Asymmetric Catalysis

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Asymmetric Syntheses of L,L- and L,D-Di-*myo*-inositol-1,1′-phosphate and their Behavior as Stabilizers of Enzyme Activity at Extreme Temperatures**

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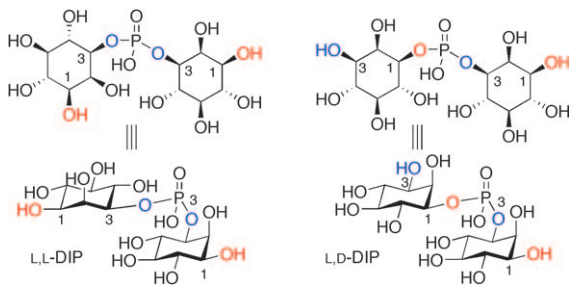
Di-*myo*-inositol-1,1′-phosphate (DIP) has been identified as a major intracellular solute in several hyperthermophilic organisms. In particular, a number of hyperthermophilic microorganisms (including archaea and the bacterium *Thermotoga maritima*) accumulate DIP as an osmolyte when grown above 75 °C;[1,2] the concentration of DIP increases dramatically at temperatures greater than 80 °C.[3] The stereoisomeric nature of naturally occurring DIP has been a matter of discussion in the literature, with reports supporting either the chiral L,L-DIP form, or its isomer, the *meso* L,D-DIP form. Presently, most of the available data, including biosynthetic[4] as well as biochemical and bioinformatic[5]



studies, support the assignment of the L,D isomer of DIP as a natural product. Importantly, it is also possible that different organisms produce different stereoisomers as a part of their individual metabolic capabilities. Through classical synthesis, van Boom and co-workers suggested the L,L isomer could be the naturally occurring form of DIP in *Pyrococcus woesei*, based on comparison of the optical rotations of natural and synthetic DIP.[6] On the other hand, in 2007 Santos and co-workers suggested the natural material produced by *Archaeoglobus fulgidus* is the L,D isomer of DIP based on ^{13}C labeling and NMR spectroscopy studies.[4] One question of interest, independent of the actual stereochemical assignment for any material of natural origin isolated to date, is whether or not the two stereoisomers are distinct functionally. That is, do the two isomers exhibit differential abilities to exhibit enzyme stabilization properties under extremophilic conditions when examined with a unique enzyme derived from a common organism? We now address this matter directly through efficient, independent state-of-the-art chemical syntheses of pure samples of known stereochemical identity, along with preliminary biochemical studies with an appropriate enzyme at high temperatures.

Previous research from our group has demonstrated that histidine-based peptides can catalyze various enantioselective phosphorylation reactions.[7] Shown in Scheme 1, the triol 2,4,6-tri-*O*-benzyl-*myo*-inositol (**2**) can be subjected to desymmetrization using either of two enantiodivergent catalysts (**4** or **5**) and diphenyl chlorophosphate to provide phosphates **1** or **3** in good yields and excellent enantioselectivity. We felt that catalysts **4** and **5** could thus be critical tools for the independent synthesis of L,L-DIP and L,D-DIP, thus enabling unambiguous access to these stereoisomers, and therefore allowing direct comparison of their differential prowess as stabilizers of enzyme activity under conditions of high temperature.

Our retrosynthetic plan was therefore quite straightforward (Scheme 2). In our approach, each inositol unit would be subjected to phosphorylation in a sequential fashion, thus allowing for rational control of O–P bond formation at either the 1-position (red) or the 3-position (blue). This plan requires the synthesis of an optically pure, fully protected inositol-derived phosphoryl chloride (**6**). Compound **6** could then be coupled to **2**, thus forming the desired phosphate bond under the control of chiral catalysts such as **4** or **5**.

Critically, phosphoryl chloride **6** contains a stereogenic phosphorus atom. As a result, catalytic phosphorylation reactions with peptide catalysts **4** and **5** create a fascinating, but complex situation of double asymmetric induction, with stereochemical preferences exerted by the catalyst and the substrate.[8] Thus, we needed to establish if catalysts **4** and **5** could overcome any inherent substrate control in the stereochemical outcome of the reactions (Scheme 3).

