Synthesis of *myo*-Inositol 1-Phosphorothioate 4,5-Bisphosphate: Preparation of a Fluorescently Labelled *myo*-Inositol 1,4,5-Trisphosphate Analogue

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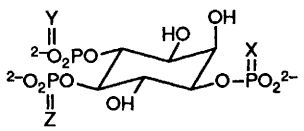
Synthesis of *myo*-inositol 1-phosphorothioate 4,5-bisphosphate, a novel analogue of the second messenger *myo*-inositol 1,4,5-trisphosphate allows, for the first time, the attachment of a fluorescent reporter group at the 1-position.

D-*myo*-Inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], **1** is a ubiquitous second messenger, which couples agonist stimulation of a wide variety of cell surface receptors to the mobilisation of intracellular calcium.[1,2] The gene coding for the Ins(1,4,5)P_3 receptor has now been cloned[3] and the ability of this transmembrane protein to gate calcium in response to Ins(1,4,5)P_3 has been demonstrated.[4] Realisation of the fundamental cellular role played by Ins(1,4,5)P_3 and the acceptance of the polyphosphoinositide signal transduction mechanism has led to a massive increase in biological[1,2] and, latterly, chemical[5,6] effort to unravel the details of this complex pathway. Ins(1,4,5)P_3 has now been synthesized by many groups and chemical emphasis in this field must now surely lie with the synthesis of novel structurally modified inositol phosphate analogues, enzyme inhibitors and antagonists to facilitate pharmacological intervention.

Few biologically potent Ins(1,4,5)P_3 analogues have yet been synthesized,[5,6] although recent reports on analogues modified at the 2-position[7] and on the synthesis of an active 5-methylene phosphonate analogue[8] have appeared. We have reasoned that, by virtue of their metabolic stability, phosphorothioate analogues will prove to be of significant importance in this field.[6,13] We previously reported the first synthesis of the trisphosphorothioate **2** [Ins(1,4,5)PS_3],[9] a potent inhibitor of human erythrocyte Ins(1,4,5)P_3 5-phosphatase,[10] and the specifically modified 5-phosphorothioate analogue **3** [Ins(1,4,5)P_3-5S].[11] Both of these highly potent analogues are already finding numerous biological applications.[6,12,13]

The few structure–activity studies which have been performed show that the vicinal 4,5-bisphosphate moiety of Ins(1,4,5)P_3 is essential for Ca^{2+}-releasing activity,[13] the 1-phosphate group being thought to provide enhanced affinity for the receptor. Semisynthetic Ins(1,4,5)P_3 analogues with modifications at the 1-phosphate position have been prepared from the deacylated polyphosphoinositide phospholipid and are biologically potent.[14] However, no reporter groups have yet been attached to the 1-phosphate group. We propose here that introduction of the nucleophilic sulphur of a 1-phosphorothioate group into the Ins(1,4,5)P_3 molecule should permit the facile attachment of reporter groups to Ins(1,4,5)P_3, such as photoaffinity labels, spin labels and fluorescent probes.



1; X = Y = Z = O
2; X = Y = Z = S
3; X = Y = O; Z = S
4; Y = Z = O; X = S

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Fluorescent labelling methodology has already shown its versatility in the nucleic acid field,[15] where it is already established as a valuable alternative to the use of radioactivity. Consequently, we have devised a synthesis of the Ins(1,4,5)P_3 analogue *myo*-inositol 1-phosphorothioate 4,5-bisphosphate **4** and demonstrate its use in the fluorescent labelling of Ins(1,4,5)P_3 (Scheme 1).

