

Fluorescent probe: complexation of Fe^{3+} with the *myo*-inositol 1,2,3-trisphosphate motif†

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Natural *myo*-inositol phosphate antioxidants containing the 1,2,3-trisphosphate motif bind Fe^{3+} in the unstable penta-axial conformation.

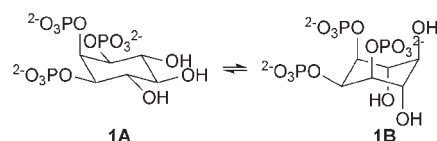
The natural product *myo*-inositol 1,2,3-trisphosphate (**1**)¹ ($\text{Ins}(1,2,3)\text{P}_3$, cellular concentration $\leq 10 \mu\text{M}$ ¹) is a potent iron-chelator (apparent $K_i = 9.0 \times 10^{-19} \text{ M}$) and antioxidant, completely inhibiting iron-catalysed hydroxyl radical (HO^\bullet) formation.^{2,3} Our recent potentiometric studies demonstrate that, unlike the more abundant *myo*-inositol hexakisphosphate ($\text{InsP}_6 \approx 20 \mu\text{M}$ ⁴), $\text{Ins}(1,2,3)\text{P}_3$ fulfils the requirements of a safe, low molecular weight biological Fe^{3+} shuttle.^{5,6} Under conditions of excess Mg^{2+} (e.g. cytosolic and nuclear regions of mammalian cells), a negligible proportion of InsP_6 is bound to Fe^{3+} .⁷ In contrast, Fe^{3+} remains fully complexed with $\text{Ins}(1,2,3)\text{P}_3$, both under Mg^{2+} -rich conditions and in acidic, Ca^{2+} -rich media such as in lysosomes.⁵ It was also predicted that iron bound to $\text{Ins}(1,2,3)\text{P}_3$ must be Fe^{3+} , since its affinity for Fe^{2+} is not high enough to allow interaction in the presence of excess cellular Mg^{2+} . Although the biological relevance of $\text{Ins}(1,2,3)\text{P}_3$ binding to iron is clear, the structure of its complex is uncertain and forms the subject of this paper. We have previously shown that the crystal structure of cyclohexylammonium $\text{Ins}(1,2,3)\text{P}_3$ adopted the expected stable penta-equatorial chair conformation (**1A**).⁸ Here using density functional calculations at the UB3LYP/6-31+G* level,⁹ we have modelled **1** as the hexamethyl phosphoester, to represent protonation and counterion/solvent screening effects. From the optimal geometries, we have estimated that **1A** is

8 kcal mol⁻¹ more favourable in energy than the penta-axial conformation **1B** (Scheme 1). This energetic preference increases to 30 kcal mol⁻¹ for the hexaanionic forms of **1A** and **1B**.

It has been proposed by Phillippy and Graf that the complex of Fe^{3+} with $\text{Ins}(1,2,3)\text{P}_3$ adopts the penta-axial conformation (**1B**):¹⁰ the phosphate groups are orientated closer together, allowing $\text{Ins}(1,2,3)\text{P}_3$ to complex to Fe^{3+} with hexa-coordination using two terminal oxygens from each phosphate. This structure was based on the observation that azide has no effect on the visible absorption spectrum of $\text{Ins}(1,2,3)\text{P}_3\text{-Fe}^{3+}$, in contrast to the marked shift observed for $\text{Ins}(1,2,6)\text{P}_3\text{-Fe}^{3+}$ due to water displacement.¹⁰ To explore this further, we employed ¹H NMR studies to confirm the penta-equatorial conformation of $\text{Ins}(1,2,3)\text{P}_3$ in the absence of Fe^{3+} . However, no structural information on its Fe^{3+} complex could be obtained due to peak broadening. An alternative experimental approach to conformational studies of **1** was therefore required.

We have recently developed a fluorescent probe based on the acid-triggered conformational flip of an orthoformate constrained *myo*-inositol ring using pyrene excimer fluorescence.^{11,12} This methodology inspired the design of a fluorescent probe to study the $\text{Ins}(1,2,3)\text{P}_3$ motif, using Fe^{3+} complexation as the potential trigger for conformational change. 4,6-Bis-*O*-pyrenoyl-*myo*-inositol 1,2,3,5-tetrakisphosphate (**2**) has been synthesised as a fluorescent probe to monitor whether the ring flip of *myo*-inositol occurs upon association with Fe^{3+} (Scheme 2). If Fe^{3+} binds to the more stable penta-equatorial chair conformation (**2A**), a monomer emission signal of the pyrene groups would be expected. If Fe^{3+} binds to the less stable penta-axial conformation (**2B**) this will favour π - π stacking of the pyrene groups promoting excimer fluorescence (Scheme 2).