Execution of the synthetic plan began with preparation of phosphoryl chloride **6**, and the recognition that it would not likely be accessed in diastereomerically pure form. Furthermore, since *myo*-inositol phosphate esters with unprotected hydroxy groups are prone to undesired cyclizations,[9] we endeavored to alkylate the free hydroxy groups of **1**

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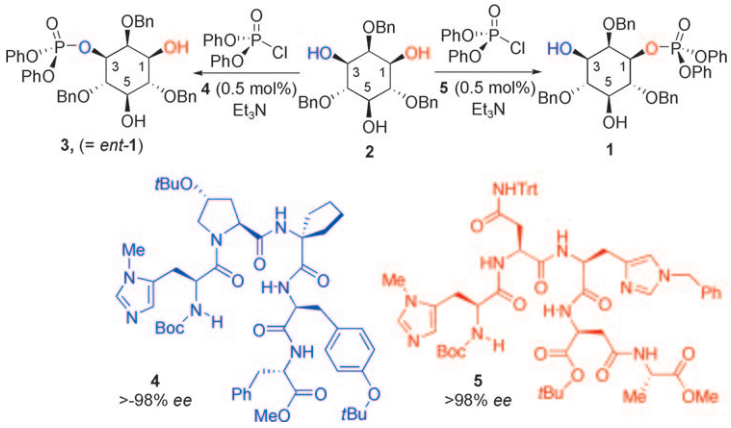
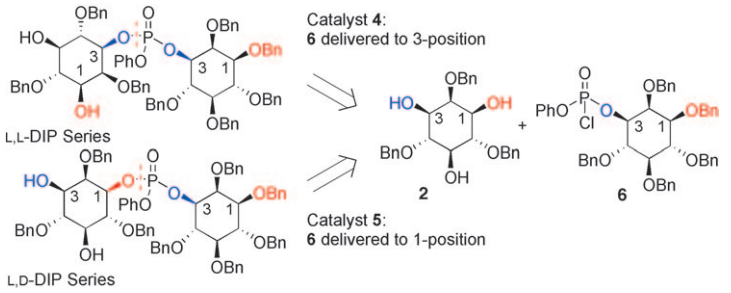
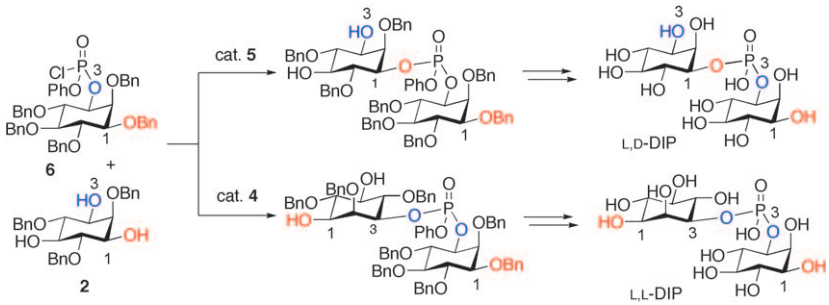


Scheme 1. Desymmetrization of *meso*-triol **2**. Boc = *tert*-butoxycarbonyl, Bn = benzyl, Trt = trityl.



Scheme 2. Retrosynthetic analysis of DIP stereoisomers.



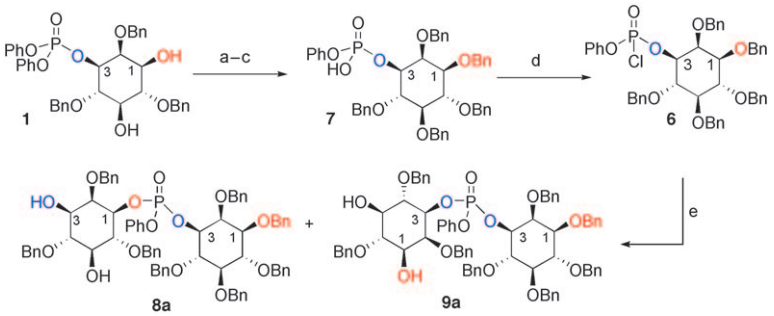
Scheme 3. Projected peptide-catalyzed coupling reactions.

