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Adenophostin A and analogues modified at the adenine moiety: synthesis, conformational analysis and biological activity

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The synthesis of adenophostin A (**2**) and two analogues [etheno adenophostin (**4**) and 8-bromo adenophostin (**5**)] modified at the adenine moiety, is reported. A combination of NMR analysis and molecular modelling was used to compare their structures in solution and determined that they all adopt very similar conformations. The analogues were tested for their ability to mobilise Ca^{2+} from DT40 cells expressing recombinant Type 1 rat Ins(1,4,5)P_3R which reveals etheno adenophostin as a high affinity fluorescent probe of the Ins(1,4,5)P_3R. 8-Bromo adenophostin was only slightly less potent. The biological results support our current hypothesis regarding the binding mode of adenophostin A at the Ins(1,4,5)P_3R, *i. e.* that a cation–π interaction between the base moiety and Arg 504 of the receptor in combination with H-bonding may be responsible for the high potency of adenophostin A relative to Ins(1,4,5)P_3.

Introduction

D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] (**1**) (Fig. 1) is a second messenger involved in the release of Ca^{2+} from intracellular stores upon interaction with its specific receptor [Ins(1,4,5)P_3R].[1]

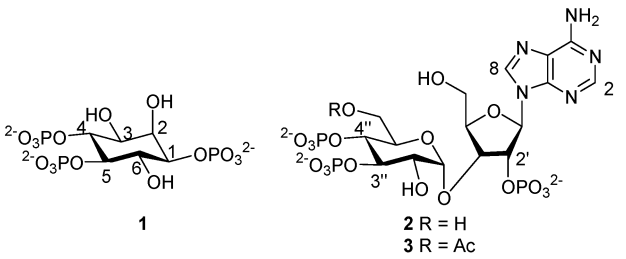

Fig. 1 Structures of D-*myo*-inositol 1,4,5-trisphosphate (**1**), adenophostin A (**2**) and adenophostin B (**3**).

Due to the biological importance of Ca^{2+} release[2] a number of analogues of Ins(1,4,5)P_3 have been synthesised and evaluated to determine the structural features required for interaction at its receptor.[3] However, to date, none of these synthetic analogues has improved upon Ins(1,4,5)P_3 in terms of receptor binding affinity or Ca^{2+} release,[3] with the exception of the dimeric species synthesised in our laboratories.[4]

Since their discovery[5] and structural elucidation[6,7] in 1993, adenophostin A 2 and B 3 have been the focus of much interest. Both molecules have been shown to exhibit a 10-fold greater affinity for the Ins(1,4,5)P_3R[8–13] than Ins(1,4,5)P_3 itself and are currently the most potent agonists of Ins(1,4,5)P_3R.[7] These interesting properties have led to the widespread use of adenophostin A as a biological tool in Ca^{2+} signalling studies and prompted many groups, including our own, to develop synthetic routes to 2 and its analogues.[9,10,14–32] None of these synthetic analogues has exceeded the potency of adenophostin A, but a great deal of information has been learned about adenophostin's structure–activity relationship at the Ins(1,4,5)P_3R (see Fig. 2).

It has been demonstrated that the adenine base (or equivalent) of adenophostin A is an important structural feature necessary for high potency, although the exact reason for this has still to be determined.[8,15,20,33,34] Our current explanation for this arose from modelling studies[20] based on the crystal structure of the binding domain of type 1 Ins(1,4,5)P_3R,[35] showing a possible cation–π interaction between the adenine ring and Arg504 when adenophostin A binds in the *anti*-conformation. This interaction may enhance binding affinity and improve the orientation of the 2′-phosphate resulting in an increase in potency. In an attempt to further elucidate these binding interactions, we report here the synthesis and conformational analysis of two analogues 4 and 5 (Fig. 3) modified at the adenine moiety.