Isomerisation of the 1-allyl group of 1-*O*-allyl-2,3,6-tri-*O*-benzyl-*myo*-inositol[16] to the corresponding prop-1-enyl derivative **5** [mixture of *ca.* 1 : 5 *cis* : *trans* prop-1-enyl isomers] was accomplished using tris(triphenylphosphine)rhodium(I) chloride catalyst in the presence of triethylenediamine (DABCO) in 7 : 3 : 1 ethanol : benzene : water. Phosphitylation of the 4,5-vicinal diol was effected using bis(2-

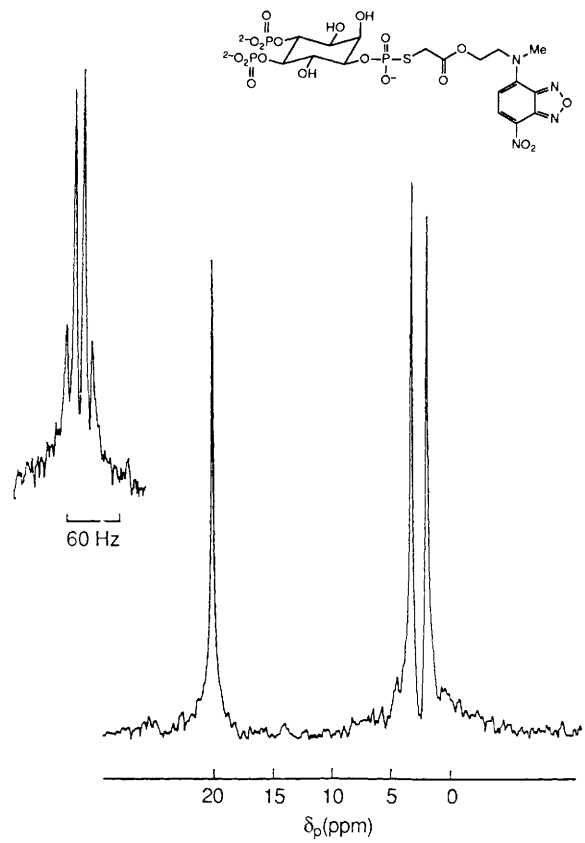
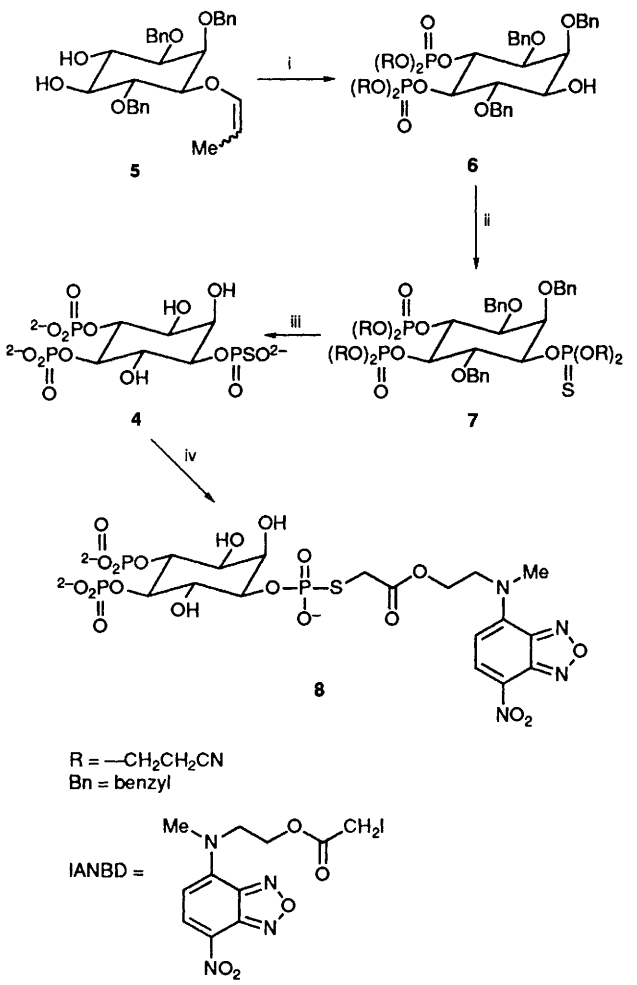


