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Inframolecular studies of the protonation of 1D-1,2,4/3,5-cyclopentanepentaol 1,3,4-trisphosphate, a ring-contracted analogue of 1D-*myo*-inositol 1,4,5-trisphosphate

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Abstract

The protonation process of the individual phosphate groups of 1D-1,2,4/3,5-cyclopentanepentol 1,3,4-trisphosphate has been investigated by ^{31}P NMR titration experiments. The results are compared with those of 1D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]. From the NMR titration curves it can be shown that P3 and P4 strongly interact, whereas P1 behaves independently. In addition, P3 and P4 are much less influenced by their vicinal hydroxyls than P1. Accordingly, the calculated microscopic protonation constants indicate a higher basicity for P3 and P4 compared with the basicity of P1. The vicinal bisphosphate of the cyclopentane-based compounds seems to adopt a similar conformation to that of Ins(1,4,5)P_3, although an analogous interaction with a hydroxyl group is not observed. Two cyclopentanepentaol trisphosphates were examined for their potency to release intracellular Ca^{2+} from rat hepatocytes. Both were found to be low-affinity agonists in this regard. The relationship of this activity to the physicochemical data is discussed for one example.

Keywords: Inositol phosphates; Second messengers; Inositol phosphate analogues; Inframolecular; Protonation; Microscopic constants; NMR titrations

1. Introduction

1D-*myo*-Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3 (**1**)], generated upon hydrolysis of phosphatidylinositol 4,5-bisphosphate by a receptor-linked phospholipase C in the cell membrane, plays a pivotal role as a second messenger in transmembrane signalling. Ins(1,4,5)P_3 acts by mobilising intracellular Ca^{2+} stores, thereby initiating important physiological responses [1–4]. The key role played by Ins(1,4,5)P_3 in Ca^{2+} signalling prompted the synthesis of many analogues, which provided a good understanding of the structure-recognition characteristics at the Ins(1,4,5)P_3 receptor [5]. Recently, chiral cyclopentane derivatives, including 1D - 1,2,4/3,5 - cyclopentanepentaol 1,3,4-trisphosphate (**2**) [6a] and the related

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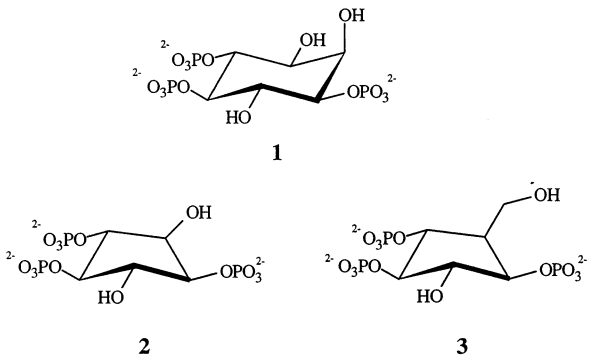


Fig. 1. Structure of Ins(1,4,5)P_3 (**1**) and cyclopentanepentaol analogues **2** and **3**.

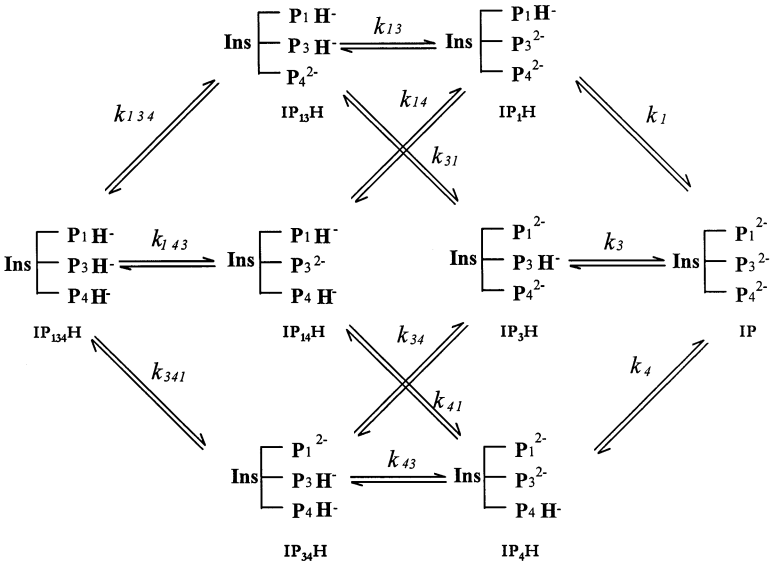
analogue **3** [6b] (Fig. 1), were prepared in order to investigate how a smaller ring size may interact with the receptor. Compound **2** corresponds to a ring-contracted Ins(1,4,5)P_3 with C-2 and its associated hydroxyl group deleted. This position of Ins(1,4,5)P_3 is the least important with respect to receptor binding and activation [5]. Compound **3** possesses an additional methylene group at the pseudo 3-position with respect to Ins(1,4,5)P_3.

