Synthesis of dimeric analogs of adenophostin A that potently evoke Ca\textsuperscript{2+} release through IP\textsubscript{3} receptors†

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Inositol 1,4,5-trisphosphate receptors (IP\textsubscript{3}R) are tetrameric intracellular channels through which many extracellular stimuli initiate the Ca\textsuperscript{2+} signals that regulate diverse cellular responses. There is considerable interest in developing novel ligands of IP\textsubscript{3}R. Adenophostin A (AdA) is a potent agonist of IP\textsubscript{3}R and since some dimeric analogs of IP\textsubscript{3}R ligands are more potent than the corresponding monomer; we considered whether dimeric AdA analogs might provide agonists with increased potency. We previously synthesized traizolophostin, in which a simple triazole replaced the adenine of AdA, and showed it to be equipotent to AdA. Here, we used click chemistry to synthesize four homodimeric analogs of triazolophostin, connected by oligoethylene glycol chains of different lengths. We evaluated the potency of these analogs to release Ca\textsuperscript{2+} through type 1 IP\textsubscript{3}R and established that the newly synthesized dimers are equipotent to AdA and triazolophostin.

Introduction

Inositol 1,4,5-trisphosphate (IP\textsubscript{3}, 1, Fig. 1) is an important secondary messenger that evokes Ca\textsuperscript{2+} release from intracellular stores through its interaction with IP\textsubscript{3} receptors (IP\textsubscript{3}R) in the endoplasmic reticulum.\textsuperscript{1} IP\textsubscript{3}R are large tetrameric proteins, within which IP\textsubscript{3} binding to each of the four subunits is required to initiate opening of the Ca\textsuperscript{2+}-permeable channel.\textsuperscript{2} High-resolution structures of the IP\textsubscript{3}-binding core (IBC, residues 224–604) have defined the interactions of IP\textsubscript{3} with IP\textsubscript{3}R.\textsuperscript{3} More recently, structures of the N-terminal region (residues 1–604)\textsuperscript{4} alongside a structure of the complete IP\textsubscript{3}R derived from cryo-electron microscopy have begun to suggest how IP\textsubscript{3} binding might trigger the opening of the intrinsic pore of IP\textsubscript{3}R.\textsuperscript{5}

There is continuing interest in the development of potent agonists and antagonists of IP\textsubscript{3}R.\textsuperscript{6} The fungal metabolite, adenophostin A (AdA, 2, Fig. 1), binds to IP\textsubscript{3}R with greater affinity than IP\textsubscript{3} and it is more potent than IP\textsubscript{3} in evoking Ca\textsuperscript{2+} release.\textsuperscript{7} AdA analogs with a nucleobase or base-surrogate are also more potent than IP\textsubscript{3}.\textsuperscript{8} Molecular docking\textsuperscript{9m,}\textsuperscript{8} and mutation studies\textsuperscript{10} suggest that a cation–π interaction between the adenine moiety of AdA and Arg504 within the IBC contributes to the increased affinity of AdA. We recently reported synthesis of a library of active AdA analogs, triazolophostins, by using a click chemistry approach.\textsuperscript{11} These potent analogs have substituted triazoles as adenine surrogates. The simplest analog, triazolophostin (3, Fig. 1) was equipotent with AdA.

Multimeric ligands often have greater affinity than monomeric ligands.\textsuperscript{12} This can be due to simultaneous binding to more than one binding site or a statistical effect arising from the local increase in ligand concentration.\textsuperscript{13} The former is unlikely for IP\textsubscript{3}R because the orientation of the IP\textsubscript{3}-binding sites within the tetrameric IP\textsubscript{3}R is unlikely to allow simultaneous binding of two ligands linked by a short tether.\textsuperscript{4b,}\textsuperscript{14}