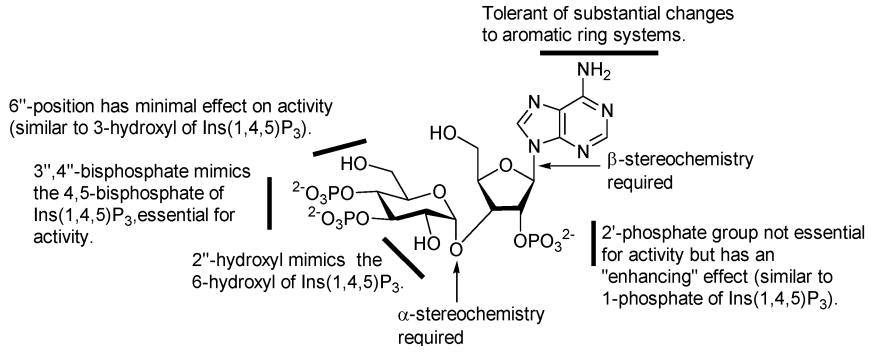

Fig. 2 Summary of the structure–activity relationships of adenophostin A at the Ins(1,4,5)P_3 receptor.

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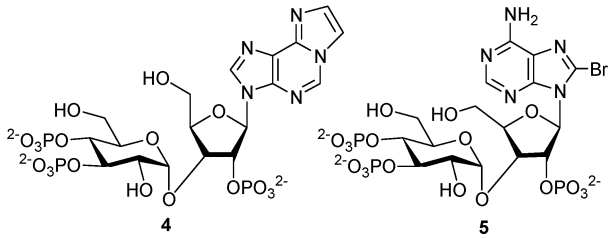


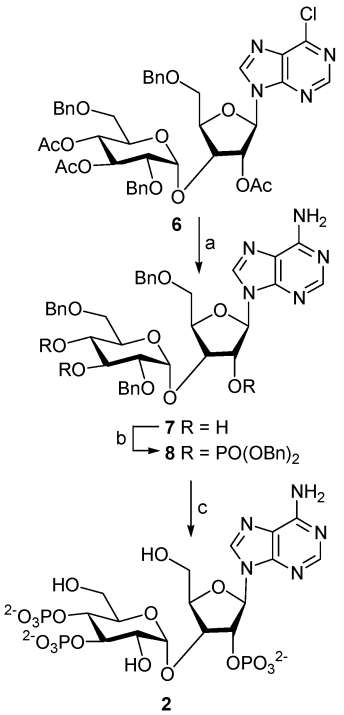
Fig. 3 Structures of etheno adenophostin (**4**) and 8-bromo adenophostin (**5**).

The etheno analogue **4** should provide an easily accessible fluorescent probe, which could be used for investigation of Ins(1,4,5)P_3Rs. A study by Rosenberg *et al.*[20] demonstrated that the Ins(1,4,5)P_3R is tolerant to the introduction of large hydrophobic groups at N-6 of adenophostin. This, coupled with work by Leonard and co-workers[36,37] showing that etheno derivatives bind like endogenous substrates to several proteins, indicated that **4** should be a good replacement for adenophostin A, hopefully retaining its high affinity binding to the Ins(1,4,5)P_3R. Earlier modelling studies by Hotoda *et al.*[33] suggest that adenophostin binds to the Ins(1,4,5)P_3R in the *syn*-conformation as opposed to *anti* as devised from our docking experiments.[20] The 8-brominated analogue **5** is sterically constrained in the *syn*-conformation and might therefore exhibit a similar potency to adenophostin, or even higher, if the *syn*-conformation is maintained upon binding to the Ins(1,4,5)P_3R. The 8-bromo analogue also provides the versatility to produce potentially useful photoaffinity labels by either introducing an azido functionality directly at the 8-position or *via* an alkyl spacer. These compounds could be used for further investigation of Ins(1,4,5)P_3Rs.

