crystallized in excellent yield (**C**, Scheme 2).

This transformation allowed the P-anhydride bond to be set up in a one-pot reaction starting from both hexaphosphates **14** and **15**, respectively. It underscores the multiple utilities of the new chiral phosphate protecting group and is the first example for the application of P^{III} chemistry in the generation of phosphoanhydrides on the way to diphosphoinositol polyphosphates. It opens up the possibility of introducing, for example, thiopyrophosphates (see the Supporting Information for proof of concept, compound **17b**) and boranopyrophosphates as chemical probes for inositol metabolism and provides a highly streamlined access to protected PP-InsP_5 derivatives.

A global deprotection of phosphoanhydrides **16** and **17** by hydrogenation[7a] led to the natural products **2** and **3**, respectively, after recrystallization from water/acetone in only ten steps. No further purification was necessary, as testified by the recorded NMR spectra (Supporting Information). Treatment of the material with ion exchange resin (Dowex Na^+) usually led to a significant improvement of the recorded spectra that tend to show very broad resonances both in the ^{31}P NMR as well as in the ^{1}H NMR spectra, blurring impurities that can be problematic in biological studies.[6c,7a] The high quality of this material will be helpful in understanding the binding specificity of X-PP-InsP_5 to proteins and the IP6Ks in unprecedented detail.

Next, application of the same chemistry to the synthesis of the two enantiomorphous 4- and 6-PP-InsP_5 isomers (**4**, **5**) was envisaged. Analysis of orthogonally protected derivatives with free hydroxy functions in the 4- and 6-positions revealed *ortho*-formates as suitable starting materials. These compounds are accessible from *myo*-inositol (**1**) by *ortho*-ester formation followed by selective silyl protection of the equatorial hydroxy function.[14] This procedure leaves the axial 4- and 6-positions available for desymmetrization as in **20** (Scheme 3).

After phosphitylation with P-amidite **7**, the desired products **21** and **22** were obtained as a 1.0:0.8 mixture, which were separated by FC, yielding both monophosphorylated isomers **21** and **22** in excellent diastereomeric ratios (>99:1). Single crystals of diastereomer **21** were obtained and an X-ray diffraction analysis[15] (Scheme 2, Supporting Information) revealed that phosphorylation had taken place at the 4-position and thus allowed assignment of the other isomer as the 6-modified phosphate triester **22**. Upon treatment with catalytic amounts of acid, first the TES group was smoothly removed, releasing the 2-OH group. It is well documented that this group is involved in the cleavage mechanism of *ortho*-esters of inositols.[16] Thus, in a second event, the *ortho*-ester was cleaved through the intermediacy of a 2 formyl-modified scaffold that eventually released the 4- and 6-



Scheme 3. a) $HC(OMe)_3$, *p*TsOH, DMSO, then recryst.; b) TES-Cl, lutidine, DCM, then recryst.; c) 3 equiv **20**, 1 equiv **7**, DCI in MeCN, 0 °C, then *t*BuOOH (5.5 M in nonane) 1.0:0.8 mixture of **21** and **22**, separated by FC and recryst.; d.r. > 99:1; excess starting material recovered, yield based on **7**; d) cat. *p*TsOH, MeOH/DCM, then recryst.; e) 10 equiv *XEP*-amidite, 15 equiv DCI, 0 °C, MeCN, then 10 equiv *m*CPBA, 0 °C, then recryst. Abbreviations: DCI = 4,5-dicyanoimidazole; TES-Cl = triethylsilyl-chloride.

modified phosphate triesters **23** and **24**, respectively. The products were exhaustively phosphitylated with *XEP*-amidite followed by in situ oxidation yielding hexaphosphates **25** and **26**.

The P-anhydride was established as previously described for the 1- and 3-isomers **16** and **17** in a one-pot sequence (Scheme 2). The protected natural products **18** and **19** were unveiled by hydrogenations yielding the corresponding 4- and 6-PP inositol pentaphosphates **4** and **5** in good quality after strong anion exchange chromatography in only seven consecutive steps.

X-PP-InsP_5 are metabolized by different enzymes, for example, phosphatases that cleave the pyrophosphate group. The assignment of the regioisomeric preference of diadenosine and diphosphoinositol polyphosphate phosphohydrolase 1 from yeast (Ddp1) is an interesting application of synthetic X-PP-InsP_5.[17] A modified malachite green assay (Supporting Information)[17c] to measure phosphate release from the isomers **2**–**5** in the presence or absence of recombinant purified Ddp1 was employed (Figure 1). This data shows, that Ddp1 is a selective 1-PP-InsP_5 phosphatase. Ddp1 dephosphorylates the product of Vip1,[17c] a kinase that phosphorylates InsP_6 in the 1/3 position. More recently, the human diphosphoinositol pentakisphosphate kinases PPIP5K (Vip1 is the yeast homolog) were assigned to be InsP_6 1-kinases.[3k] The obtained data support this assignment, as only one out of the four analyzed isomers, namely 1-PP-InsP_5, is a substrate of Ddp1.

In summary, a novel approach for a unified synthesis of unsymmetric diphosphoinositol polyphosphates has been

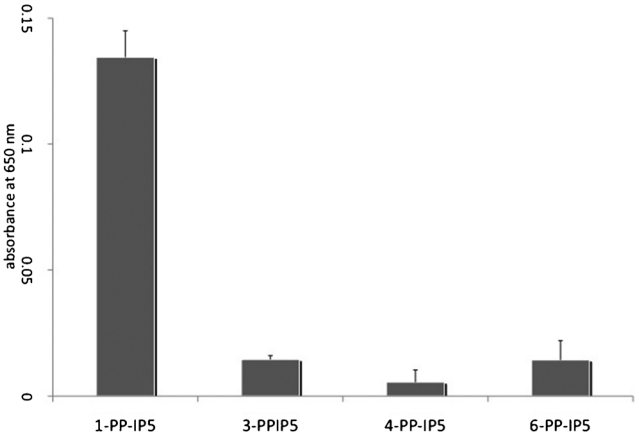


Figure 1. Measurement of phosphate release from nonsymmetric X-PP-IP_5 in the presence of recombinant 6×His-Ddp1 from yeast. 100 μM solutions of **2**–**5** were incubated with 100 ng Ddp1 at 37 °C for 10 min. Absorbance was quantified at 650 nm after addition of malachite green reagent. All assays were conducted in triplicate and corrected for nonenzymatic hydrolysis of **2**–**5** under the assay conditions.

developed. The application of one single C_2-symmetric phosphoramidite allows inositol to be desymmetrized, accompanied by the direct introduction of an orthogonally protected phosphate triester, in all four relevant positions. It was shown that this approach is complementary to usually applied desymmetrizations with camphor acetals and is more efficient as a phosphate group is installed directly. Moreover, the auxiliary was cleanly removed upon treatment with base under very mild conditions, as expected from its kinship to the β-CE protecting group, allowing an efficient one-pot synthesis of the vital P–anhydride bond. This method can readily be adapted for the introduction of chemical modifications such as, for example, thiophosphates. The availability of this set of natural/unnatural products in unprecedented purity and the opportunity to access interesting derivatives will help to decipher the enigmas associated with X-PP-$InsP_5$-mediated signaling in greater detail than previously possible.

Received: February 6, 2013
Revised: March 29, 2013
Published online: May 27, 2013

Keywords: chiral auxiliaries · cyclitols · natural products · phosphorylation · polyanions

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