![Fig. 1. The structures of IP\textsubscript{3} (1), adenophostin A (AdA, 2) and triazolophostin (3).](image-url)
A few multimeric ligands of IP₃R have been reported. Before the location of the IP₃-binding sites within IP₃R was known, clustered bi- and tetra-dentate analogs of ribophostin (4, Fig. 2A) were synthesized, anticipating that if the spacing between the linked ligands was appropriate they might bind simultaneously to the four IP₃-binding sites. However, the potencies of the monomeric and polymeric ligands were rather similar. Several homodimeric and heterodimeric ligands of IP₃ (5–10, Fig. 2B), particularly those with short linkers, were shown to bind to IP₃R with increased affinity. Very recently, dimers of 2-O-Bt-IP₄/IP₅ (11, Fig. 2C) were shown to be antagonists of IP₃Rs. These results demonstrate that dimeric IP₃R ligands can provide useful tools, some of which have greater affinity than the monomeric ligands. We therefore considered whether dimers of AdA might be more potent than AdA.

**Results and discussion**

As the synthesis of AdA dimers is challenging, we decided to make oligoethylene glycol-tethered dimers of triazolophostin (Fig. 3). We envisaged that use of click reaction with a linker connected to alkyne at both termini would ensure both formation of triazole and link the two monomers in one step. Previous studies suggested that short linkers were most likely to improve the affinity of homodimers. We therefore selected spacers smaller than hexaethylene glycol. The linkers 14a–d were synthesized by slightly modifying previously reported procedures. The oligoethylene glycols were first co-evaporated with toluene and then treated with sodium hydride in the presence of excess propargyl bromide to get dipropargyl polyethylene glycols 14a–d in good to excellent yields. The azide 13 was synthesized from glucose and xylose by several protection-deprotection reactions followed by phosphorylation as reported.

![Fig. 2](image1.png)  
*Fig. 2* The representative structures of (A) ribophostin dimer 4, (B) homo and hetero dimers of IP₃ (5–10) and (C) dimers of 2-Bt-IP₄/IP₅ 11.

![Fig. 3](image2.png)  
*Fig. 3* The structure of dimeric analogs of triazolophostin 12.

Scheme 1  
Synthesis of triazolophostin dimers. Reagents and conditions: (a) ref. 11; (b) Cu, CuSO₄, H₂O : BuOH (1 : 1, v/v), rt, 24 h; (c) Pd(OH)₂/C, cyclohexene, MeOH : H₂O (10 : 1, v/v), 80 °C, 4 h; (a), n = 2; (b), n = 3; (c), n = 4; (d), n = 6.
Products were purified by chromatography. The dimeric ligands were no more potent than the corresponding monomer (3). This suggests that whereas dimeric derivatives of IP₃ have reduced efficacy but improved affinity,¹⁰,²¹ dimerization of AdA analogs does not improve their affinity.

Experimental section

General methods

The chemicals were purchased from commercial sources and used as received. The TLC plates were visualized under UV light and by dipping plates into either phosphomolybdic acid in MeOH or sulphuric acid in ethanol, followed by heating. All NMR experiments were carried out on a 500 MHz NMR spectrometer and at room temperature. Tetramethylsilane (TMS, δ 0.0 ppm) or the solvent reference (CDCl₃, δ 7.26 ppm; D₂O, δ 4.79 ppm) relative to TMS were used as the internal standard. The data are reported as follows: chemical shift in ppm (δ) (multiplicity [singlet (s), doublet (d), doublet of doublet (dd), triplet (t), quartet (q), and multiplet (m)], coupling constants [Hz], integration and peak identification). All NMR signals were assigned on the basis of ¹H NMR, ¹³C NMR, COSY and HMQC experiments. ¹³C NMR spectra were recorded with complete proton decoupling. Carbon chemical shifts are reported in ppm (δ) relative to TMS with the respective solvent resonance as the internal standard. The concentration of the compounds for ¹H and ¹³C NMR was 5 mg per 0.5 mL and for ¹³C NMR it was 20 mg per 0.5 mL for protected compounds and 5–7 mg per 0.5 mL for final compounds in case of ¹H and ¹³C NMR. Modified Brigg’s phosphate assay was employed to quantify each triazolophosphin dimer. Silica gel 230–400 mesh was used to perform flash column chromatography.

