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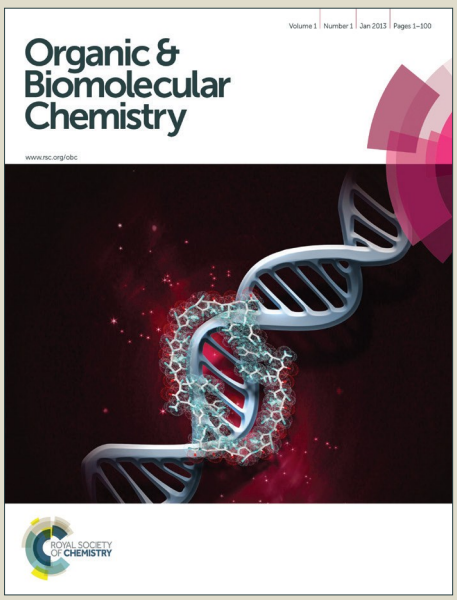

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COMMUNICATION

Synthesis of 2-Diphospho-*myo*-inositol 1,3,4,5,6-Pentakisphosphate and a Photocaged Analogue

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Diphosphoinositol polyphosphates (inositol pyrophosphates, X-InsP$_7$) are a family of second messengers with important roles in eukaryotic biology. Their chemical synthesis and modification remains a challenging task due to the high density of phosphate groups arranged around the *myo*-inositol core. Here, a novel approach is presented that facilitates the incorporation of the diphosphate in the 2-position (2-InsP$_7$) and that enables the introduction of a photocage subunit.

myo-Inositol pyrophosphates are a family of ubiquitous messenger molecules with broad impact on different cellular functions.[1] Since their initial discovery in 1993[2] it has been shown, for example, that they are involved in the regulation of telomere length, tumor growth, insulin secretion, weight gain, and monitoring of cellular energy homeostasis.[3] They provide a link between signalling and metabolism[4] and are specifically regulated by a diverse set of kinases and phosphatases.[5] While in principle six different diphosphoinositol pentakisphosphates (X-InsP7, X position of the diphosphate on the *myo*-inositol ring, see Figure 1) could exist, only three of them have been identified in nature so far, namely 1-InsP$_7$ **1**, 5-InsP$_7$ **5** and 6-InsP$_7$ **6**. Addition of yet another phosphate group gives rise to InsP$_8$ of which 1,5-InsP$_8$ and 5,6-InsP$_8$ as well as the triphosphate containing 5-InsP$_8$ are occurring in nature.[6] Even so, there remains uncertainty as to whether the family of naturally occurring inositol pyrophosphates has been exhaustively described, as their structural assignment is difficult and material for analysis is scarce.

There are several examples in which synthetic material has been used to assign the naturally occurring regioisomers. Moreover, a more detailed understanding of the specificities of the dedicated

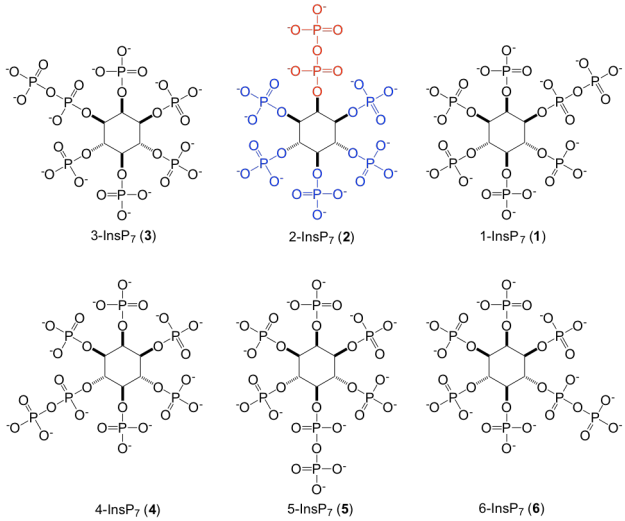

Figure 1. All six possible X-InsP$_7$ (**1**-**6**)

kinases and phosphatases has been achieved with such material. [5a, 6c, 7] Previous synthetic work to obtain the inositol pyrophosphates has exclusively relied on hydrogenations in order to remove up to thirteen protecting groups in the last step without cleaving the P-anhydride.[7b, 7d, 8] However, such conditions are not compatible with a wide range of modifications that would be useful for studies into the function of the inositol pyrophosphates. Consequently, there is a dearth of synthetic tools for chemical biology applications, with non-hydrolysable analogues as the only exceptions that have been reported and applied by the groups of Fiedler and Potter.[9]