Results and discussion

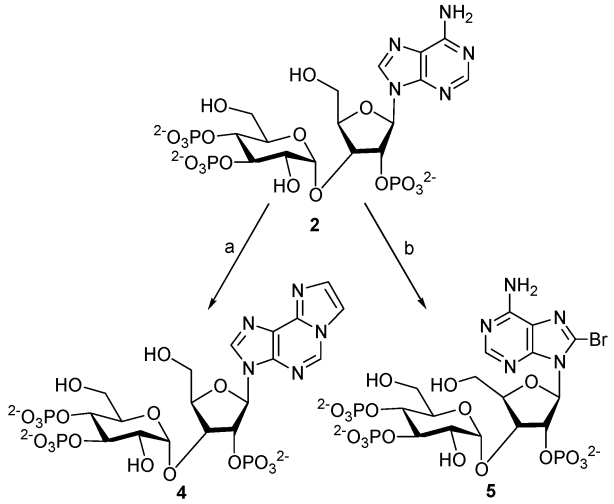
Synthesis

The synthesis of adenophostin A **2** and analogues **4** and **5** employs the 6-chloropurine building block **6** (Scheme 1), which we have previously shown to be a versatile intermediate. This has been reacted with a range of nucleophiles to produce a variety of N-6 base substituted adenophostin analogues[20] and is a convenient precursor for an improved route to adenophostin A. Treatment of **6** with ethanolic ammonia[38] gave the required 6-amino functionality with concomitant removal of the acetate protecting groups to afford **7** in 92% yield.



Scheme 1 *Reagents and conditions*: (a) EtOH, NH_3, 60 °C, 4 days; (b) i) $(BnO)_2PN^iPr_2$, imidazolium triflate, DCM, RT, 1.5 h; ii) mCPBA, −78 °C, 10 min; (c) moist 20 % $Pd(OH)_2/C$, cyclohexene–MeOH–H_2O, 80 °C, 3 h.

The introduction of the protected phosphates was achieved using freshly prepared imidazolium triflate and bis(benzyloxy)(diisopropylamino)phosphine.[16,39] Oxidation of the trisphosphite with *m*CPBA furnished, after purification, the fully protected adenophostin intermediate **8** in 53% yield. Deprotection was achieved using catalytic transfer hydrogenation[16,40] with the product being purified by ion exchange chromatography to give **2** in 75% yield. ^{1}H, ^{13}C, ^{31}P NMR and FAB mass spectral analysis were concurrent with those previously reported.[16,32,41] Having synthesised adenophostin A, our attention now turned to its modification to both the etheno **4** and 8-bromo **5** analogues (Scheme 2).



Scheme 2 *Reagents and conditions*: (a) chloroacetaldehyde (50% aq.), NaOAc, 2 days; (b) 0.5 M NaOAc, Br_2, 60 h.

The synthesis of etheno nucleotides and nucleosides has been accomplished numerous times using slight modifications of a general procedure.[36,37,42–45] Thus, adenophostin A was dissolved in a 50% aqueous solution of chloroacetaldehyde (pH 4.0–4.5) and was stirred for 48 h at room temperature. Purification using an MP1 AG ion exchange column gave the fluorescent etheno adenophostin analogue **4** showing characteristic doublets for H-10 and H-11 in the ^{1}H NMR spectrum at 8.22 and 8.87 ppm.

Like the etheno derivatives, a number of groups have previously synthesised 8-bromo analogues of nucleotides and nucleosides[46–50] with the critical point being the need for a pH of between 4.0–4.5 for the reaction to proceed efficiently. This can be achieved using a sodium acetate buffer. In our case, an optimum concentration of 0.5 M sodium acetate was found to be sufficient, with the pH changing by only *ca*. 0.2 units over the course of the reaction. Adenophostin A was dissolved in 0.5 M sodium acetate buffer and a solution of bromine in buffer was slowly added. After stirring for 60 h, purification of the reaction mixture was achieved using an MP1 AG ion exchange column eluting with 150 mM TFA to give the desired 8-bromo analogue showing only one singlet in the ^{1}H NMR spectrum at 8.01 ppm representing H-2. The purity of analogues **4** and **5** was determined by HPLC showing >96% and >99% purity, respectively.

Conformational analysis in solution

Following the procedure of Hotoda *et al.*,[33] the ^{1}H and NOESY spectra of compounds **2**, **4** and **5** were recorded in 50 mM phosphate buffer (p^2H 6.8) at 25 °C in an attempt to explore their conformation in solution. The results obtained for adenophostin