Since pH influences the binding of inositol phosphates to Ins(1,4,5)P_3 receptors [7,8], determination of the acid–base properties of the phosphate groups would contribute to the knowledge of recognition mechanisms. In previous studies, we described the acid–base properties of various inositol phosphates at an inframolecular level, i.e., for each individual phosphate group [9–13]. These studies elucidated the intrinsic basicity of the phosphates, their protonation cooperativity and the influence of the configuration of neighbouring functional groups. All these studies have concerned six-membered rings; compound **2** affords the first possibility of extending these studies to a trisphosphate in a five-membered ring, in which the dihedral angles between the substituents are different from those in six-membered rings. Of particular interest is the phosphate group at position 4 (P4), which might interact both with its neighbouring phosphate P3 and with P1 above the plane of the ring. The present study was designed to determine the microscopic acid–base properties of **2**, along with the cooperative effects between the phosphates, to compare effects in a cyclopentane with those in a cyclohexane. The studies were performed by potentiometry and ^{31}P NMR titrations in 0.2 mol dm^{-3} KCl solution at 37 °C near physiological ionic strength and temperature. We also report on the biological activity for two such five-membered inositol polyphosphate analogues.

2. Materials and methods

Materials.—1D-1,2,4/3,5-cyclopentanepentol 1,3,4-trisphosphate (**2**) [6a] and the related analogue **3** [6b] were synthesised as previously described [6]. Before the titrations, the ligand **2** in a triethylammonium salt form was converted into its acid form by ion exchange using an Amberlite IRN-77(H^+) resin. The resulting solution (at a concentration of about 7×10^{-3} mol dm^{-3}) was used the same day for both potentiometric and NMR measurements. The base used as titration reactant was KOH. All the solutions were prepared with fresh double-distilled demineralised water.

Potentiometric and ^{31}P NMR titrations.—The macroscopic protonation constants were determined as previously described [9,10,14] either from the potentiometric titrations using the program SUPERQUAD [15] or from the ^{31}P NMR titration curves using the program HYPNMR [16]. For the determination of the microscopic protonation constants, the experiments were carried out in two steps in which the same initial solution of the ligand is successively subjected to potentiometric and ^{31}P NMR titrations. The protonation fraction curves resulting from the $\delta_i^{obs} = f(\mathrm{pH})$ curves were analysed by non-linear regression with the curve-fitting option of Easyplot or Enzfitter (Elsevier–Biosoft) in order to yield the microscopic protonation constants. ^{31}P NMR spectra were recorded at 81.015 MHz on a Bruker AC200 Fourier transform spectrometer. Chemical shifts were measured relative to an external 85% orthophosphoric acid reference. Field frequency lock was achieved using 10% ^2H_2O. Phosphorus resonance peaks of **2** were assigned by performing 2D ^{31}P–^{1}H chemical shift correlation experiments at pH 3.3 and 7.5. The proton resonances were assigned on the basis of ^{1}H^{1}H-COSY experiments.



Scheme 1. Microscopic protonation scheme for compound **2**.

