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Poly(ethylene glycol)-linked dimers of D-*myo*-inositol 1,4,5-trisphosphate

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The first poly(ethylene glycol)-linked dimers of D-*myo*-inositol 1,4,5-trisphosphate have been synthesised as probes for multi-subunit binding proteins of this ubiquitous second messenger.

D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] acts as an intracellular second messenger in almost all mammalian cells by evoking the release of Ca^{2+} ions from intracellular stores through Ins(1,4,5)P_3-gated ion channels[1] [Ins(1,4,5)P_3 receptors, IP_3Rs] located in the endoplasmic reticulum. IP_3Rs are tetramers, composed of four subunits, each with a single Ins(1,4,5)P_3 binding site, surrounding the central ion channel. This suggests the possibility of designing multivalent ligands for IP_3Rs, in which two or more copies of the natural ligand connected by a linker may be able to access multiple binding sites simultaneously, potentially giving enhanced potency, selectivity or other novel effects. To this end, a synthesis of bivalent and tetravalent analogues has been reported in which two or four molecules of a synthetic carbohydrate-based Ins(1,4,5)P_3 mimic were directly attached to a small hydrophobic hub.[2] As the IP_3R is large (12 nm width, estimated from electron microscopy[3]) and the locations of the Ins(1,4,5)P_3 binding sites are unknown, a more promising and versatile approach may be to use bivalent ligands with hydrophilic polymeric linkers in a realistic range of lengths. This strategy has been successfully employed, using very simple chemistry, to identify super-potent dimeric ligands for tetrameric cyclic nucleotide gated (CNG) channels of photoreceptor and olfactory neurones by using a series of dimers of the 8-thio derivative of the natural ligand (cGMP) linked by PEG chains.[4] These PEG-linked dimers of cGMP also showed partial agonist properties at photoreceptor CNG channels, and some were membrane-permeant.

Application of this approach to Ins(1,4,5)P_3 is, however, synthetically much more difficult. Nevertheless, studies have shown that bulky groups may be attached to the axial 2-oxygen atom of Ins(1,4,5)P_3 with minimal reduction in affinity for the IP_3R,[5] suggesting that at the Ins(1,4,5)P_3 binding sites of the IP_3R this area may be open to solvent. We therefore chose to synthesise Ins(1,4,5)P_3 dimers linked *via* the 2-position (Fig. 1). The synthetic strategy was first explored using a short PEG linker derived from hexa(ethylene glycol) (estimated[4] r.m.s. length 1.5 nm), and then three larger dimers were synthesised from PEGs with average molecular weights 1450, 3350 and 8000 (estimated rms lengths 3, 5 and 8 nm, respectively).

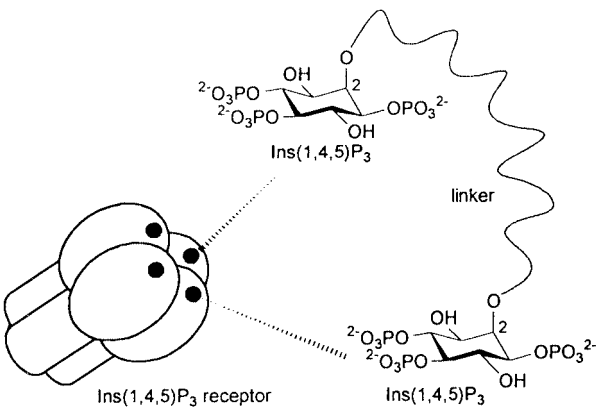
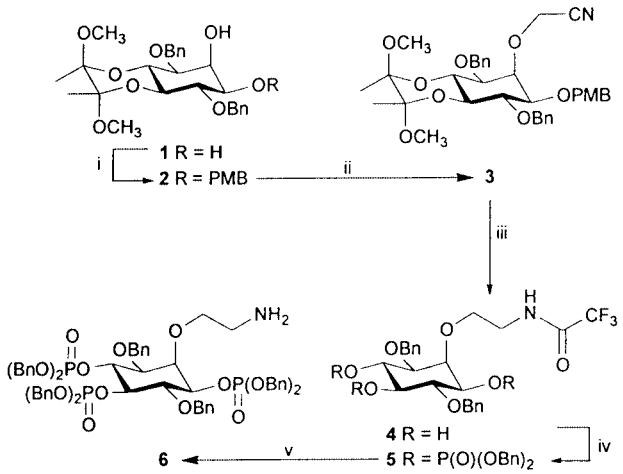


Fig. 1 Ins(1,4,5)P_3 dimers could target multiple receptor binding sites.