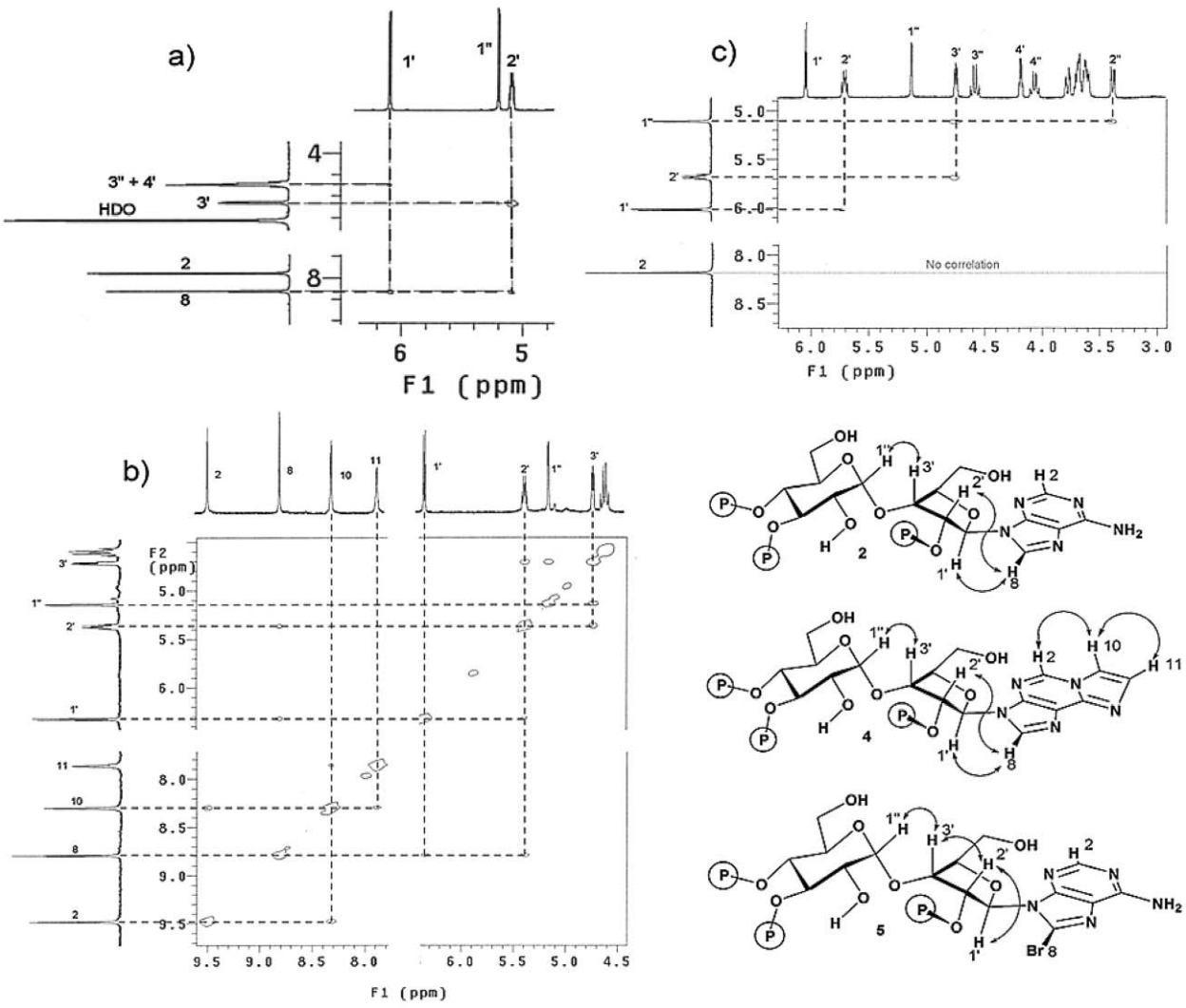


Fig. 4 NOESY spectra of (a) synthetic adenophostin A[a] **2**; (b) etheno adenophostin **4**[b]; (c) 8-bromo adenophostin **5**[b]. The structures of each compound are shown on the bottom right with the important NOE correlations highlighted. [a] Spectrum recorded in 50 mM phosphate buffer in D_2O (p^2H 6.8) at 600 MHz with a mixing time of 800 ms at 298 K. [b] Spectra recorded in DMSO-d_6 at 400 MHz with a mixing time of 800 ms at 298 K.

Table 1 Selected coupling constants of adenophostin A **2**, and analogues **4** and **5** and their calculated C-2′ *endo* percentage[a].

