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Synthesis of Densely Phosphorylated Bis-1,5-Diphospho-*myo*-Inositol Tetrakisphosphate and its Enantiomer by Bidirectional P-Anhydride Formation**

Samanta Capolicchio,
Department of Chemistry, University of Zürich (UZH), Winterthurerstrasse 190, 8057 Zürich (Switzerland)

Dr. Huanchen Wang,
Inositol Signaling Group, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC (USA)

Divyeshsinh T. Thakor,
Department of Chemistry University of Zürich (UZH) Winterthurerstrasse 190, 8057 Zürich (Switzerland)

Dr. Stephen B. Shears, and
Inositol Signaling Group, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC (USA)

Dr. Henning J. Jessen
Department of Chemistry University of Zürich (UZH) Winterthurerstrasse 190, 8057 Zürich (Switzerland)

Henning J. Jessen: Henningjacob.jessen@chem.uzh.ch

Abstract

The ubiquitous mammalian signaling molecule bisdiphosphoinositol tetrakisphosphate (1,5-$(PP)_2$-myo-$InsP_4$, or $InsP_8$) displays the most congested three-dimensional array of phosphate groups found in nature. The high charge density, the accumulation of unstable P-anhydrides and P-esters, the lack of UV absorbance, and low levels of optical rotation constitute severe obstacles to its synthesis, characterization, and purification. Herein, we describe the first procedure for the synthesis of enantiopure 1,5-$(PP)_2$-myo-$InsP_4$ and 3,5-$(PP)_2$-myo-$InsP_4$ utilizing a C_2-symmetric P-amidite for desymmetrization and concomitant phosphitylation followed by a one-pot bidirectional P-anhydride-forming reaction that combines sixteen chemical transformations with high efficiency. The configuration of these materials is unambiguously shown by subsequent X-ray analyses of both enantiomers after being individually soaked into crystals of the kinase domain of human diphosphoinositol pentakisphosphate kinase 2.

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Correspondence to: Henning J. Jessen, Henningjacob.jessen@chem.uzh.ch.

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Keywords

chiral auxiliaries; inositol; kinases; phosphorylation; second messengers

Cells propagate and integrate information by using phosphorylated second messengers based on the *myo*-inositol scaffold.[1] Their polar character and relatively low abundance often necessitate tedious isolation procedures, which is especially true for the family of inositol pyrophosphates.[2] The need to have ready access to inositol pyrophosphates for biological studies motivated the development of a facile, scalable, and stereoselective synthesis of these molecules.

Inositol pyrophosphates, such as 5-PP-*myo*-$InsP_5$ (**1**), 1-PP-*myo*-$InsP_5$ (**2**, *myo*-$InsP_7$), and 1,5-$(PP)_2$-*myo*-$InsP_4$ (**3**, *myo*-$InsP_8$; Figure 1), can signal by two distinctly different processes, that is, by either binding to a specific receptor[3] or by non-enzymatic transfer of the β-phosphate to phosphorylated serine residues in proteins (transphosphorylation).[4] In vivo, both processes contribute to complex signaling networks that are far from being understood.[5]

Inositol pyrophosphates and their dedicated kinases (IP6Ks: inositolhexakisphosphate kinases; PPIP5Ks: diphosphoinositol pentakisphosphate kinases)[6] and phosphatases (DIPPs: diphosphoinositol polyphosphate phosphohydrolases)[7] have been linked to a range of biological phenomena, such as insulin signaling,[8] telomere length regulation,[9] pleckstrin homology (PH) domain regulation,[10] cellular energy homeostasis,[11] apoptosis,[12 and regulation of the innate immune system.[13] Some of these studies have been facilitated by the use of chemically synthesized *myo*-$InsP_7$ and derivatives thereof.[4b,8b,12,13b] In contrast, the specific biological functions of *myo*-$InsP_8$ remain largely unexplored, mainly because a method to chemically synthesize these molecules in sufficient purity has not been reported. Nonetheless, it has been established that concentrations of *myo*-$InsP_8$ in mammalian cells are regulated in a stimulus-dependent fashion increasing severalfold following osmotic stress or thermal challenge, whereas bioenergetic stress significantly decreases levels of *myo*-$InsP_8$.[14]

In mammals, the structure of the *myo*-$InsP_8$ isomer has been identified as the 1,5 isomer **3**.[15] Chemical synthesis of this isomer would greatly aid studies into its function. Having access to both enantiomers **3** and **4** would be even more useful for functional studies, in which biological specificity could be tested.