The synthesis begins with diol **1**[6] (Scheme 1). Stannylene-mediated regioselective alkylation of the equatorial 1-OH group with *p*-methoxybenzyl chloride in the presence of CsF gave the alcohol **2** in 80% yield. Alkylation of **2** with bromoacetonitrile and sodium hydride in acetonitrile at reduced temperatures[7] then gave the 2-*O*-cyanomethyl derivative **3** {mp 120–121 °C, $[\alpha]_D^{18}$ +56 (*c* 1, $CHCl_3$)} in 83% yield. The nitrile was smoothly reduced with LAH in THF to the primary amine, which was not isolated but temporarily protected as the trifluoroacetamide by reaction with ethyl trifluoroacetate in THF at room temperature.[8] Finally, the acid-labile butanediacetal (BDA) and PMB protecting groups were cleaved using TFA, exposing the hydroxy groups at positions 1, 4 and 5. The trifluoroacetyl protection was not affected under these conditions, and the triol **4** {mp 130–131 °C, $[\alpha]_D^{18}$ +3 (*c* 1, $CHCl_3$)} was obtained in 77% overall yield from **3**. Phosphitylation using bis(benzyloxy)(*N*,*N*-diisopropylamino)phosphine and 1*H*-tetrazole followed by *in situ* oxidation with MCPBA gave crystalline **5** {mp 85–87 °C, $[\alpha]_D^{20}$ −6 (*c* 1, $CHCl_3$)}.



Scheme 1 *Reagents and conditions*: i, (a) Bu_2SnO, MeOH, 4 Å sieves, Soxhlet, reflux, 16 h; (b) PMBCl, CsF, DMF, 50 °C, 5 h, 84%; ii, NaH, $BrCH_2CN$, CH_3CN, −20 to −40 °C 5 h then room temp., 16 h, 83%; iii, (a) LAH, THF, room temp, 1 h, (b) ethyl trifluoroacetate, THF, room temp., 1 h, (c) TFA–CH_2Cl_2–H_2O (19:20:1), room temp., 30 min, 77% from **3**; iv, $(BnO)_2PNPr^i_2$, 1 *H*-tetrazole, CH_2Cl_2, room temp., 1 h, then MCPBA, −78 °C to room temp., 30 min (96%); v, LiOH·H_2O (10 equiv.) in THF–MeOH–H_2O (2:2:1), room temp., 1 h, 91%. Bn = benzyl, PMB = *p*-methoxybenzyl.

It was now necessary to expose the primary amine by selective removal of the trifluoroacetyl group. Treatment of **5** with methanolic ammonia or K_2CO_3 in aqueous methanol was only partially successful; long reaction times were required, leading to the formation of various polar products, presumably from partial cleavage of benzylphosphate esters. However, it was found that the trifluoroacetyl group could cleanly be removed by treatment with LiOH in THF–MeOH–H_2O[9] for 1 h.

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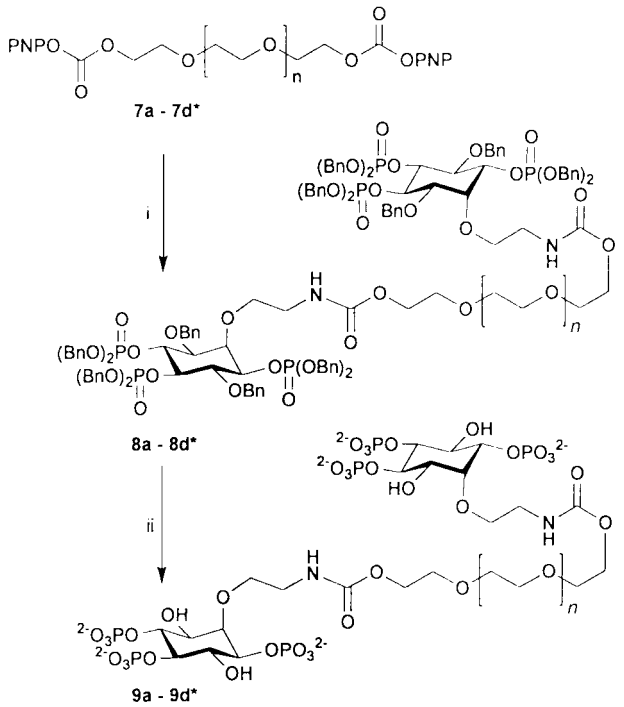

Scheme 2 *Reagents and conditions*: i, **6** (3–4 equiv.), DMF, room temp., 24 h, 31–58%; ii, Pd–C, H_2, 50 psi, room temp., 24 h, 52–65%. Bn = benzyl, PNP = *p*-nitrophenyl. ***a**, n = 4; **b**, $n \approx 30$; **c**, $n \approx 75$; **d**, $n \approx 180$.

Within this time there was little effect on the benzylphosphate groups. The amine **6** was found to be unstable, and was therefore freshly prepared for each cross-linking reaction and used immediately.