General procedure for syntheses of fully protected triazolophosphin dimers

To a solution of azide 13 (0.144 mmol) and dialkynyl PEG 14a–d (0.072) in H₂O/BuOH (1/1, v/v, 2 mL) was added Cu (0.036 g, 0.57 mmol) and CuSO₄ (8 mg, 0.028 mmol) and stirred at room temperature for 24 h. The reaction was monitored by TLC. When the TLC showed complete disappearance of the azide 13, the mixture was filtered through a Celite bed and was partitioned between ethyl acetate and water. The organic layer was washed with brine. The organic layer was dried over anhyd. sodium sulphate, filtered and concentrated under reduced pressure.

Conclusions

In conclusion, based on several previous reports that dimeric IP₃R ligands can be more potent than the corresponding monomers, we anticipated that dimers of AdA might have increased potency. We used click chemistry to synthesize dimers of a potent analog of AdA (triazolophosphin) linked by spacers of different length. In assays of Ca²⁺ release through IP₃R, the dimeric ligands were no more potent than the monodentate fashion.

Earlier,¹⁰ the azide 13 was then treated with dialkynyl polyethylene glycols 14a–d in the presence of Cu(i) catalyst to get fully protected triazolophosphin dimers 15a–d in good yields. The debenzylolation of protected triazolophosphin dimers 15a–d was carried out using transfer hydrogenolysis in the presence of palladium and cyclohexene under reflux condition and the products were purified by ion-exchange chromatography to yield dimers 12a–d, in excellent yields (Scheme 1).

The dimeric ligands 12a–d were screened for their abilities to evoke Ca²⁺ release through IP₃R (Table 1, Fig. 4). All four dimers were full agonists of IP₃R, more potent than IP₃, but similar in their potency to AdA and the monomer, triazolophosphin. The similar potencies of 12a–d irrespective of their tether length suggest that these ligands might be interacting with IP₃R in monodentate fashion.

Experimental section

General methods

The chemicals were purchased from commercial sources and used as received. The TLC plates were visualized under UV light and by dipping plates into either phosphomolybdic acid in MeOH or sulphuric acid in ethanol, followed by heating. All NMR experiments were carried out on a 500 MHz NMR spectrometer and at room temperature. Tetramethylsilane (TMS, δ 0.0 ppm) or the solvent reference (CDCl₃, δ 7.26 ppm; D₂O, δ 4.79 ppm) relative to TMS were used as the internal standard. The data are reported as follows: chemical shift in ppm (δ) (multiplicity [singlet (s), doublet (d), doublet of doublet (dd), triplet (t), quartet (q), and multiplet (m)], coupling constants [Hz], integration and peak identification). All NMR signals were assigned on the basis of ¹H NMR, ¹³C NMR, COSY and HMQC experiments. ¹³C NMR spectra were recorded with complete proton decoupling. Carbon chemical shifts are reported in ppm (δ) relative to TMS with the respective solvent resonance as the internal standard. The concentration of the compounds for ¹H and ¹³C NMR was 5 mg per 0.5 mL and for ¹³C NMR it was 20 mg per 0.5 mL for protected compounds and 5–7 mg per 0.5 mL for final compounds in case of ¹H and ¹³C NMR. Modified Brigg’s phosphate assay was employed to quantify each triazolophosphin 12a–d. Silica gel 230–400 mesh was used to perform flash column chromatography.

General procedure for syntheses of fully protected triazolophosphin dimers

To a solution of azide 13 (0.144 mmol) and dialkynyl PEG 14a–d (0.072) in H₂O/BuOH (1/1, v/v, 2 mL) was added Cu (0.036 g, 0.57 mmol) and CuSO₄ (8 mg, 0.028 mmol) and stirred at room temperature for 24 h. The reaction was monitored by TLC. When the TLC showed complete disappearance of the azide 13, the mixture was filtered through a Celite bed and was partitioned between ethyl acetate and water. The organic layer was washed with brine. The organic layer was dried over anhyd. sodium sulphate, filtered and concentrated under reduced pressure.
pressure. The residue thus obtained was purified by flash column chromatography using a mixture of acetone, diethyl ether and petroleum ether (4 : 2 : 15 v/v/v) as eluent to get pure 15a-d as a colourless gum.