Determination of macroscopic and microscopic protonation constants.—The overall protonation constants of the ligand β_y refer to the following equilibria:

$$L^{6-} + yH^+ \Leftrightarrow H_yL^{(6-y)-}$$

$$\beta_y = \frac{[H_yL^{(6-y)-}]}{[L^{6-}][H^+]^y}$$

A stepwise protonation process can also be defined by K_y characterising the equilibrium:

$$H_{y-1}L^{(7-y)-} + H^+ \Leftrightarrow H_yL^{(6-y)-}$$

$$K_y = \frac{[H_yL^{(6-y)-}]}{[H_{y-1}L^{(7-y)-}][H^+]}$$

However, the protonation state of each individual phosphate group can only be investigated by resolving the protonation process at an inframolecular level as described in Scheme 1. As shown previously [10], such resolution becomes possible by performing ^{31}P NMR titrations provided that the observed chemical shift δ_i^{obs} corresponds to the weighted average of shifts for the possible protonated and deprotonated forms. This preliminary condition is checked by the calculation, separately from both potentiometric and ^{31}P NMR titrations, of the mean number of protons bound per molecule of **2** versus pH curves. If the two resulting curves are satisfactorily superimposed as is the case for **2** (curves not shown), the fraction of protonation of the phosphates in position i can easily be calculated from Eq. (1):

$$f_{i,p} = \frac{\delta_i^{obs} - \delta_{i,d}}{\delta_{i,p} - \delta_{i,d}} \quad (1)$$

where $\delta_{i,p}$ and $\delta_{i,d}$ are the chemical shifts of the protonated and deprotonated phosphates, respectively.

$f_{i,p}$ can also be expressed as a function of the macro and microprotonation constants and the proton concentration. For instance, the protonation fraction of the phosphate in position 1 ($f_{1,p}$) is defined as:

$$f_{1,p} = \frac{\beta_3[H^+]^3 + (k_{13}k_1 + k_{14}k_1)[H^+]^2 + k_1[H^+]}{\beta_3[H^+]^3 + \beta_2[H^+]^2 + \beta_1[H^+] + 1} \quad (2)$$

The desired microconstants are obtained from a non-linear least-squares fit of a given ^{31}P NMR protonation fraction curve, while keeping constant in Eq. (2) the values of β_1, β_2 and β_3 previously determined by ^{31}P NMR.

From the microscopic constants the protonated microspecies distribution curves ($\alpha = f(pH)$) and the interactivity parameters can be

calculated. These parameters account for the basicity change at one phosphate group when another phosphate group takes up a proton. For instance, $\Delta \log k_{m-n,0p}$ and $\Delta \log k_{m-n,0d}$ represent the interaction between phosphates in positions m (P_m) and n (P_n), P_0 being protonated and deprotonated, respectively.

Ca^{2+} release from permeabilised rat hepatocytes.—Hepatocytes (10^6 cells ml^{-1}), permeabilised with saponin (10 μg mL^{-1}) were loaded to steady-state with $^{45}Ca^{2+}$ (16 μCi mL^{-1}) at 37 °C in cytosol-like medium (CLM) with a free [Ca^{2+}] of 200 nM [17]. After 5 min, cells were diluted five-fold into similar medium at 37 °C but without ATP and supplemented with thapsigargin (1.25 μM) to inhibit the Ca^{2+} pumps of the intracellular Ca^{2+} stores. After 15 s, appropriate concentrations of the ligand were added, and after a further 60 s, the $^{45}Ca^{2+}$ contents of the intracellular stores were determined after rapid filtration using a Brandel receptor-binding harvester [17]. Because responses to even the lowest concentrations of Ins(1,4,5)P_3 are complete within 30 s [18], the 60 s incubations used here allow the extent of the Ca^{2+} release by each of the agonists to be determined. CLM had the following composition: 140 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 1 mM ethyleneglycol bis(β-aminoethyl ester) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 300 μM $CaCl_2$ (free [Ca^{2+}] = 200 nM), 10 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 7.5 mM ATP, 20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 7.0 at 37 °C.

Concentration–response relationships were fitted to a four-parameter logistic equation using Kaleidegraph software (Synergy Software, PA, USA) from which the maximal response, half-maximally effective agonist concentration (EC_{50}) and Hill slope (h) were determined. All results are expressed as means ± S.E.M. Ins(1,4,5)P_3 was from American Radiolabeled Chemicals.