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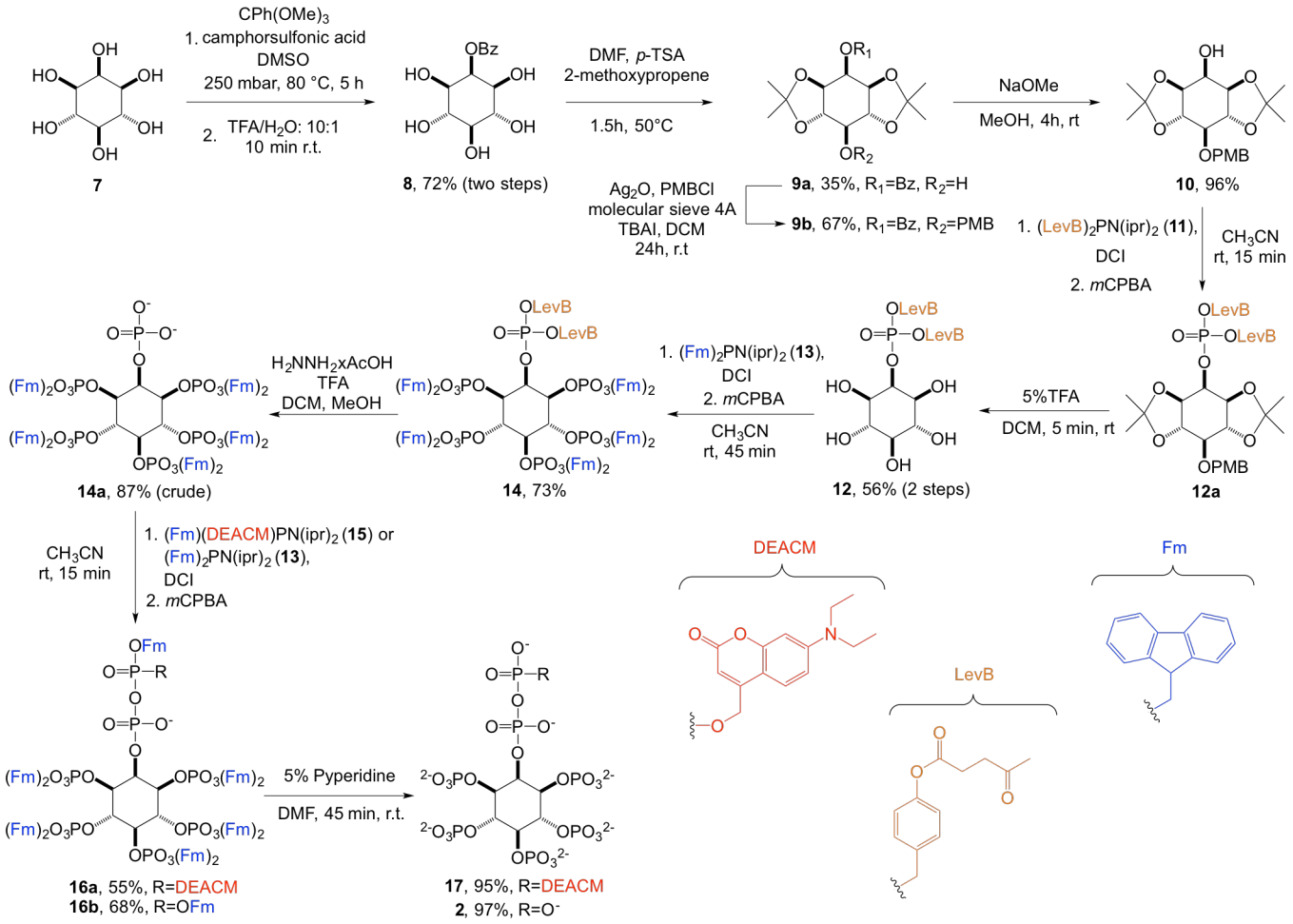