**Protected triazolophostin dimer 15a.** Click reaction of azide 13 (0.2 g, 0.144 mmol) with diyne 14a (0.011 g, 0.072 mmol) gave the protected dimer 15a (0.18 g, 85%) as a colourless gum. 1H NMR (500 MHz, CDCl3) δ: 3.47–3.57 (m, 18H, H-2”, H-4”, H-6”A, and DEG-H), 3.73–3.75 (m, 2H, H-5”), 4.20–4.23 (m, 2H, PhCH2), 4.27–4.32 (m, 8H, H-5’A and PhCH2), 4.30–4.45 (m, 10H, H-3”, H-5”, H-6”B and PhCH2), 4.57–4.59 (m, 2H, PhCH2), 4.63–4.66 (m, 4H, PhCH2), 4.68–4.73 (m, 6H, PhCH2), 4.80–4.93 (m, 16H, H-3”, H-4” and PhCH2), 5.11 (d, 2H, J = 3.2 Hz, H-1”), 5.26–5.28 (m, 2H, H-2”), 6.24 (d, 2H, J = 5.0 Hz, H-1”), 7.00 (d, 4H, J = 7.0 Hz, Ar-H), 7.05–7.19 (m, 82H, Ar-H), 7.26 (d, 4H, J = 7.0 Hz, Ar-H), 7.60 (s, 2H, H-5’). 13C NMR (125 MHz, CDCl3) δ: 64.2, 68.3, 69.1, 69.2, 69.3, 69.5, 69.6, 69.7, 69.9, 70.1, 70.4, 71.9, 73.3, 73.5, 74.1, 78.0, 78.5, 82.8, 90.1, 95.7, 121.6, 127.6, 127.7, 127.9, 128.0, 128.1, 128.3, 128.4, 135.2, 135.7, 135.8, 136.1, 137.3, 137.5, 138.0, 145.2; 13P NMR (202.4 MHz, CDCl3) δ: −1.482, −1.928, −2.146; HRMS (ESI) mass calc for C158H166N6O39P6 [M]+ 3133.0669, found 3133.0665.

**General procedure for synthesis of triazolophostin dimers 12a-d**

The protected triazolophostin dimers 15a-d (0.15–0.175 g, 0.05–0.055 mmol) were treated with cyclohexene (3 mL) and Pd(OH)2 (20% on carbon, 50 mg) in a mixture of methanol and water (9 : 1 v/v, 10 mL) at 80 °C for 4 h. The reaction mixture was then cooled, filtered through a membrane filter, washed successively with methanol and water. The combined filtrate was evaporated under reduced pressure. The crude product thus obtained was purified by ion-exchange column chromatography on Q-Sepharose matrix using 0–1.0 M TEAB as eluent to get pure triazolophostin dimers 12a-d.

**Triaiozolophostin dimer 12a.** The global debenzylation of 15a (0.15 g, 0.05 mmol) gave 46 mg (69%) of triazolophostin dimer 12a as a white hygroscopic solid: 1H NMR (500 MHz, D2O) δ: 3.63–3.65 (m, 8H, DEG-H), 3.70–3.83 (m, 12H, H-5’A and H-5’B, H-6’A and DEG-H), 4.09–4.10 (m, 2H, H-5’), 4.41 (bs, 2H, H-4’), 4.48 (bs, 2H, H-5’), 4.62–4.65 (m, 6H, H-3” and H-4’ and H-5”), 5.16 (bs, 2H, H-2’), 5.24 (bs, 2H, H-1”), 6.36 (bs, 2H, H-1”), 8.22 (s, 2H, H-5’); 13C NMR (125 MHz, D2O) δ: 60.1, 60.7, 62.8, 68.9, 70.5, 71.5, 72.8, 73.7, 76.4, 77.9, 83.8, 90.9, 97.9, 124.3, 144.1; 31P NMR (202.4 MHz, D2O) δ: 3.504, 3.583, 4.301; HRMS (ESI) mass calc for C31H35N6O4P6 [M]+ 1336.1165, found: 1336.1169.