Cross-linking of two molecules of **6** was first attempted using the bis(*p*-nitrophenylcarbonate) derivative† **7a** of hexa(ethylene glycol) (Scheme 2). Reaction of **7a** with 3 equivalents of **6** in DMF gave the protected PEG-linked dimer **8a**, which was isolated in moderate yield (58% based on **7a**) yield after purification by flash chromatography on silica gel. The ^{1}H NMR spectrum of **8a** confirmed that it was a dimer‡ and the ^{31}P NMR spectrum showed three signals, each corresponding to two equivalent phosphorus atoms in **8a**. Removal of all sixteen benzyl groups from **8a** was easily achieved by hydrogenation over Pd–C. Purification by ion-exchange chromatography on Q-Sepharose Fast Flow resin, eluting with a gradient of triethylammonium hydrogencarbonate buffer gave **9a** as the triethylammonium salt, which eluted between 0.7 and 0.9 mol dm^{-3} buffer. The structure of **9a** was confirmed by ^{1}H and ^{31}P NMR spectroscopy, and by negative ion FAB mass spectrometry§ before accurate quantification by total phosphate assay.[10] The larger protected dimers **8b**, **8c** and **8d** were then synthesised by reaction of **6** with bis(*p*-nitrophenylcarbonate)-PEGs **7b**, **7c** and **7d** under the conditions established for **7a**. In each case, the product was purified by flash chromatography and its dimeric structure was confirmed by ^{1}H NMR spectroscopy before deprotection as for **8a**. Finally, purification of each dimer by ion exchange chromatography as for **9a** gave **9b**, **9c** and **9d** as their triethylammonium salts, which were all freely soluble in water.

Thus we have demonstrated, for the first time, a viable synthetic route to high molecular weight bivalent ligands as potential pharmacological tools to probe the IP_3R. Other proteins are known to have multiple binding sites[11] for Ins(1,4,5)P_3 or phosphatidylinositol 4,5-bisphosphate and the Ins(1,4,5)P_3 dimers could also be used to investigate these. A particularly attractive application might be to use Ins(1,4,5)P_3 metabolising enzymes to convert Ins(1,4,5)P_3 dimers into dimers of other inositol phosphates, which may have their own intracellular targets proteins.

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Notes and references

† **7a**: this was synthesised by reaction of hexa(ethylene glycol) with 6 equivalents of bis(*p*-nitrophenyl) carbonate in DMF in the presence of diisopropylethylamine, and was purified by flash chromatography on silica gel before use. **7b** and **7d** were synthesised in a similar way from PEGs with average molecular weights of 1450 and 8000, respectively. The bis(*p*-nitrophenyl carbonate)-PEG **7c**, derived from a PEG of average molecular weight 3350, is commercially available (Sigma).

‡ *Selected data* for **8a**–**8d**: **8a** δ_H($CDCl_3$, 400 MHz) 3.24–3.30 (4H, m, OCH_2CH_2N), 3.38 (2H, dd, *J* 10.0, 2.1 Hz, 3-H), 3.50 (4H, s, OCH_2CH_2N), 3.55–3.58 (16H, m, 8 × PEG CH_2), 3.66–3.70 (4H, m, 2 × PEG CH_2), 4.00 (2H, dd, *J* 9.7, 9.4 Hz, 6-H), 4.04 (2H, br s, 2-H), 4.10–4.16 (4H, m, 2 × PEG CH_2), 4.18 (2H, ddd, *J* 9.7, 7.3, 2.3 Hz, 1-H), 4.48 (2H, ddd, *J* 9.4, 9.4, 9.1 Hz, 5-H), 4.47, 4.57 (4H, AB_q, J_{AB} 11.7 Hz, OCH_2Ph), 4.62–4.68 (2H, 0.5 of AB_q with $^3J_{HP}$ coupling, J_{AB} 11.7, J_{HP} 8.5 Hz, $POCH_2Ph$), 4.74–5.07 (28H, m, 4-H and 6.5 AB systems of OCH_2Ph), 5.60 (2H, br t, *J* 5.3 Hz, NH), 6.96–6.98 (4H, m, Ph), 7.08–7.36 (76H, Ph); protected dimers **8b**–**8d** had similar ^{1}H NMR spectra to **8a**, except; **8b**: δ 3.55–3.70 (approx. 130H, m, CH_2 of PEG), **8c**: δ 3.55–3.70 (approx. 300H, m, CH_2 of PEG); **8d**: δ 3.55–3.70 (approx. 700H, m, CH_2 of PEG).

§ *Selected data* for **9a**–**d**: **9a**: δ_H(CD_3OD, 400 MHz) δ *ca.* 3.3 [4H, m (buried), OCH_2CH_2N], 3.61–3.70 (22H, m, 3-H and 10 × PEG CH_2), 3.77–3.85 (2H, m, $OCHHCH_2N$), 3.92–4.00 (6H, m, 5-H, 6-H and $OCHHCH_2N$), 4.01–4.06 (4H, m, 1-H and 2-H), 4.14–4.20 (4H, m, 2 × CH_2 of PEG), 4.32 (2H, ddd, *J* 9.4, 8.9, 8.6 Hz, 4-H); δ_P(CD_3OD, 162 MHz) 2.06 (2 P), 3.19 (2 P) and 3.66 (2 P); MS *m/z* (−ve ion FAB, relative intensity); 1281 (90%), 1259 [M^-, 80%], 97 [$H_2PO_4^-$, 100%]; Accurate mass FAB^-: calc. for $C_{30}H_{61}N_2O_{39}P_6^-$, 1259.127; found 1259.122. Dimers **9b**–**d** had similar ^{1}H NMR spectra to that of **9a**, except for the increasing integral of the signal at δ *ca.* 3.60–3.70 corresponding to CH_2 of PEG. Their ^{31}P NMR spectra were also similar to that of **9a**.

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