Scheme 1. Synthesis of 2-InsP$_7$ **2** and photocaged DEACM 2-InsP$_7$ **17** based on fluorenylmethyl (Fm) protection in combination with a recently developed phosphate protecting group (LevB). Abbreviations: TFA: trifluoroacetic acid. DCM: dichloromethane. DCI: 4,5-dicyanoimidazole. *m*CPBA: *meta*-chloro perbenzoic acid. Fm: fluorenylmethyl. DEACM: [7-(diethylamino)-coumarin-4-yl]methyl. DMF: dimethylformamide. *p*TSA: *para*-toluene sulfonic acid. DMSO: dimethyl sulfoxide. TBAI: tetrabuthylammonium iodide. PMB: *para*-methoxybenzyl.

Recently, a novel approach has been disclosed and enabled the synthesis of 5-InsP$_7$ **5** and a photocaged analogue without the need for a hydrogenation in the last step.[10] Moreover, initial studies have demonstrated how such molecules can be transported into living cells to study their biological function.[10-11] Here, it is shown that the novel synthetic approach can be applied to the synthesis of 2-InsP$_7$ **2** with the diphosphate moiety in the axial position, a challenge that has previously only been mastered by *Falck* and coworkers in 1997.[8d] Additionally, it is shown that also fluorescent photocaged[12] 2-InsP$_7$ **17** can be prepared due to the novel deprotection conditions applied in the last step. This opens up the possibility to introduce a range of different alternative residues for functional and control studies, which were previously inaccessible.

The synthesis commenced with a selective benzoylation of the 2-position of *myo*-inositol via the *ortho*-benzoate as published previously by *Potter* and coworkers (see Scheme 1).[13] Bis-acetonide formation, *para*-methoxybenzyl (PMB) protection and cleavage of the benzoate furnished alcohol **10**,[8e] which was phosphitylated with the novel P-amidite **11**. This P-amidite contains two LevB protecting groups that link a levulinate (Lev) protecting group via a benzyl adapter (B) unit to the P-atom. The adapter enables the use of acyl protecting groups, such as Lev, that could otherwise not be stably connected to the phosphate moiety in **12a** that results after oxidation of the phosphite intermediate. Both acetonides and the PMB groups were then removed under acidic conditions, which did not affect the LevB protecting groups on the phosphate. The ensuing pentaol was exhaustively phosphitylated with bis-Fm-P-amidite **13** containing the fluorenylmethyl (Fm)[14] protecting group and the phosphite triesters were oxidized with *meta*-chloroperoxybenzoic acid (*m*CPBA). Despite the significant molecular crowding it was possible to isolate hexakisphosphate **14** in 73% yield, underlining the efficacy of P-amidite chemistry.[15] The phosphate triester in the axial 2-position was then deprotected

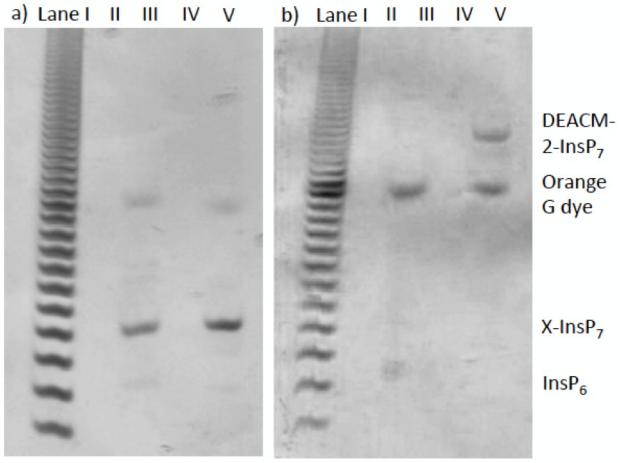

Figure 2. Purity analysis of 2-InsP$_7$ and DEACM-2-InsP$_7$ by gel electrophoresis (PAGE) and DAPI staining. Gel a) Lane I: PolyP marker. Lane II: empty. Lane III: 5-InsP$_7$ (control). Lane IV: empty. Lane V: 2-InsP$_7$ **2**. Gel b) Lane I: PolyP marker. Lane II: empty. Lane III: orange G dye. Lane IV: empty. Lane V: DEACM-2-InsP$_7$ **17**. PolyP serves as a standard for the quality of separation and Orange G dye to track migration before staining. Gels were converted to grey-scale.