**Triaiozolophostin dimer 12b.** The global debenzylation of 15b (0.15 g, 0.055 mmol) gave 51 mg (72%) of triazolophostin dimer 12b as a white hygroscopic solid: 1H NMR (500 MHz, D2O) δ: 3.56–3.60 (m, 12H, TETEAG-H), 3.69–3.74 (m, 12H, H-5’A, H-5’B, H-6’A and TETEAG-H), 4.06 (bs, 2H, H-5” and H-5”), 4.36 (bs, 2H, H-4’), 4.44 (bs, 2H, H-5’), 4.50–4.60 (m, 6H, H-3” and H-4’ and H-5”), 5.12 (bs, 2H, H-2’), 5.18 (bs, 2H, H-1”), 6.31 (bs, 2H, H-1”), 8.18 (s, 2H, H-5’); 13C NMR (125 MHz, D2O) δ: 60.1, 60.7, 62.8, 68.9, 70.5, 71.5, 72.8, 73.7, 76.4, 77.8, 83.8, 90.8, 97.9, 124.3, 144.1; 31P NMR (202.4 MHz, D2O) δ: 3.451 (2 × P), 4.224; HRMS (ESI) mass calc for C31H35N6O4P6 [M]+ 1308.1427, found: 1308.1420.

**Triaiozolophostin dimer 12c.** The global debenzylation of 15c (0.16 g, 0.052 mmol) gave 64 mg (85%) of triazolophostin dimer 12c as a white hygroscopic solid: 1H NMR (500 MHz, D2O) δ: 3.57–3.61 (m, 16H, TriazaEG-H), 3.69–3.74 (m, 12H, H-5’A, H-5’B, H-6’A and TETEAG-H), 4.05 (bs, 2H, H-5”), 4.37 (bs, 2H, H-4’),

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Triazolophosphin dimer 12d. The global debenzylation of 15d (0.175 g, 0.055 mmol) gave 65 mg (77%) of triazolophosphin dimer 12d as a white hygroscopic solid: $^1$H NMR (500 MHz, D$_2$O) $\delta$: 3.58–3.72 (m, 2H, HEG-H), 3.77–3.81 (m, 12H, H-5$'$, H-2$, H$-2$, H-6$'$ and HEG-H), 4.01 (bs, 2H, H-5$'$), 4.38–4.48 (m, 4H, H-4$'$ and H-5$'$), 4.58–4.63 (m, 6H, H-3$'$, H-3$''$ and H-4$''$), 5.12 (bs, 2H, H-2$''$), 5.20 (bs, 2H, H-1$''$), 6.32 (bs, 2H, H-1$'$), 8.19 (s, 2H, H-5$''$); $^{13}$C NMR (125 MHz, D$_2$O) $\delta$: 60.2, 60.8, 62.9, 68.9, 69.4, 69.5, 70.8, 71.7, 72.6, 73.7, 76.3, 77.4, 83.8, 90.9, 97.9, 124.2, 144.2; $^{31}$P NMR (202.4 MHz, D$_2$O) $\delta$: 3.482 (2 $\times$ P), 4.258; HRMS (ESI) mass calcd for C$_{40}$H$_{74}$N$_{6}$O$_{14}$P$_{6}$ [M$^+$], 1512.2214; found: 1512.2210.

Biological assay
Ca$^{2+}$ release from the intracellular stores of saponin-permeabilized DT40 cells expressing only type 1 IP$_3$Rs was measured using a low-affinity Ca$^{2+}$ indicator (Mag-fluo-4) trapped within the endoplasmic reticulum as described previously.$^{11}$

Briefly, Ca$^{2+}$ uptake was initiated by addition of 1.5 mM MgATP in cytosol-like medium (140 mM KCl, 20 mM NaCl, 1 mM EGTA, 20 mM PIPES, pH 7.0, free [Ca$^{2+}$]$_0$~220 nM after addition of ATP) containing p-trifluoromethoxyphenylhydrazine (FCPP) to inhibit mitochondria. After about 120 s, the triazolophosphin analogs were added with cyclopiazonic acid (10 $\mu$M) to inhibit further Ca$^{2+}$ uptake. Ca$^{2+}$ release was assessed 10–20 s after addition of the analog, and expressed as a fraction of the ATP-dependent Ca$^{2+}$ uptake.

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Notes and references