The 1,2,3,5-tetrakisphosphate *myo*-inositol derivative was chosen over the $\text{Ins}(1,2,3)\text{P}_3$ derivative due to the significantly shorter synthesis of the fluorescent probe. We are confident that *myo*-inositol 1,2,3,5-tetrakisphosphate is a good model of the 1,2,3-trisphosphate motif as it possesses similar anti-oxidant properties and Fe^{3+} affinity to $\text{Ins}(1,2,3)\text{P}_3$.² The synthesis of



Scheme 1 Penta-equatorial (**1A**) and penta-axial (**1B**) conformations of $\text{Ins}(1,2,3)\text{P}_3$.

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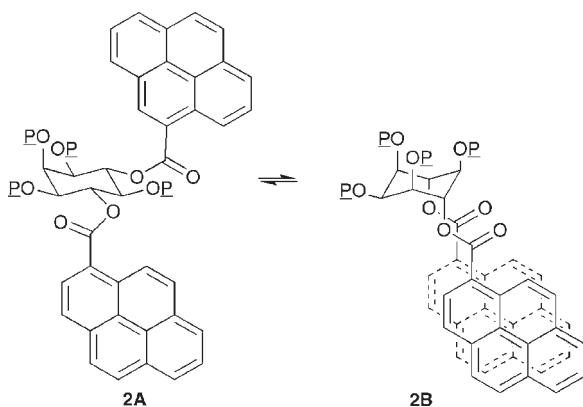
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† Electronic supplementary information (ESI) available: Coordinates and absolute energies for **7A** and **7B**. Crystal data for **3-CDCl₃**. CCDC 690016. For ESI and crystallographic data in CIF or other electronic format, see DOI: 10.1039/b809238a



Scheme 2 Penta-equatorial (**2A**) and penta-axial (**2B**) conformations of 4,6-bispyrenoyl Ins(1,2,3,5)P₄. P = PO₃²⁻.

(**2**) (Scheme 3) commences with 4,6-bispyrenoyl-*myo*-inositol 1,3,5-orthoformate (**3**), the excimer fluorescent properties of which have been reported by our group.^{11,12}

Fortuitously, crystals of (**3**·CDCl₃) were obtained and its X-ray structure is described (Fig. 1).[‡] The crystal structure elegantly demonstrates the π - π stacking of the pyrene rings, and hence rationalises its fluorescent properties. Bond angles at orthoformate oxygen atoms O1, O3 and O5 are approximately tetrahedral in **3**, but the angle at O2, which bears the bulky silyl group, is opened to 122.35(10)°, even wider than the value of 121.01(2)° for the corresponding angle in the related feruloyl derivative.¹³ In contrast to the feruloyl derivative, the two large pyrene rings of **3** are efficiently stacked (Fig. 1A), the dihedral angle between ring planes being only 3.29(3)°. The pyrene rings of **3** resemble a clamshell hinged along the C18...C20 and C35...C37 side (Fig. 1B). C18 and C20 are only 3.307(2) and 3.276(2) Å out of the least-squares plane through all ring carbon atoms from C32 to C47, but on the far side the distance from this plane increases to 3.578(2) and 3.592(2) Å for C25 and C27. Since the stacking separation exceeds the theoretical 2.52 Å between adjacent identical axial substituents on an undistorted cyclohexane ring, and the average 2.54 Å distance between axial H atoms observed