	$J_{1',2'}$ (Hz)	$J_{3',4'}$ (Hz)	[C-2′ endo]% $J_{1',2'}/(J_{1',2'}+J_{3',4'})$	$J_{1'',2''}$ (Hz)	$J_{2'',3''}$ (Hz)	$J_{3'',4''}$ (Hz)	$J_{4'',5''}$ (Hz)
2	6.2	3.3	65%	3.7	9.5	9.5	9.5
4	5.7	3.5	62%	3.2	9.1	9.1	9.1
5	7.4	5.3	58%	3.8	9.4	9.4	9.4

[a] All values were recorded in 50 mM phosphate buffer in D_2O (p^2H 6.8) at 400 MHz.

A (Table 1) paralleled those of Hotoda,[33] confirming: a chair conformation of the glucose ring with 2 equatorial phosphates; the close proximity of H-1″ to H-3′ as determined by NOE correlation; a preference for the C-2′-*endo* conformation of the ribose ring and a *syn*-orientation of the adenine moiety as determined from NOE correlations between H-8 and both H-1′ and H-2′ (Fig. 4a).

In order to model adenophostin A in solution, its structure (based on ^1H and NOESY NMR data) was surrounded by a "layer" of water molecules and energy minimisation calculations were performed to determine the lowest energy conformation. The model obtained (Fig. 5a) supports the data from the ^1H NMR experiments, showing a chair conformation of the glucose ring with 2 equatorial phosphates linked to a C-2′ *endo* puckered ribose moiety. This is the major difference to the Hotoda *in vacuo* study which found the C-3′ *endo* conformation in their lowest energy structure (although they state that the C-2′ *endo* conformation is more likely). The NOESY experimental data is also supported, with the H-1″ to H-3′ distance being 2.37 Å, suitably close for a strong NOE correlation. The torsional angle about the N-glycosidic bond (χ†) is 6.4°, showing a *syn*-conformation of the adenine moiety which is required for the NOE correlations between H-8 and H-1′ and H-2′. Although there is a difference in χ between our model and Hotoda's (56°), both show the expected *syn*-conformation of the adenine moiety. This is observed because the energy barriers preventing rotation around the N-glycosidic bond are not high when compounds are in the C-2′ *endo* conformation.[51]

Etheno adenophostin **4** gave markedly similar results to adenophostin A (Table 1), with the ribose moiety showing a preference for the C-2′ *endo* conformer as determined by the Davies and Danyluk equation.[52] The NOESY spectrum (see Fig. 4b) shows correlations between H-8 of the base with H-1′ and H-2′ of the ribose moiety suggesting a *syn* conformation in solution, similar to that of adenophostin A. Further NOE's show the expected interactions of H-10 with both H-11 and H-2

† The torsional angle about the N-glycosidic bond (C1′–N) is the angle between the C1′–O bond and the N9–C4 bond. Therefore, when $\chi = 0°$ the O–C1′ bond is eclipsed by the N9–C4 bond. When $\chi = 0 \pm 90°$ the base is said to be in the *syn* conformation with the *anti* conformation being $\chi = 180 \pm 90°$.

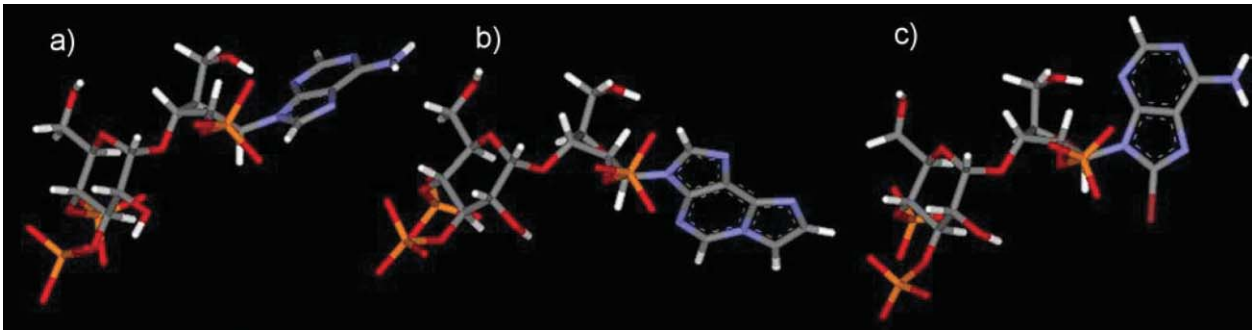


Fig. 5 Minimum energy solution phase conformations of (a) adenophostin A **2**; (b) etheno adenophostin **4**; (c) 8-bromo adenophostin **5**. For ease of viewing the water molecules have been removed.

