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SYNTHESIS OF *MYO*-INOSITOL 1,2,4,5-TETRAKISPHOSPHATE, A Ca^{2+}-MOBILISING TETRAKISPHOSPHATE WITH A POTENCY SIMILAR TO *MYO*-INOSITOL 1,4,5-TRISPHOSPHATE

Stephen J Mills[1], Stephen T Safrany[2], Robert A Wilcox[2], Stefan R Nahorski[2] and Barry V L Potter[1]*

**[1]School of Pharmacy & Pharmacology and Institute for Life Sciences
University of Bath
Claverton Down, Bath BA2 7AY, UK**

**[2]Department of Pharmacology and Therapeutics
University of Leicester
Leicester LE1 9HN, UK**

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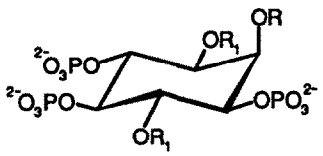
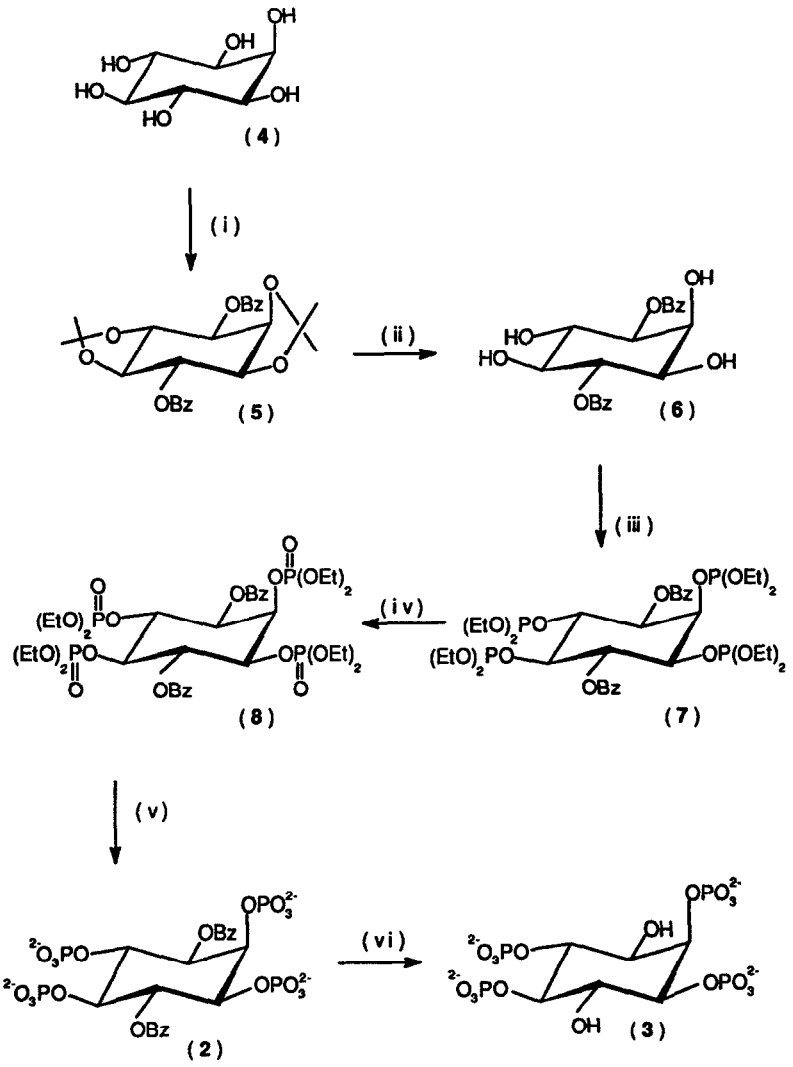
Abstract: The synthesis of *myo*-inositol 1,2,4,5-tetrakisphosphate from inositol is described; this tetrakisphosphate is a highly potent Ca^{2+}-mobilising agonist at the $Ins(1,4,5)P_3$ receptor.