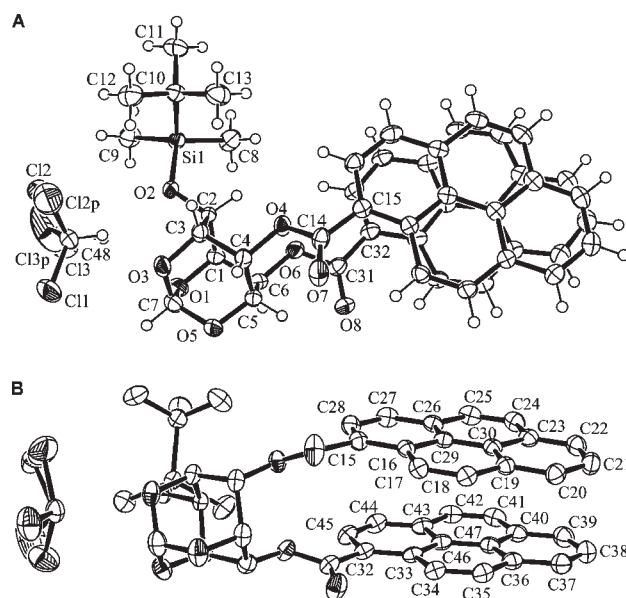


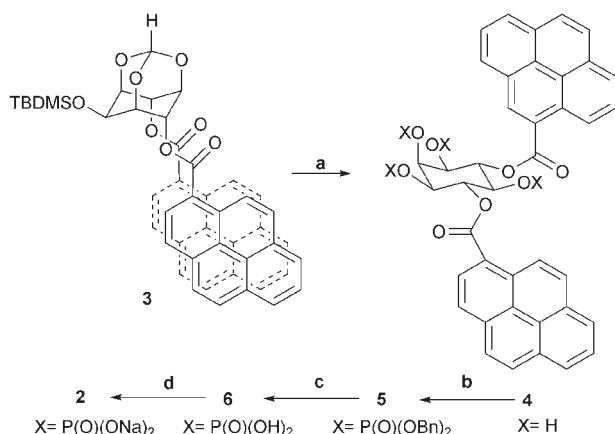
Fig. 1 Complementary ORTEP¹⁶ diagrams of (**3**·CDCl₃) showing the atom numbering scheme. Ellipsoids are drawn at the 50% probability level. In **B** the H atoms and selected atom labels are omitted for clarity.

in cyclohexane,¹⁴ adjustments are required. These start with the splaying effect of expansion above the tetrahedral angle in C4-C5-C6 to 113.75(13)°, C5-C4-O4 to 110.65(12)° and C5-C6-O6 to 114.51(14)°. Twisting about the three rotatable bonds between C4 and C15 and those between C6 and C32 results in a steady increase in separation distance from 2.565(2) Å for C4...C6 to 2.804(2) Å for O4...O6 and 3.616(2) Å for C14 and C31 that still allows the pyrene rings to be nearly parallel.

The orthoformate and *tert*-butyldimethylsilyl protecting groups in **3** were removed simultaneously in the presence of *p*-toluenesulfonic acid¹⁵ to give tetrol **4**, which was treated with dibenzyl *N,N*-diisopropylphosphoramidite, followed by oxidation with *m*-chloroperoxybenzoic acid to give **5**. Benzyl deprotection of **5** was achieved by catalytic hydrogenation to generate the free acid **6**, which was converted to the sodium salt (**2**) (Scheme 3).§

The fluorescence emission spectra of **2** were recorded in the absence and presence of Fe³⁺ (Fig. 2). The emission spectrum of **2** alone gave rise to blue fluorescence observed at 386 nm and attributed to the locally excited state of the pyrene monomer. The presence of Fe³⁺ caused a marked change in fluorescence, with the observation of substantial quenching of the fluorescence of the locally excited state at 386 nm accompanied by the appearance of a new broad emission band at 510 nm (green fluorescence). This was attributed to the excimer emission of the two closely located pyrene groups achieved in the penta-axial conformation (**2B**).

Due to the quenching properties of Fe³⁺, the fluorescence emission spectrum of **2** was also recorded in the presence of Ga³⁺ (d¹⁰), a non-quenching analogue of Fe³⁺ (high-spin d⁵).¹⁷⁻¹⁹ Both cations have the same charge, similar ionic radii and are known to form isomorphous hexa-coordinate complexes.²⁰ Based on this, Ga³⁺ was predicted to behave similarly to Fe³⁺ with respect to its binding to the fluorescent probe. This was indeed the case and a similar emission spectrum was recorded in the presence of Ga³⁺



Scheme 3 Synthesis of the fluorescent probe 4,6-bis-pyrenoyl-*myo*-inositol 1,2,3,5-tetrakisphosphate (**2**): (a) *p*-toluenesulfonic acid, THF : MeOH (2 : 1); (b) i, dibenzyl *N,N*-diisopropylphosphoramidite, 0.45 M 1*H*-tetrazole, MeCN; ii, *m*-CPBA, DCM; (c) H₂/Pd-C, ethanol; (d) Dowex 50-X8 resin, mesh 20–50, Na⁺ form, elution with water.