selectively using hydrazine acetate in acetic acid. These conditions effected hydrazone formation and thus initiated Lev cleavage followed by 1,6 elimination of the benzyl adapter as a quinone methide. The deprotected phosphate **14a** was obtained in 87% crude yield after precipitation and it was not purified further due to its amphiphilic character. Next, the phosphoric anhydride was generated through the intermediacy of a mixed P(III)-P(V) anhydride followed by oxidation.[16] Two different P-amidites **13** and **15** were applied in these couplings to generate both an exhaustively Fm protected 2-InsP$_7$ **16a** and an analogue in which one of the Fm groups was replaced by a fluorescent diethylamino coumarine photocage (DEACM) **16b**.[10, 17] All 11 or 12 Fm protecting groups were then cleaved in 5% piperidine in DMF. After complete deprotection, the piperidinium salts (11 or 12 piperidinium counterions) of 2-InsP$_7$ **2** or DEACM 2-InsP$_7$ **17** precipitated from DMF upon addition of diethylether. The precipitate was dissolved in methanol and addition of excess sodium iodide led to precipitation of the sodium salts of the products in high quality.

The quality of the material was checked by polyacrylamide gel electrophoresis (PAGE) on dense gels followed by staining with 4,6-Diamidin-2-phenylindol (DAPI).[18] The gels underline the high quality of the material with only little contamination of InsP$_6$ as hydrolysis product (Figure 2). This impurity is easier to assign by PAGE due to overlapping signals in the ^{31}P NMR. Since photocaged 2-InsP$_7$ **17** contains a chromophore, the quality was additionally checked by reversed phase HPLC and only one peak was found in this analysis (see the supplementary information for the HPLC trace).

The synthetic material was then used in an enzymatic assay to further study the specificity of diadenosine and diphosphoinositol polyphosphate phosphohydrolase 1 from yeast (Ddp1). This phosphatase has been shown previously to have a high preference for cleavage of the diphosphate in 1-InsP$_7$ **1** over all other positions but it has not been studied concerning the cleavage of 2-InsP$_7$ **2**. [19] Recombinant purified Ddp1 was used in a modified malachite green assay that measures phosphate release from the inositol pyrophosphate according to literature precedence.[8b] No significant phosphate release from 2-InsP$_7$ **2** was measured within 10 minutes incubation in the presence of 100 ng Ddp1 at 37 °C, whereas 1-InsP$_7$ **1** was cleaved efficiently (data not shown). Thus, 2-InsP$_7$ **2** is no substrate of Ddp1, underlining the specificity of the phosphatases involved in inositol pyrophosphate turnover, which was also recently demonstrated for the phosphatase domain of Asp1 from *Schizosaccharomyces pombe*.[20]

Conclusions

This paper describes a twelve-step synthesis of 2-InsP$_7$ **2** and a photocaged analogue **17** that does not rely on a hydrogenation in the last step. Consequently, the approach is highly modular and allows for the generation of previously inaccessible analogues. The application of fluorenylmethyl protection of phosphates facilitates basic deprotection, which is compatible with the presence of a wide range of chemical functionalities. Specifically, it is shown that a DEACM photocaging subunit can be installed on the β-phosphate of the axial diphosphate of 2-InsP$_7$ **2**. Such DEACM photocages are very useful tools as they are intrinsically fluorescent, can thus be tracked in living cells and cleaved by irradiation with UV light. Applications of such analogues to biological questions will be described elsewhere. The synthetic 2-InsP$_7$ **2** was used in a modified malachite green assay to study the specificity of a dedicated inositol pyrophosphate phosphatase from yeast (Ddp1). 2-InsP7 **2** is no substrate, underlining the exquisite specificity of these enzymes. More generally, access to 2-InsP$_7$ **2** will be helpful to assign substrates and products of the enzymes involved in inositol pyrophosphate turnover. In addition, the new synthetic strategy in combination with stereoselective approaches[21] has the potential to also make accessible modified unsymmetric inositol pyrophosphates for chemical biology applications.

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