and also the correlation between H-3′ and H-1″. The proton–proton coupling constants of the glucose ring confirm the presence of a chair conformation with two equatorial phosphate groups. Modelling studies were performed in the same manner as described previously for adenophostin A, resulting in the low energy structure seen in Fig. 5b. The carbohydrate backbone of the molecule remains essentially unchanged; however, there is a substantial change in χ from 6.4 to −76.0° probably resulting from the increase in electron density of the aromatic system being repelled by the C-2′ phosphate. Despite this large change, the adenine moiety remains in the *syn*-conformation which would allow the observed NOE correlations of H-8 with H-1′ and H-2′.

8-Bromo adenophostin (**5**), not unexpectedly, follows the same pattern as adenophostin A (**2**) and etheno adenophostin (**4**) (Table 1), assuming the same conformation in terms of glucose and ribose moieties. The NOESY spectrum (Fig. 4c) shows no visible correlations between H-2 and any other protons suggesting the same *syn*-conformation of the base moiety. Modelling of the 8-bromo analogue (Fig. 5c) confirms the retention of conformation within the glucose and ribose moieties, as expected from NMR data, but again a change in χ is observed from 6.4° in **2** to 63.2° in **5**. This rotation about the N-glycosidic linkage is caused by steric clashes between the bulky bromine atom and the ribose moiety making the *syn*-conformation more energetically favourable. Similar observations were made in the crystal structure of 8-bromo adenosine.[53]

Biological evaluation

Compounds **4** and **5** were evaluated in DT40 cells, expressing recombinant Type 1 rat Ins(1,4,5)P_3R, for their ability to release Ca^{2+} from intracellular stores (Table 2). The results for Ins(1,4,5)P_3 (**1**) and adenophostin A (**2**), synthesised by this present route, are also shown and all values are shown relative to adenophostin A measured in parallel experiments for a more accurate comparison.

The EC_{50} value of adenophostin A was *ca.* 10 times lower than that of Ins(1,4,5)P_3 (EC_{50} 24.8 and 2.1 nM, respectively), consistent with previous results and confirming its higher potency. The drop in potency of the etheno analogue **4** was modest, its EC_{50} being 65% that of adenophostin A. Interestingly, the 8-bromo compound **5** was still half as potent as adenophostin A (**2**), with a 4 fold increase in potency over Ins(1,4,5)P_3 (**1**).

Despite the *syn*-conformation of adenophostin A in solution, our proposed model for its binding to the Ins(1,4,5)P_3R[20] places the adenine moiety of adenophostin in the *anti*-conformation allowing the possibility of a cation–π interaction between the base and the guanidinium sidechain of Arg 504 (Fig. 6a).

Cation–π interactions have been observed numerous times,[54–56] with calculations predicting that the interaction may