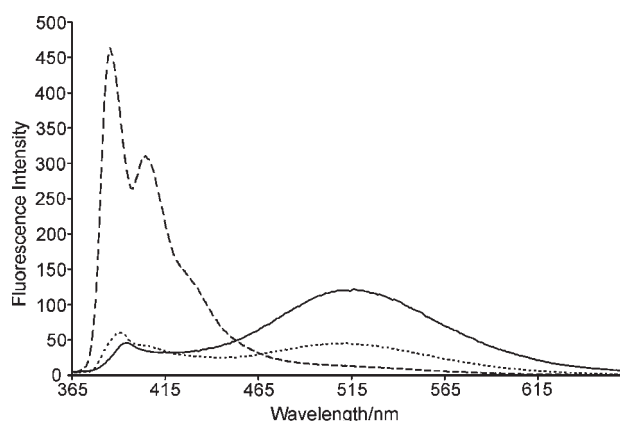


Fig. 2 Emission spectra of 4,6-bispyrenoyl Ins(1,2,3,5)P₄ (**2**) in the absence (---) and presence (···) of Fe³⁺ (1 equiv.) and the presence (—) of Ga³⁺ (1 equiv.). Spectra were recorded at a concentration of 1 mM in methanol at 20 °C. Excitation and emission slit widths were 3 nm (absence of metal) and 5 nm (presence of metal).

(Fig. 2). The excimer peak was observed at 515 nm with an intensity more than double that observed with Fe^{3+} . This evidence supports the general principle that Fe^{3+} binds to the penta-axial conformation.¹⁰

To obtain detailed structural information, calculations on high-spin Fe^{3+} complexes of penta-axial hexamethyl phosphoester at the UB3LYP/6-31 + G* level were performed. Conforming to the 1 : 1 Ins(1,2,3) P_3 - Fe^{3+} stoichiometry suggested by potentiometric titrations⁵ and the absence of water within the coordination sphere implied by azide competition experiments,¹⁰ we obtained after conformational searching two essentially isoenergetic Fe^{3+} -coordinating penta-axial geometries, **7A** and **7B** (Fig. 3). Distinct from the structure suggested by Phillippy and Graf,¹⁰ for which we were unable to detect a stable minimum, both **7A** and **7B** involve inositol *phosphoester* oxygens as well as terminal oxygens in the coordination of Fe^{3+} . Similar Fe^{3+} -coordination is predicted for hexa-anionic Ins(1,2,3) P_3 . Structure **7A** adopts a distorted tetrahedral coordination involving one phosphoester and three terminal oxygens in the coordination of Fe^{3+} . Structure **7B**, which is 0.1 kcal mol⁻¹ higher in energy than **7A**, adopts a distorted trigonal bipyramidal coordination around Fe^{3+} involving participation of two phosphoester and three terminal oxygens. Further experimental studies are required to unambiguously establish the structure of the Ins(1,2,3) P_3 - Fe^{3+} complex.

This study provides evidence that Fe^{3+} binding to *myo*-inositol phosphates possessing the 1,2,3-trisphosphate

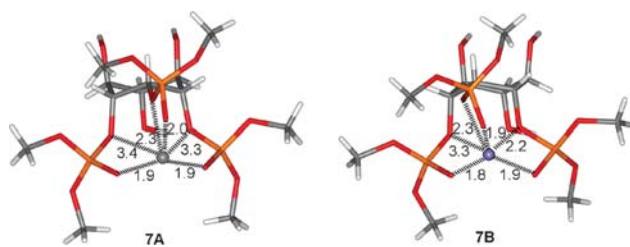


Fig. 3 Optimal UB3LYP/6-31+G* geometries (Å) of the hexamethyl phosphoester of Ins(1,2,3)P₃-Fe³⁺ (**1**) in the high-spin state.

motif is achieved by adopting the penta-axial conformation. Interest surrounds the precise biological function of Ins(1,2,3)P₃ (**1**), and in future research the fluorescent probe may be of use to study the behaviour of **1** in cells.

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Notes and references

‡ Crystal data for **3-CDCl₃**: C₄₇H₃₈O₈Si·CDCl₃, *M* = 880.25, triclinic, space group *P1*, *a* = 11.1679(9), *b* = 11.6838(9), *c* = 16.9249(13) Å, *α* = 72.420(2), *β* = 81.387(2), *γ* = 87.634(2)°, *V* = 2081.5(3) Å³, *Z* = 2, *T* = 150 K, *Z* = 2, *D* = 1.404 (calc.), *μ* = 0.306 mm^{−1}, 18 993 reflections measured, 9307 unique (*R*_{int} = 0.0075); after final refinement *R* = 0.041 for 6716 reflections with *F*_o > 4σ(*F*_o), w*R*(*F*²) = 0.114 for all 9307 reflections.