Different isomers and analogues of *myo*-$InsP_7$ have been accessed by chemical synthesis, but it is still a challenge to obtain reasonably pure material.[16] The method must be mild enough to protect the delicate P-anhydride, while enforcing the generation of a highly congested structure. The more intense molecular crowding in 1,5-*myo*-$InsP_8$ presents even more complex synthetic challenges. In fact, the chemical synthesis of only one *myo*-$InsP_8$ isomer is reported, namely the unnatural and symmetric *meso* 2,5-isomer; its published purity of approximately 10% underscores the technical demands in synthesizing these molecules.[17]

Synthetic access to enantiopure nonsymmetric *myo*-$InsP_7$ can be achieved by using a C_2-symmetric P-amidite through the desymmetrization of a protected analogue of the *meso* compound *myo*-inositol.[18] The efficiency of this approach is based on the fact that the chiral auxiliary also serves as an orthogonal protecting group that can be cleaved under very mild conditions. As all positions in *myo*-$InsP_8$ are phosphorylated, desymmetrization by phosphorylation[18a, 19] significantly decreases the required number of steps. Consequently, extension of this approach to synthesize *myo*-$InsP_8$ derivatives **3** and **4** could potentially provide efficient access to these molecules.

The synthesis commenced with *myo*-inositol (**5**) that was converted[18a] (Scheme 1) into benzylidene-protected intermediate **6** with a free 5-OH group by the method of Holmes et al. [20] The 5-axial hydroxy group in alcohol **6** was phosphitylated with a bis-fluorenyl-protected P-amidite **7**,[21] oxidized, and the benzylidene acetal was selectively cleaved yielding *meso*-diol **8**.

Diol **8** was subjected to phosphitylation with C_2-symmetric P-amidite **9**, followed by oxidation of the P^{III} intermediate. The obtained mixture of diastereomers **10a/b** was then separated by repeated flash chromatography (FC) until an enrichment of either diastereomer, **10a** or **10b**, to a purity of 90% to 95%, was achieved. Note that with this approach, a total of 2.7 g of the diastereomeric mixture was separated, providing access to more than 1 g of each of the purified diastereomers (diastereomeric ratio, d.r. >9:1). After cleavage of the *para*-methoxybenzyl (PMB) protecting groups under acidic conditions and crystallization, both diastereomers **11a** and **11b** were obtained in highly enriched form (d.r. ≥ 98:2), as evidenced by mixing of the separated compounds and ^{31}PNMR spectroscopic analysis (see the Supporting Information). Consequently, enrichment of the diastereomers **10a** or **10b** prior to cleavage of the PMB groups is sufficient to obtain essentially diastereopure material in the next step. The diastereopure tetraols **11a** and **11b** were then phosphitylated with an *o*-xylylene-derived phosphoramidite **12**[16g] and oxidized in situ. The obtained unsymmetric hexakisphosphates **13a** and **13b** were again analyzed for diastereomeric purity by mixing experiments (d.r. ≥ 98:2; Supporting Information).

In the key step, the hexakisphosphates **13a** and **13b** were subjected to a bidirectional "telescoping" P-anhydride-forming reaction (Scheme 2). This step involved the introduction of both protected pyrophosphates in the 1 or 3 and 5 positions in a single, one-pot reaction without workup of the reaction product between steps. As this process combined sixteen discrete chemical reactions, precise control over every single step was required. Overall, four protecting groups (Fm and chiral auxiliaries R^{χ}) were removed by replacement with more labile trimethylsilyl groups (TMS) under mild, basic conditions in the presence of the TMS donor *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA). Subsequently, all four TMS groups were cleaved by methanolysis and the nucleophilic dibasic phosphates formed were stabilized by mono-protonation with trifluoroacetic acid (TFA). The monobasic phosphates obtained were then phosphitylated with bis-benzyl P-amidite **14**, oxidized in situ, and the generated bis-P-anhydrides **15a** and **15b** were isolated in almost pure form by simple precipitation. It was mandatory to obtain the bis-P-anhydrides **15a** and **15b** in high quality, as they were not stable enough for further purification.