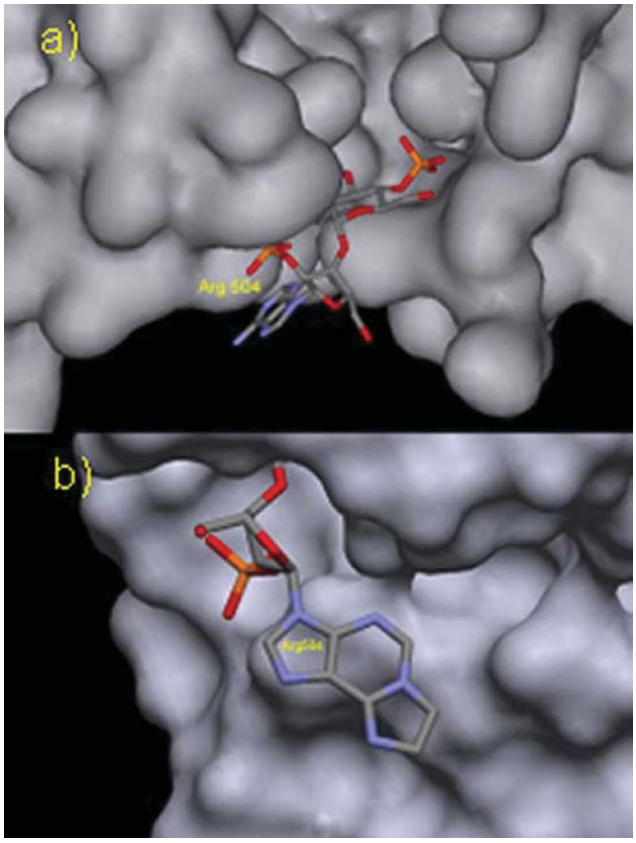


Fig. 6 Predicted binding modes of (a) adenophostin A and (b) etheno adenophostin with the Ins(1,4,5)P_3R[20]. The adenine moiety (or equivalent) is in the *anti*-conformation showing a possible cation–π interaction with Arg 504. Adapted from ref. 20

Table 2 $^{45}Ca^{2+}$ Release data for **1**, **2** **4** and **5** from DT40 cells[a]

	EC_{50}/nM	max. Response/%	*h*	EC_{50} with respect to **2**[b]	*n*
1	24.8 ± 2.1	78 ± 2	1.21 ± 0.06	0.09 ± 0.01	11
2	2.1 ± 0.6	76 ± 1	1.54 ± 0.13	1.0	12
4	4.0 ± 0.6	80 ± 1	1.69 ± 0.16	0.65 ± 0.05	3
5	5.4 ± 0.6	79 ± 3	1.93 ± 0.11	0.49 ± 0.05	4

[a] Results (means ± SEM of *n* independent experiments) show the concentration of each compound causing half the maximal response (EC_{50}), the Hill coefficient (*h*) and the maximal response (% of intracellular Ca^{2+} stores released) for each of the four compounds. [b] Results are expressed relative to the effect of **2** in parallel, with values lower than 1 denoting compounds of lower potency than **2**.

be stronger than a typical salt bridge in an exposed aqueous environment[57] such as we see in the Ins(1,4,5)P_3R. The crystal structures of the $NADP^+$-dependent aldehyde dehydrogenase,[58] tropinone reductase I[59] and 6-phosphogluconate dehydrogenase[60] are of particular interest due to the structural similarities of parts of the cofactor for these enzymes and adenophostin A. All three examples show interactions of an arginine residue with both the adenine moiety (cation–π) and the 2′-phosphate group (from $NADP^+$ for the first two and 2′-AMP for the last), corresponding well with our current model. Earlier work on ribophostin,[61] an adenophostin analogue without the base, had shown that the adenine moiety (or equivalent) is essential for high potency with the ribophostin EC_{50} for Ca^{2+} release increasing compared to that of Ins(1,4,5)P_3. This suggests that a synergistic effect of the 2′-phosphate group and the adenine moiety interacting with Arg 504, combined with H-bonding at N-3 from Arg 269, may be the reason for the 10-fold increase in potency of adenophostin over Ins(1,4,5)P_3. The energy gain from these strong interactions should be more than sufficient to overcome the weak energy barriers imposed upon interconversion from a *syn*- to *anti*-conformation.[51]

The slight decrease in potency of etheno adenophostin **4** relative to **2** may be caused by the increase in electron density of the base repelling the C-2′ phosphate therefore disrupting its optimal orientation in the receptor. A more likely explanation is that the additional ring of the etheno analogue disrupts the electrostatic potential of the base moiety which, in turn, lowers the energy of the cation–π interaction. Studies concerning these interactions have shown that aromatics with areas of high electrostatic potential at their centre form the strongest cation–π interactions and that electron rich heterocycles are quite poor cation–π binders.[62,63] However, docking experiments have shown a virtually identical orientation of the etheno analogue (Fig. 6b) when compared to adenophostin A which would allow cation–π interactions with Arg 504. Fig. 6b illustrates the space available around the proposed adenine binding pocket for occupation by modified bases, such as etheno adenine.