(Scheme 4). Reaction conditions that avoid formation of cyclic phosphates were found to be limited to neutral, acidic, or mild basic conditions. In this vein, benzylation according to the conditions of Poon and Dudley proved uniquely successful.[10] Hydrolysis to the mono(acid) **7** was then achieved with lithium hydroxide. Preparation of the inositol-based phosphoryl chloride was then achieved using oxalyl chloride and DMF. Within 5 minutes, chloride **6** was observed as a mixture of diastereomers (2:1) that were epimeric at the phosphorous center, as was revealed in the ^{31}P NMR spectrum.

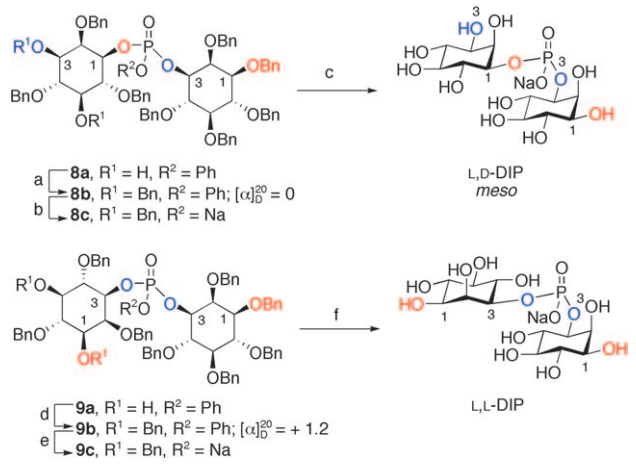
We then turned our attention to the identification of efficient reaction conditions for the coupling of **2** and **7**. Numerous reaction parameters were evaluated (solvent, base, reagent ratios, temperature, reaction time, and order of addition) before settling on addition of phosphoryl chloride **6** to triol **2** under the conditions shown in Scheme 4. These studies were initially conducted with DMAP as an achiral nucleophilic catalyst to define both optimized conditions, and the inherent regioselectivity of the process. The optimized conditions provided a mixture of the two DIP adducts **8a** and **9a** (3:1) in a combined yield of 57%.

Each isomer (**8a** and **9a**) was separated and carried forward to the corresponding isomer of DIP in order to assign stereochemical identity. Notably, reaction at the 1-position of **2** led to **8a**, the precursor to *meso* L,D-DIP, whereas reaction at the 3-position of **2** led to **9a**, the precursor to optically active L,L-DIP. As shown in Scheme 5, the remaining two hydroxy groups of **8a** and **9a** were converted into benzyl ethers to allow access to the *meso* or optically active material. Optical rotation and ^{1}H NMR data were then used to determine that intermediate **8b** was *meso* ($[\alpha]_D^{20} = 0$, $c = 0.4$, D_2O) and intermediate **9b** was optically active ($[\alpha]_D^{20} = +1.2$, $c = 0.4$, D_2O), thus establishing the absolute stereochemistry of products **8a** and **9a**. Hydrolysis of the phenyl phosphate group was then achieved using lithium hydroxide. Exchange to the sodium salt and subsequent hydrogenolysis yielded each isomer of the DIP product. The final steps were exceptionally clean, enabling isolation of pure DIP with a minimum of late-stage purification.

Having established the absolute configurations of products **8a** and **9a**, a preliminary study of chiral catalysts (e.g., **5** and **4**) used in the coupling reaction was undertaken to assess if catalyst control might lead to optimized yields of **8a** and **9a**, respectively (Table 1). Achiral nucleophilic catalysts such as DMAP and NMI (entries 1 and 2), defined the degree of substrate control during the formation of the DIP precursors (entries 1 and 2; L,L adduct/L,D adduct ≈3–



Scheme 4. Conditions and reagents: a) 2-benzyloxy-1-methyl-pyridinium triflate, MgO, $C_6H_5CF_3$; 73%; b) LiOH, THF/H_2O (1:1); c) Dowex 50X2-200; quant. (over 2 steps); d) $(COCl)_2$ (2 equiv), DMF (1 equiv), CH_2Cl_2, 5 min, RT; e) Et_3N (4 equiv), DMAP (1 equiv), **2** (2 equiv), RT, 12 h, 57%. DMAP = 4-dimethylaminopyridine, DMF = *N*,*N*-dimethylformamide, THF = tetrahydrofuran.