Rapid deprotection by high-pressure hydrogenation of **15a** or **15b** led to **3** or **4**, respectively (Scheme 2), and precipitation from solution gave material of suitable purity for biological studies. The presence of trace impurities (10%) of *myo*-$InsP_7$ (e.g. **1** and **2**) was detected by ^{31}P NMR spectroscopy and PAGE analysis[22] (Scheme 2, dotted box; Supporting Information). Even though α-unprotected P-anhydrides[23] are less prone to hydrolysis compared to P-anhydrides that are fully protected,[16g, 17] complete preservation of the moiety could not be achieved.

It is difficult to assign the absolute configuration of inositolphosphates exclusively by measurement and comparison of optical rotations, as these values are usually very small and strongly depend on the pH value.[19] Optical rotation values of *myo*-$InsP_8$ have not been reported. However, the naturally occurring isomer of *myo*-$InsP_8$, **3**, has previously been identified in crystal complexes of the kinase domain of human PPIP5K2 ($PPIP5K2^{KD}$).[15] New crystal complexes of $PPIP5K2^{KD}$ were now prepared, into which either *myo*-$InsP_8$ enantiomer **3** or **4** were individually soaked (Figure 2). The electron-density maps allowed for an unequivocal assignment of the absolute configuration of each of the *myo*-$InsP_8$ enantiomers. It is interesting to note that the unnatural enantiomer **4** is also accommodated in the catalytically active site of the enzyme. In addition to the active site of $PPIP5K2^{KD}$ accommodating the natural (**3**) and unnatural (**4**) enantiomers, both were also substrates for the phosphatase enzyme DIPP1 (**3**: V_{max} = 124.9 ± 5.5 $nmolmg^{-1}$ min^{-1}; **4**: V_{max} = 239.9 ± 9.3 $nmolmg^{-1}$ min^{-1}, n = 3; measured by inorganic phosphate (P_i) release as described previously (see Ref. [24] and the Supporting Information).

Overall, this study unveils a new strategy to synthesize 1,5- and 3,5-*myo*-$InsP_8$ **3** and **4** in scalable quantities, based on desymmetrization by phosphitylation on a gram-scale, followed by a bidirectional P-anhydride synthesis in a one-pot telescoping procedure. The absolute configuration was deduced by cocrystallization with $PPIP5K2^{KD}$, allowing for the subsequent unambiguous assignment of each enantiomer. This study provides novel strategies to construct the most congested and highly charged three-dimensional assemblies of phosphate groups occurring in nature. These strategies can be applied to target other regioisomers and functionalized analogues and will help to unravel the enigmas associated with the inositol pyrophosphate signaling network.

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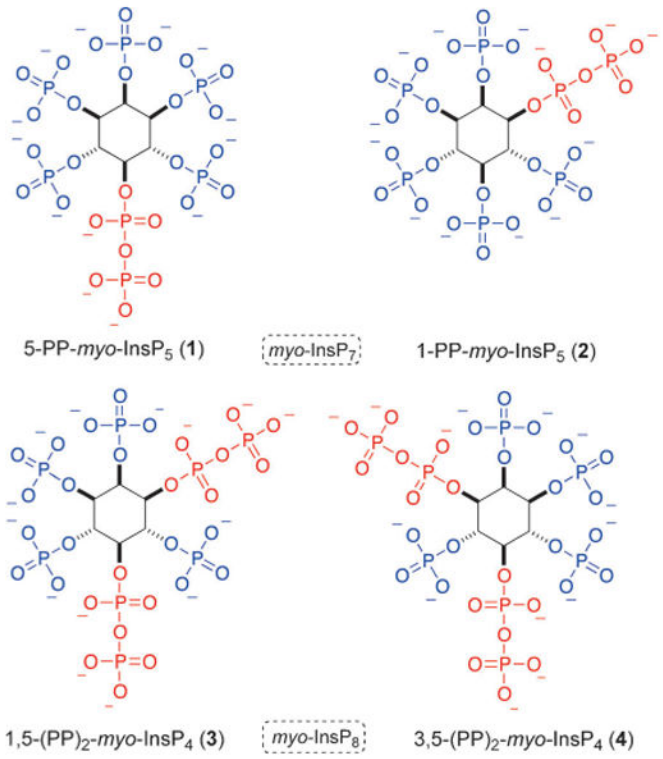


Figure 1.