§ For 2, δ_{H} (500 MHz, D₂O, water supp.): 9.12 (2H, d, J 9.4 Hz, Pyr), 8.90 (2H, d, J 7.6 Hz, Pyr), 8.76–8.25 (14H, m, Pyr), 6.04 (2H, t, J_{AA} 9.9 Hz, H-4/6), 5.34 (1H, br d, J_{PH} 10.1 Hz, H-2), 4.91 (1H, dt \approx br q, $J_{\text{AA}} \approx J_{\text{PH}}$ 8.7 Hz, H-5), 4.79–4.68 (2H, br t, $J_{\text{AA}} \approx J_{\text{PH}}$ 7.2 Hz, H-1/3). δ_{P} (121 MHz, D₂O): 1.79 (1P), –0.09 (2P), –1.08 (1P). IR data: ν_{max} /cm^{–1} 1703 (C=O); 1257 (m), 1226 (m), 1195 (m), (P=O); 1082 (s), 1048 (s), 1018 (s) (P–O–Bn).

- 1 (a) C. J. Barker, P. J. French, A. J. Moore, T. Nilsson, P. O. Berggren, C. M. Bunce, C. J. Kirk and R. H. Michell, *Biochem. J.*, 1995, **306**, 557–564; (b) F. M. McConnell, S. B. Shears, P. J. L. Lane and M. S. Scheibel, *Biochem. J.*, 1992, **284**, 447–455.
- 2 I. D. Spiers, C. J. Barker, S.-K. Chung, Y.-T. Chang, S. Freeman, J. M. Gardiner, P. H. Hirst, P. A. Lambert, R. H. Michell and D. R. Poyner, *Carbohydr. Res.*, 1996, **282**, 81–99.
- 3 I. D. Spiers, S. Freeman, D. R. Poyner and C. H. Schwalbe, *Tetrahedron Lett.*, 1995, **36**, 2125–2128.
- 4 A. J. Letcher, M. J. Schell and R. F. Irvine, *Biochem. J.*, 2008, DOI: 10.1042/BJ20081417.
- 5 N. Veiga, J. Torres, D. Mansell, S. Freeman, S. Dominguez, C. J. Barker, A. Díaz and C. Kremer, *J. Biol. Inorg. Chem.*, DOI: 10.1007/s00775-008-0423-2.
- 6 C. J. Barker, J. Wright, P. J. Hughes, C. J. Kirk and R. H. Michell, *Biochem. J.*, 2004, **380**, 465–473.
- 7 J. Torres, S. Dominguez, M. F. Cerdá, G. Obal, A. Mederos, R. F. Irvine, A. Díaz and C. Kremer, *J. Inorg. Biochem.*, 2005, **99**, 828–840.
- 8 I. D. Spiers, S. Freeman and C. H. Schwalbe, *J. Chem. Soc., Chem. Commun.*, 1995, 2219–2220.
- 9 M. J. Frisch and co-workers, *Gaussian 03*, 2004. See ESI for full citation†.
- 10 B. Q. Phillippy and E. Graf, *Free Radical Biol. Med.*, 1997, **22**, 939–946.
- 11 M. Kadirvel, E. V. Bichenkova, A. D’Emanuele and S. Freeman, *Chem. Lett.*, 2006, **35**, 868–869.
- 12 M. Kadirvel, B. Arsic, S. Freeman and E. V. Bichenkova, *Org. Biomol. Chem.*, 2008, **6**, 1966–1972.
- 13 A. Hosoda, Y. Ozaki, A. Kashiwada, M. Mutoh, K. Wakabayashi, K. Mizuno, E. Nomura and H. Taniguchi, *Bioorg. Med. Chem.*, 2002, **10**, 1189–1196.
- 14 R. Kahn, R. Fourme, D. Andre and M. Renaud, *Acta Crystallogr., Sect. B: Struct. Crystallogr. Cryst. Chem.*, 1973, **29**, 131–138.
- 15 T.-H. Kim and A. B. Holmes, *J. Korean Chem. Soc.*, 2006, **50**, 129–136.
- 16 C. K. Johnson, *ORTEP-II*, Report ORNL-5138, Oak Ridge National Laboratory, Oak Ridge, TN, USA, 1976.
- 17 B. Bodenant, F. Fages and M. H. Delville, *J. Am. Chem. Soc.*, 1998, **120**, 7511–7519.
- 18 F. Fages, B. Bodenant and T. Weil, *J. Org. Chem.*, 1996, **61**, 3956–3961.
- 19 F. Fages, S. Leroy, T. Soujanya and J.-E. Sohna, *Pure Appl. Chem.*, 2001, **73**, 411–414.
- 20 B. A. Borgias, S. J. Barclay and K. N. Raymond, *J. Coord. Chem.*, 1986, **15**, 109–123.