Scheme 5. Conditions and reagents: a) 2-benzyloxy-1-methyl-pyridinium triflate, MgO, $C_6H_5CF_3$; 35%; b) LiOH, THF/H_2O (1:1); Chelex 100 Na form; 69% (over 2 steps); c) H_2, $Pd(OH)_2$/C, EtOAc/MeOH (1:1); 96%; d) 2-benzyloxy-1-methyl-pyridinium triflate, MgO, $C_6H_5CF_3$; 27%; e) LiOH, THF/H_2O (1:1; Chelex 100 Na form; 47% (over 2 steps); f) H_2, $Pd(OH)_2$/C, EtOAc/MeOH (1:1); 82%.

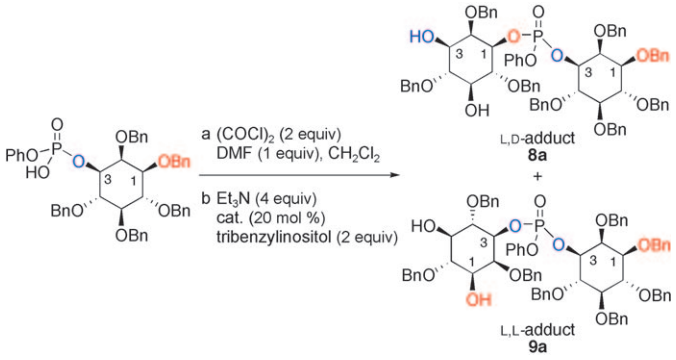
4:1). The simple amino acid catalyst derived from histidine (entry 3; with the π-nitrogen methylated, Pmh) revealed a similar product ratio (3:1) as the achiral catalysts. However, catalyst **5** (entry 4) showed a modest reversal (1:1.25) of the inherent selectivity to favor the L,L-isomer (**9a**) slightly. On the other hand, catalyst **4** (entry 5) exerted little stereochemical influence and showed similar ratios in comparison to the achiral catalysts. Investigation of 20 other catalysts[11] led to the identification of a different catalyst (**10**) that gave substantial selectivity for the L,D-isomer **8a** (entry 6; 13:1), and another (**11**) that more significantly reversed selectivity in favor of the L,L-isomer **9a** (entry 7; 1:2.5). These results bode well for further study, and amplified instances of improved peptides that could exhibit even higher degrees of catalyst control in these reactions. It is further of note that these differential ratios are achieved in a coupling reaction involving a diastereomeric mixture of phosphoryl chlorides, epimeric at the phosphorus atom, which necessarily contributes to the complexity of these processes. To our knowledge, preparation of diastereomerically pure phosphoryl chlorides of this type is not yet reported in the literature.

With a robust synthesis of L,L-DIP and L,D-DIP secured, we turned our attention to assessing the differential ability of each isomer to contribute to thermoprotection in the context of a key enzyme found in a unique extremophile. *Archaeoglobus fulgidus* is one such organism. Therefore, its proteins are good candidates to assess the effect of the solutes on protein thermostability. Previously, it was shown that the inositol monophosphatase (IMPase) from this organism denatures irreversibly when heated above 90 °C.[4,5,12] This enzyme thus serves as an interesting case to assess if the DIP anion can reduce the extent of thermal denaturation.

For this study, we examined the effect of KCl and NaCl as well as the glutamate salts of each cation (Figure 1) in protecting the IMPase activity after heating to 95 °C for 15 minutes. This treatment in the absence of compatible solutes rendered the enzyme nearly inactive (residual activity was 3% after this heating). Thermoprotection could be observed at 100 mM of added salt, although the effects were much stronger at higher concentrations of the salts. Of particular interest is the observation that the sodium salts were virtually ineffective in this concentration range, while KCl and K^+/glutamate were similar and much more effective at protecting IMPase activity.