Mammalian inositol pyrophosphates **1–3** and an unnatural enantiomer **4**. The dashed box indicates the generic description, with the more precise names given underneath the compounds.

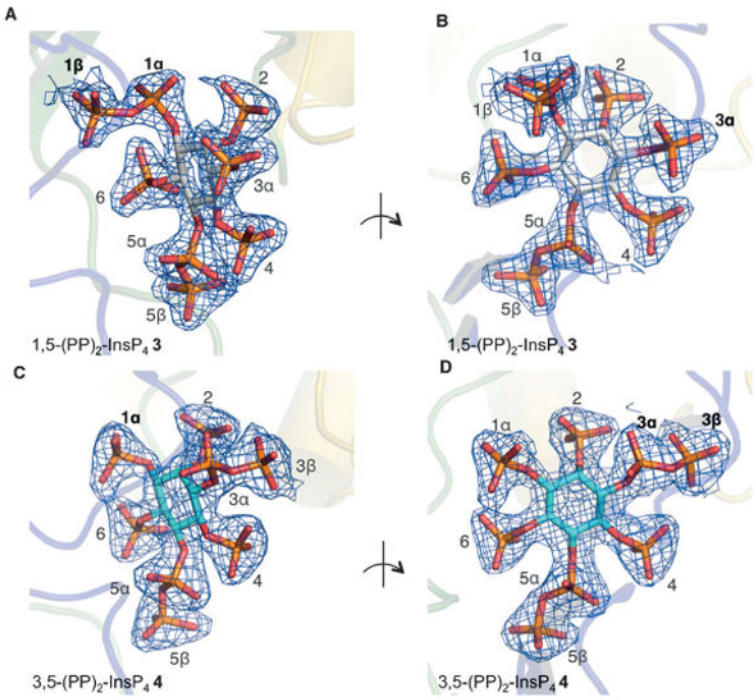
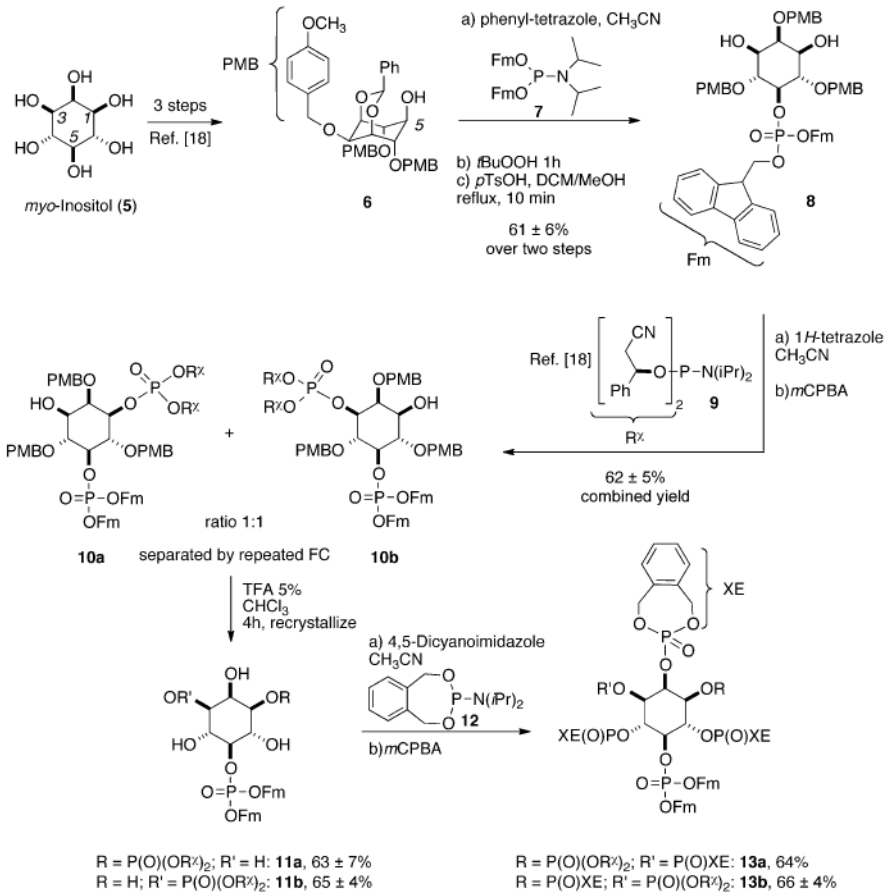