3. Results and discussion

The ^{31}P NMR titration curves of **2** and its corresponding protonation fraction versus pH are shown in Fig. 2(a) and (b). For the purpose of comparison, the curves of Ins(1,4,5)P_3 are superimposed in the solid line in Fig. 2(a). As earlier noted for the *myo*-inositol phosphates [9–13], the chemical shifts of the phosphorus nuclei of all the three phosphates undergo large upfield shifts upon protonation. Clearly the protonation of P3 and P4 appears biphasic, whereas that of P1 is monophasic. In addition, the $f_{3,p}$ and $f_{4,p}$ versus pH curves, which are almost superimposed, show the similarity of the P3 and P4 protonation processes and the strong interaction between vicinal phosphate groups, while P1 behaves as an independent phosphate. Therefore, the suggestion of an interaction between P1 and P4, which could be assumed by the consideration of a Dreiding molecular model, can be discarded. These results indicate that a slightly distorted cyclopentanepentaol ring in an envelope conformation brings the phosphate

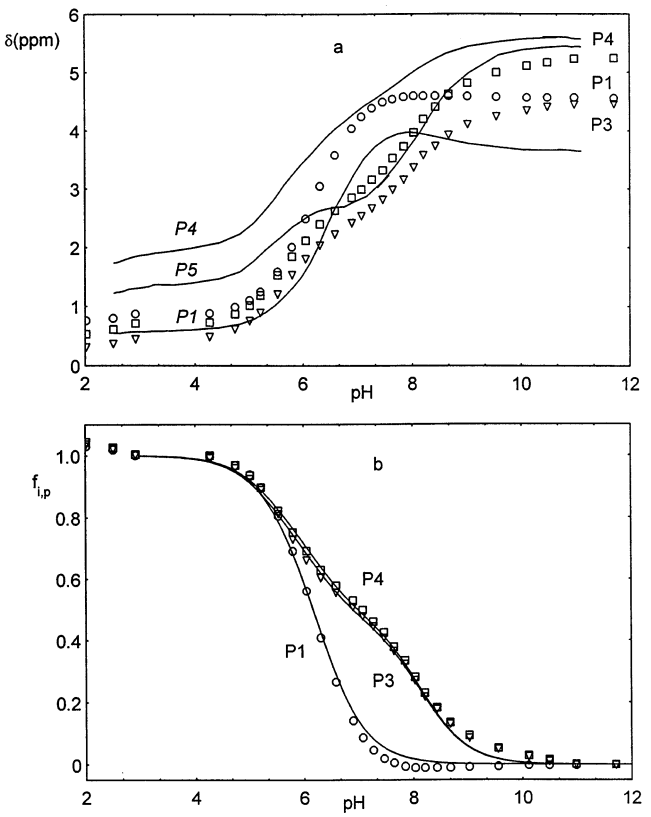


Fig. 2. Chemical shifts δ from ^{31}P NMR titrations for **2** (a) and the corresponding protonation fraction curves $f_{i,p}$ (b) as a function of pH in KCl 0.2 M at 37 °C. For the purpose of comparison, $\delta_i^{obs} = f(pH)$ for the Ins(1,4,5)P_3 (**1**) are superimposed in (a) (solid line — the assignments of the phosphates are shown in italic). The least-squares fit of $f_{i,p}$ vs. pH according to Eq. (2) is shown as the solid line in (b).

Table 1
Logarithms of the stepwise macroprotonation constants (log K_y) and microprotonation constants according to Scheme 1 for **2** [a,b]

y	log K_y [c]		i	log k_i	ii'	log $k_{ii'}$	$ii'i''$	log $k_{ii'i''}$
	Pot.	NMR						
			1	6.34	13	7.74	134	5.75
			3	7.81	14	7.68	143	5.77
1	8.21 ± 0.04	8.14 ± 0.03	4	7.82	31	6.20	431	5.77
2	6.52 ± 0.05	6.32 ± 0.06			34	6.21		
3	5.49 ± 0.07	5.34 ± 0.12			41	6.21		
					43	6.21		

[a] log k_i, log $k_{ii'}$ and log $k_{ii'i''}$ represent a general designation for, respectively, the logarithms of the first, second and third stepwise microprotonation constants. i, ii' and $ii'i''$ allow the location of the protons on the phosphates of the studied compounds to be determined. The uncertainties are estimates of the standard deviation as calculated by SUPERQUAD [15] and HYPNMR [16] for the macroconstants.