Within the last decade D-*myo*-inositol 1,4,5-trisphosphate $Ins(1,4,5)P_3$ **(1)** (Fig. 1), released by receptor-mediated phospholipase C-catalysed cleavage of phosphatidylinositol 4,5-bisphosphate has emerged as a second messenger linking the spatially separated events of receptor stimulation and release of intracellular calcium from internal stores[1,2]. $Ins(1,4,5)P_3$ acts through an intracellular receptor which has been isolated[3], cloned and sequenced[4,5] and reconstituted[6]. $Ins(1,4,5)P_3$ is metabolised *via* two pathways[7]: deactivation by a 5-phosphatase to $Ins(1,4)P_2$ or phosphorylation by a 3-kinase to $Ins(1,3,4,5)P_4$. The function of the latter still remains controversial and $Ins(1,3,4,5)P_4$ may gate a plasma membrane Ca^{2+} channel[8].



R = R_1 = H (**1**)
R = PO_3^{2-}; R_1 = Bz (**2**)
R = PO_3^{2-}; R_1 = H (**3**)

Figure 1



Scheme

Reagents and conditions:

(i) 2,2-Dimethoxypropane, DMF, PTSA, 100° C, 2hr; then, benzoyl chloride, pyridine.
(ii) 80% acetic acid, reflux 30 mins. (iii) $(EtO)_2PCl$, DIPE, DMF, (iv) 70% *tert*-BuOOH;
(v) TMSBr, CH_2Cl_2, overnight; (vi) 1M NaOH. All substituted *myo*-inositol compounds are racemic.

As part of an ongoing programme aimed to study structure-activity relationships in inositol tris- and tetrakisphosphates[9] we have been engaged in the synthesis of *myo*-inositol polyphosphates and their analogues. Ins(2,4,5)P_3 is a non-naturally occurring trisphosphate, which in racemic form has a potency in Ca^{2+} release some 30-fold lower than Ins(1,4,5)P_3[10,11], but which has found application as a metabolism-resistant analogue[12] of Ins(1,4,5)P_3 owing to the fact that it is a weak substrate for Ins(1,4,5)P_3 5-phosphatase and a very poor substrate for Ins(1,4,5)P_3 3-kinase[10,13]. Since it is not clear whether the substitution of the 2-hydroxyl group or the lack of a phosphate group at the 1-position is responsible for these properties we decided to synthesise the hybrid molecule *myo*-inositol 1,2,4,5-tetrakisphosphate, Ins(1,2,4,5)P_4 (**3**). While Ins(1,2,4,5)P_4 could be classified as an analogue of Ins(1,4,5)P_3, but with the 2-position substituted with a charged group (a number of papers have focussed upon substitution of the 2-position with neutral bulky groups[11,14]), this molecule can also, of course, be envisaged as being related to Ins(1,3,4,5)P_4, but where the 3-phosphate has been transposed onto the adjacent 2-hydroxyl group. Clearly Ins(1,2,4,5)P_4 is a key synthetic analogue relating to structure-activity studies in respect of both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 binding proteins.

Two *myo*-inositol tetrakisphosphates were synthesised and biologically evaluated, *myo*-inositol 3,6-di-*O*-benzoyl 1,2,4,5-tetrakisphosphate [3,6-dibenzoyl Ins(1,2,4,5)P_4] (**2**) and Ins(1,2,4,5)P_4 (**3**). Both of these compounds have been previously synthesised[15,16] but no biological data have been reported. Meek *et al*[15] adapted a P(III) approach to phosphorylate 1,4-di-*O*-benzoyl-*myo*-inositol, which after phosphate group deblocking gave (**2**). Further deprotection with base afforded Ins(1,2,4,5)P_4. Carless and Busia[16] synthesised Ins(1,2,4,5)P_4 (**3**) from benzene, generating the intermediate 1,4-di-*O*-benzyl-*myo*-inositol as phosphorylation precursor and using a P(V) approach for phosphorylation. Subsequent phosphorylation and deprotection furnished (**3**).

In our approach the key phosphitylation precursor (**6**) was prepared in racemic form in a three step reaction (Scheme). First, *myo*-inositol (**4**) was fully protected by heating with 2,2-dimethoxypropane, *p*-toluene sulphonic acid in DMF at 100°C for 2 hours, followed by the addition of benzoyl chloride in pyridine to furnish (**5**) according to Gigg *et al*[17] (yield 30%). Acid hydrolysis of the ketals gave racemic 1,4-di-*O*-benzoyl-*myo*-inositol (**6**) (yield 95%), which was polyphosphitylated using chlorodiethoxyphosphine to give a tetrakisphosphite derivative (**7**), which was not isolated. The intermediate polyphosphite, which exhibited two AB ^{31}P-^{31}P spin-spin coupling systems ($^5J_{1,2}$ = 1.83Hz; $^5J_{4,5}$ = 3.62Hz)[18], was oxidized with *tert*-butylhydroperoxide to provide the highly crystalline 1,2,4,5-tetrakis(diethylphosphate) (**8**) [mp 122 - 123°C] in 85% yield from (**6**). The eight ethyl groups were removed quantitatively (as judged by ^{31}P nmr) using trimethylsilylbromide to give crude (**2**). The benzoyl groups were then removed with aqueous sodium hydroxide to provide DL-Ins(1,2,4,5)P_4 (**3**) (80% yield). The deprotected phosphates (**2**) and (**3**) were purified by DEAE Sepharose ion-exchange chromatography and quantified as their glassy triethylammonium salts using the Briggs phosphate assay.