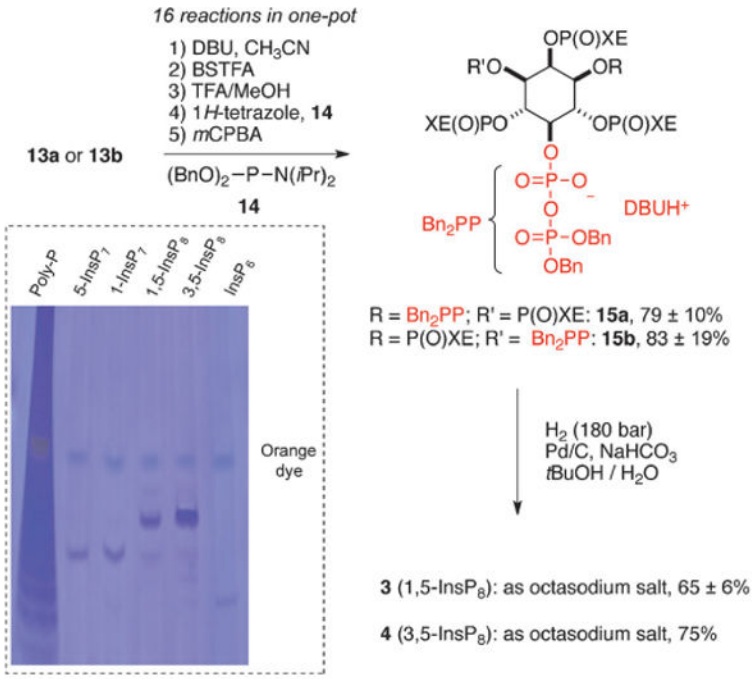
Figure 2.

Refined 2 $F_o - F_c$ maps contoured at 2.0σ (panels A, B) or 1.0σ (panels C, D) and shown in blue mesh. *Myo*-$InsP_8$ molecules are shown in a stick model. The kinase domain of PPIP5K2 is shown in a transparent representation. The phosphate groups around the inositol ring are numbered. Panels A, B: $PPIP5K2^{KD}$/ADP complexed with *myo*-$InsP_8$ (1,5-$(PP)_2$-$InsP_4$ **3**), showing two orientations. Panels C, D: $PPIP5K2^{KD}$/AMP-PNP complexed with the enantiomer 3,5-$(PP)_2$-$InsP_4$ (**4**). (PDB codes: 4Q4C and 4Q4D).



Scheme 1.

Synthesis of the unsymmetric hexakisphosphates **13a** and **13b** by phosphitylation with C_2-symmetric P-amidite **9**, followed by the gram-scale separation of the diastereomers **10a**,**b**. The chiral auxiliary (R^{χ}) serves as an orthogonal protecting group throughout the synthesis. DCM = dichloromethane, PMB = *para*-methoxybenzyl, Fm = fluorenylmethyl, *t*BuOOH = *tert*-butylhydroperoxide, *p*TsOH = *para*-toluenesulfonic acid, *m*CPBA = *meta*-chloroperbenzoic acid, TFA = trifluoroacetic acid, FC = flash chromatography, XE = o-xylylene.



Scheme 2.

One-pot synthesis of protected 1,5- or 3,5-$(PP)_2$-*myo*-$InsP_4$ (**15a**/**15b**) followed by high-pressure hydrogenation to yield enantiopure *myo*-$InsP_8$ (**3** or **4**) as octasodium salts. Dotted Box: PAGE analysis of synthetic *myo*-$InsP_8$ with synthetic standards (5-*myo*-$InsP_7$, 1-*myo*-$InsP_7$) and commercial standards (polyphosphate and *myo*-$InsP_6$). Stained with toluidine blue. BSTFA = *N*,*O*-bis(trimethylsilyl) trifluoroacetamide, DBU = 1,8-diazabicyclo[5.4.0]undecene, TFA = trifluoroacetic acid, *m*CPBA = *meta*-chloroperbenzoic acid, Bn = benzyl.