[b] The calculated interactivity parameters are: $\Delta \log k_{1-3,4d} = 0.10 \pm 0.03$, $\Delta \log k_{1-4,3d} = 0.14 \pm 0.01$, $\Delta \log k_{3-4,1d} = 1.60 \pm 0.01$, $\Delta \log k_{1-3,4p} = 0.44 \pm 0.01$, $\Delta \log k_{1-4,3p} = 0.44 \pm 0.02$, $\Delta \log k_{3-4,1p} = 1.95 \pm 0.02$. For **1** $\log K_1 = 7.85$, $\log K_2 = 6.40$, $\log K_3 = 5.31$ were previously found [19].

[c] It can be noted that log K_y also corresponds to the classical pK_a values that refer to a proton dissociation process.

groups into a pseudo-equatorial conformation rather than a pseudo-axial one, thus showing the same trend as a *myo*-inositol ring. From Fig. 2, the curves of **2** look more tightened than those of **1** and the $\delta_{i,p}$ and $\delta_{i,d}$ values are less scattered, i.e., **2** resembles the deoxy-*myo*-inositol phosphate analogues [9,11]. This indicates that in **2** P3 and P4 are less influenced by their neighbouring OH groups than P4 and P5 in a *myo*-inositol ring, leading to the shielding of chemical shifts of their protonated and deprotonated forms. Contrarily, P1, whose chemical shifts show the opposite tendency, is expected to interact more favourably with OH-2 and OH-5 than P1 does with OH-2 and OH-6 in **1** [9].

Table 1 lists the macroscopic and microscopic protonation constants for compound **2**. The stepwise macroscopic protonation constants determined by potentiometry and NMR spectroscopy agree. The slight difference may be attributed to the presence of a small percentage of migration product of **2** [6a], which affects the potentiometric measurements but not the NMR chemical shifts. Note that these values are close to those previously determined for compound **1** (see Table 1) [19]. The relative basicity of the different phosphate groups, already suggested by the titration curves, can be derived by the calculation of the concentration ratios of the monoprotonated microspecies, which are constant at any pH. Thus, $[IP_3H]/[IP_4H] = k_{43}/k_{34} = 1$ shows the same basic character for the vicinal P3 and P4 phosphates, whereas $[IP_3H]/[IP_1H] = [IP_4H]/[IP_1H] = k_{13}/k_{31} = k_{14}/k_{41} = 35$, indicates a much lower basicity for P1 with respect to P3 and P4. Furthermore, the large $\Delta \log k_{3-4,1d}$ and $\Delta \log k_{3-4,1p}$ values (see Table 1) account for the large interaction of P3 and P4, while the interactivity parameters characterizing the interaction between the latter phosphates and P1 are very low. Fig. 3 shows the distribution of the microprotonated species versus pH calculated from the microscopic constants in Table 1. These curves provide a direct observation of the protonation state of each phosphate group.

Preliminary biological studies [6] indicated that injection of compound **2** into *Xenopus*

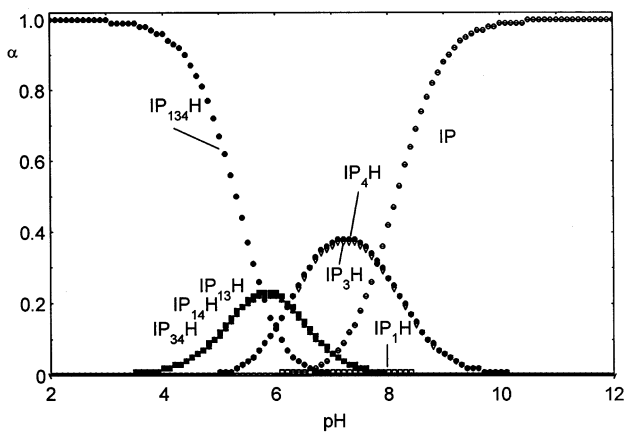


Fig. 3. Relative concentrations (α) of the protonated microspecies of **2** in KCl 0.1 M at 37 °C, plotted against pH (concentration of **2** = 1×10^{-3} mol dm^{-3}).