Racemic 3,6-di-benzoyl Ins(1,2,4,5)P_4 (**2**) and Ins(1,2,4,5)P_4 (**3**) were examined for their ability to displace [^3H]-Ins(1,4,5)P_3 from membranes prepared from bovine adrenal cortices[19]. (**3**) Displaced [^3H]-Ins(1,4,5)P_3 (15 - 30 Ci/mmol, NEN) with K_i = 26.4 $\pm$ 1.8nm, whereas (**2**) did not compete (<3μM). Likewise, (**2**) was essentially devoid of Ca^{2+}-mobilising activity from $^{45}Ca^{2+}$-preloaded SH-SY5Y cells permeabilised using saponin, as previously described for 1321NI cells[19] (< 100μM). However, (**3**) released $^{45}Ca^{2+}$ potently from intracellular stores, with EC_{50} = 165 $\pm$ 11nM, compared with EC_{50} = 52 $\pm$ 2nM for Ins(1,4,5)P_3.

Although (**2**) did not interact with the Ins(1,4,5)P_3 receptor, and inhibited Ins(1,4,5)P_3 phosphorylation by a rat brain homogenate supernatant high in Ins(1,4,5)P_3 3-kinase activity[20] with low affinity [K_i = 100 $\pm$ 30μM, (cf K_m for Ins(1,4,5)P_3 = 1.5μM)], it

inhibited the dephosphorylation of [^{3}H] Ins(1,4,5)P_3 by human erythrocyte membrane Ins(1,4,5)P_3 5-phosphatase[21] competitively, with K_i = 15.9μM [K_m for Ins(1,4,5)P_3 = 31.8μM]. **(3)**, Which did not inhibit [^{3}H]-Ins(1,4,5)P_3 phosphorylation (< 300μM), was a potent inhibitor of Ins(1,4,5)P_3 5-phosphatase, with K_i = 2.9μM. Under conditions where 10μM Ins(1,4,5)P_3 (2.5nmoles) liberated 1.4 $\pm$ 0.5 nmoles of inorganic phosphate, monitored colorimetrically as previously described[22], Ins(1,4,5)P_3 5-phosphatase was unable to liberate inorganic phosphate when incubated with **(2)** or **(3)** (10μM, 2.5nmoles). Under identical conditions, Ins(1,3,4,5)P_4 was bound with K_m = 3.9μM and liberated 0.11nmoles of inorganic phosphate. However, preliminary experiments monitoring the temporal aspects of Ca^{2+} mobilisation from saponin-permeabilised SH-SY5Y cells with the Ca^{2+}-sensitive fluorescent dye, Fluo-3 (1μM), using a method similar to that of Michelangeli[23], indicate that **(3)** mobilises Ca^{2+} in a transient manner. The rate of reuptake of Ca^{2+} was only marginally slower than that seen with Ins(1,4,5)P_3, suggesting that **(3)** is metabolised by an enzyme(s) other than Ins(1,4,5)P_3 3-kinase and 5-phosphatase.

Two tetrakisphosphates have to date been reported with Ca^{2+}-mobilising activity, namely Ins(1,3,4,5)P_4 [EC_{50} = 2.1μM][24] and Ins(1,3,4,6)P_4 [EC_{50} = 5.9μM][25]. The present data now identify **(3)** as the most potent Ca^{2+}-mobilising inositol tetrakisphosphate yet described. Assuming L-Ins(1,2,4,5)P_4 is inactive with regard to Ca^{2+} mobilisation, the introduction of a 2-phosphate group has thus only very slightly attenuated the potency of this newly described inositol tetrakisphosphate relative to Ins(1,4,5)P_3 at the Ins(1,4,5)P_3 receptor.

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