Fig. 1 36.2 MHz broad band ^{1}H-decoupled ^{31}P NMR spectrum of **8** in D_2O [15 mmol dm^{-3} solution of **8** in 50 mmol dm^{-3} TEAB, 5 mmol dm^{-3} ethylenediaminetetraacetic acid (EDTA) pH 7.7]. ^{31}P NMR parameters were: sweep width, 10 kHz; pulse width, 9 μs; collected over 8 K; no. of transients, 2500; referenced to external H_3PO_4. The inset shows part of the ^{1}H-coupled spectrum for the resonance at 19.9 ppm



Scheme 1 *Reagents and conditions*: i, (*a*) $(RO)_2PNPr^i_2$ (10 equiv.)–tetrazole (12 equiv.) in CH_2Cl_2, (*b*) Bu^tOOH (70% in H_2O), (*c*) HgO (1 equiv.)–$HgCl_2$ (1.25 equiv.) in 10:1 v/v acetone:water; ii, (*a*), $(RO)_2PNPr^i_2$ (5 equiv.)–tetrazole (6 equiv.) in CH_2Cl_2, (*b*) excess S_8–pyridine; iii, Na–liq. NH_3; iv, IANBD (1.1 equiv.)–EtOH; all inositol compounds are racemic

cyanoethyl)-*N*,*N*-diisopropylaminophosphine,[17] followed by oxidation of the protected inositol P^{III} bisphosphite to the P^V bisphosphate with Bu^tOOH. Removal of the prop-1-enyl protecting group using HgO–$HgCl_2$ gave **6**, which was phosphitylated in the same fashion and the product oxidized to the fully protected inositol 1-monophosphorothioate 4,5-bisphosphate **7** using sulphur in pyridine. Deblocking of all protecting groups was accomplished using sodium in liquid ammonia[9] to give the Ins(1,4,5)P_3 analogue **4**,‡ which was purified by ion-exchange chromatography on DEAE Sephadex A-25. Compound **4** was eluted at *ca.* 800 mmol dm^{-3} triethylammonium hydrogen carbonate (TEAB) buffer. ^{31}P NMR spectroscopy showed clearly that **4** possessed a single phosphorothioate group (δ_p, 42.1 ppm) and two phosphate groups (δ_p, 4.8, 5.0 ppm). Compound **4** was a potent mobiliser of intracellular Ca^{2+} from permeabilised cells.

‡ All new compounds exhibited satisfactory spectral data.

Nitrobenzoxadiazole (NBD) derivatives, such as the iodoacetate *N*-[{2-(iodoacetoxy)ethyl}-*N*-methyl]amino-7-nitro-2,1,3-benzoxadiazole (IANBD), are unique in having long-wavelength fluorescein-like fluorescence spectral properties, but with high environmental sensitivity of the quantum yield coupled with a relatively small molecular size.[18] Such a probe seems ideal for preliminary studies of the interactions of a fluorescently tagged Ins(1,4,5)P_3 with the intracellular receptor and the metabolic enzymes 5-phosphatase and 3-kinase. Reaction of the phosphorothioate analogue **4** with IANBD proceeded smoothly to give the adduct **8**, which was purified by ion exchange chromatography, and was eluted at *ca.* 800 mmol dm^{-3} TEAB. ^{31}P NMR spectroscopy (Fig. 1) showed clearly that the 1-phosphorothioate group (δ_p, 42.1 ppm) had been converted to the *S*-alkyl phosphorothiolate (δ_p, 19.9 ppm; $^3J_{POCH} = {}^3J_{PSCH} = 9.5$ Hz). The adduct **8** exhibited a UV spectrum consistent with the presence of an NBD chromophore and when excited at 460 nm showed the expected fluorescence at 540 nm.

Compound **8** was potent at releasing ATP-sequestered intracellular Ca^{2+} from permeabilised cells and was therefore recognized by the Ins(1,4,5)P_3 receptor. Biological results for **4** and **8** will be reported elsewhere. The synthesis of **8** thus provides the first example of a biologically active Ins(1,4,5)P_3 analogue labelled with a fluorescent reporter group, which should be of considerable utility in probing the interactions of this second messenger with proteins. These studies are now in progress.

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