Table 2
Release of Ca^{2+} from the intracellular stores of permeabilised rat hepatocytes ($n = 3$) [a]

Ligand	$^{45}Ca^{2+}$ release (% of stores)
Ins(1,4,5)P_3 (10 μM)	44 ± 2
Ins(1,4,5)P_3 (150 nM)	27 ± 2
2 (100 μM)	18 ± 3
3 (100 μM)	25 ± 2
Ins(1,4,5)P_3 (150 nM) with **2** (100 μM)	32 ± 1
Ins(1,4,5)P_3 (150 nM) with **3** (100 μM)	41 ± 6

[a] Cells loaded to steady state with $^{45}Ca^{2+}$ were added to thapsigargin, 15 s later the indicated concentrations of ligand were added, and after a further 60 s the $^{45}C^{2+}$ contents of the intracellular Ca^{2+} stores were determind. Active $^{45}Ca^{2+}$ uptake is defined as that which could be released by 10 μM ionomycin. $^{45}Ca^{2+}$ release is shown as a percentage of the actively accumulated $^{45}Ca^{2+}$ content of the control cells at the end of the 60 s incubation.

oocytes induced Ins(1,4,5)P_3-like Ca^{2+} spikes, but at higher concentrations than Ins(1,4,5)P_3 (**1**) i.e., compound **2** is an agonist but less potent than Ins(1,4,5)P_3. When the cyclopentanepentaol polyphosphates **2** and the related **3** were examined for Ca^{2+}-mobilising properties in saponin-permeabilised rat hepatocytes, they showed weak Ins(1,4,5)P_3-like activity (Table 2). Neither **2** nor **3** caused detectable Ca^{2+} release at 1 μM, nor did they antagonise the Ca^{2+} release evoked by a half-maximally effective concentration of Ins(1,4,5)P_3. While it was not possible to obtain accurate EC_{50} values for the compounds, it appears that both **2** and **3** possess similar activity, with **3** being slightly more potent. The estimated EC_{50} values are around 100 μM, making such compounds much weaker than Ins(1,4,5)P_3 and many of the other analogues synthesised to date. To test for possible antagonist properties, each ligand (**2** and **3**) was simultaneously applied at 100 μM with a submaximal concentration of Ins(1,4,5)P_3 (150 nM). Neither compound antagonised the response to Ins(1,4,5)P_3 and the greater extent of the Ca^{2+} release evoked by the compounds in combination with Ins(1,4,5)P_3 is consistent with them having similar efficacy to Ins(1,4,5)P_3. We tentatively suggest that **2** and **3** are full agonists with ~700-fold lesser affinity than Ins(1,4,5)P_3.

These results may partly be explained by considering the physico-chemical properties of **1** and the analogue **2** that we have studied in detail here. Direct comparison between compounds **1** and **2** cannot be made on the basis of the microscopic protonation constants since the initial low-field shift of P1 upon protonation prevented the calculation of these constants for **1**. However, by consideration of the ^{31}P NMR titration curves of both molecules, some similarities and differences appear. Thus for **2**, the D-*threo* vicinal phosphate pair, P3 and P4, seems to adopt a very similar conformation to the corresponding system in Ins(1,4,5)P_3, which is essential for binding to the Ins(1,4,5)P_3 receptor. However, OH-5 of compound **2**, which was assumed to be equivalent to OH-6 for **1**, in fact behaves very differently. In particular, there is apparently no interaction between P4 and OH-5 (corresponding to the P5–OH-6 interaction in **1** [19]), which seems crucial in the complex dependency of the protonation process to the setting of intramolecular hydrogen bonds. Interestingly, the $f_{i,p}$ versus pH curves of D-6-deoxy-Ins(1,4,5)P_3, in which OH-6 was deleted, are strikingly close to those of **2** [20]. D-6-Deoxy-Ins(1,4,5)P_3 binds very poorly to the Ins(1,4,5)P_3 receptor [21] and we would predict a similar binding affinity for **2**. Analogue **3** may be a slightly more potent agonist than **2** because the hydroxymethyl group is better able to mimic either the 3-OH or 2-OH groups of Ins(1,4,5)P_3, but the effects are marginal and did not therefore warrant a full physico-chemical evaluation of this compound.

Before discarding the cyclopentanepentaol triphosphates as candidates for potent Ins(1,4,5)P_3 mimics, ways to restore the important OH-5 function should be considered. For example, synthesis of an analogue of **2** where OH-5 is replaced by a hydroxymethyl group is one possibility.

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