To get a measure of what each stereoisomer of the DIP anion might contribute to thermoprotection, we examined the effects of the Na^+ salts of DIP (Figure 1, inset). We examined

Table 1: Examination of catalysts for selective synthesis of **8a** and **9a**.



Entry	Catalyst sequence	L,D Adduct/ L,L adduct
1	4-dimethylaminopyridine (DMAP)	3:1
2	*N*-methylimidazole (NMI)	4:1
3	Boc-Pmh-OMe	3:1
4	**5**: Boc-Pmh-Asn(trt)-His(*t*Bu)-Asp(O*t*Bu)-Ala-OMe	1:1.25
5	**4**: Boc-Pmh-Hyp(*t*Bu)-Sp5-Tyr(*t*Bu)-Phe-OMe	3.6:1
6	**10**: Boc-D-Pmh-Pro-Aib-Trp(Boc)-Phe-OMe	13:1
7	**11**: Boc-Pmh-D-Pro-Aib-D-Phe-D-Phe-OMe	1:2.5

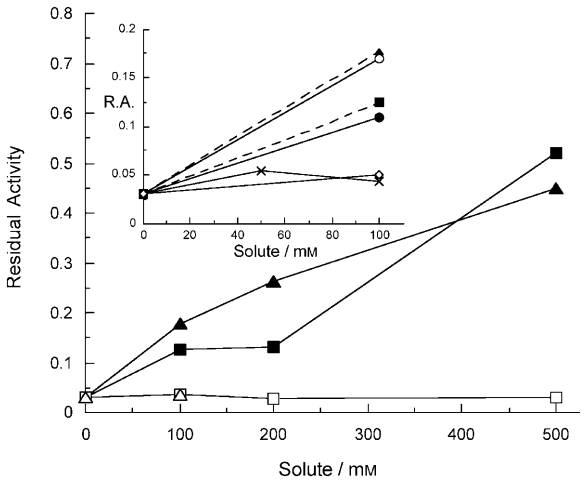


Figure 1. Effect of solutes on the residual activity of *Archaeoglobus fulgidus* inositol monophosphatase, heated to 95 °C for 15 minutes, then cooled and assayed at 85 °C: ▲, KCl; △, NaCl; ■, K^+/glutamate; □, Na^+/glutamate. The inset shows the effect of the sodium salts of L,L- (○) and L,D- (●) DIP at 100 mM, as well as dimethylphosphate (DMP; ×) and *myo*-inositol (◇) compared to the K^+/salts solutes (dashed lines) shown in the main figure.

100 mM of each Na^+/DIP on IMPase activity after heating. As a control, *myo*-inositol at 100 mM was also examined for any thermoprotection effects on this enzyme. Surprisingly, both Na^+/L,L-DIP and Na^+/L,D-DIP were effective in producing a recovery of activity (17% and 11%, respectively). In comparison to the thermoprotection provided by KCl (18% recovery of activity), it is particularly notable the DIP sodium salts offer protection, when NaCl is completely ineffective, therefore further pointing to the thermoprotective properties of the DIP anions. Inositol alone had no effect. In addition, the Na^+ salt of dimethylphosphate (DMP) offered no thermoprotective capacity, thus indicating that each DIP anion on its own is unique and has significant thermoprotective effects.

The modest differences in the thermoprotective influence of the two DIP stereoisomers are intriguing. At least in *A. fulgidus* as well as in *Thermotoga maritima*,[13] the natural product appears to be the L,D-DIP isomer. Yet, the fact that the alternative stereoisomer also offers protective effects raises fascinating questions about how the DIP anion stabilizes proteins to thermal denaturation. While the molecular details are not understood, the extended and rigid network of the hydroxy groups on the inositol rings could help to organize the water around the protein and stave off (to some degree) irreversible unfolding.[14] The apparent lack of a strong stereospecificity for these effects with the isomers of DIP,[15] which have been elucidated by combined chemical synthesis and biochemical studies, may provide an intriguing and potentially unusual case of chirality in nature that is more a matter of biosynthetic convenience rather than a matter of evolutionary optimization. Our hope is that these chemical and biochemical approaches will now set the stage for detailed, high precision study of these intriguing phenomena.

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