The lower activity of 8-bromo adenophostin **5** relative to **2** was not expected based on Hotoda's study[33] but corresponds well with our model. However, an EC_{50} value higher than Ins(1,4,5)P_3 (**1**) was surprising due to the requirement of an *anti*-conformation for high affinity binding and the steric restraints imposed to prevent this. This suggests that the ligand–receptor complex could be of sufficiently low energy to overcome the steric repulsion in the transition between the *syn*- and *anti*-conformations. The energy required to overcome this transitional barrier leads to a loss in potency, with **5** being approximately half as active as adenophostin A. Similar findings have been observed for ligand–receptor complexes of 8-Br$ADP^{64,65}$ and 8-BrNAD^+,[65] both ligands binding as the *anti*-conformer despite being in the *syn*-conformation when unbound. Interestingly, protein bound adenosine derivatives binding in the *syn*-conformation have been found, however, there are only a few reported cases.[66–68]

Fluorescence properties

The fluorescence emission of etheno adenophostin in 0.1M HEPES buffer at varing pH is shown in Fig. 7. The fluorescence emission is greatest at pH 7.0, forming a single unresolved band with a maximum at 412 nm, indicating that etheno adenophostin could be a valuable tool for the investigation of Ins(1,4,5)P_3Rs at physiological pH.

Conclusions

We have synthesised two adenophostin analogues **4** and **5**, modified at the adenine moiety. Etheno adenophostin **4** is a high affinity fluorescent probe which could be used at physiological pH for the investigation of Ins(1,4,5)P_3Rs. 8-Bromo adenophostin **5** has

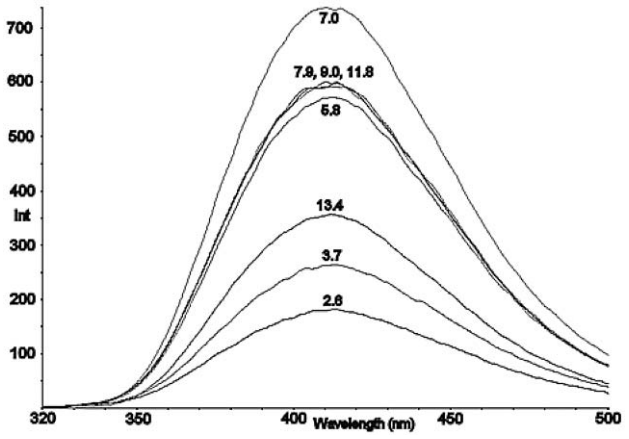


Fig. 7 Variation in fluorescence emission intensity of etheno adenophostin **4** with pH in 0.1 M Hepes buffer. Excitation at 296 nm.

further applications in the synthesis of potential photoaffinity analogues and other 8-modified adenophostin analogues. We propose that two conformations of adenophostin A exist dependent on whether in solution or bound to the Ins(1,4,5)P_3R with NMR and modelling data supporting this. We suspect that the proposed cation–π interaction between the base moiety of adenophostin and its analogues with Arg 504 is at least partly responsible for their increased potency relative to Ins(1,4,5)P_3 and this model warrants further investigation.

Experimental

General

Chemicals were purchased from Acros, Aldrich, Sigma and Fluka. All anhydrous solvents were purchased from either Aldrich or Fluka. RPMI 1640 medium, L-glutamine, 2-mercaptoethanol and G-418 were from Invitrogen, sera were from Sigma and Mag-fluo-4AM was from Molecular Probes. TLC was performed on precoated plates (Merck aluminum sheets silica 60 F_{254}, Art. No. 5554). Products were visualised under UV light and by dipping into phosphomolybdic acid in MeOH followed by heating or by dipping into anisaldehyde in EtOH followed by heating. Radial-band chromatography (RBC) was performed using a Chromatotron (7924T, TC Research, UK) with Adsorbosil Plus-P (6–15 μm) (Alltech) as the adsorbent. Ion exchange chromatography was performed on an LKB-Pharmacia medium pressure ion-exchange chromatograph using MP1 AG ion-exchange resin and a gradient of 0–100% 150 mM TFA as eluent. ^{1}H NMR, NOESY and ^{13}C spectra were recorded on JEOL JMN GX-270, EX-400 or Varian Inova-600 NMR spectrometers. ^{31}P NMR spectra were recorded on JEOL JMN GX-270 or EX-400 NMR spectrometers and chemical shifts were measured in ppm and denoted positive downfield from external 85% H_3PO_4. All coupling constants are quoted in Hz. Melting points were determined using a Reichert-Jung Therm Galen Kofler block and are uncorrected. Low resolution mass spectra were recorded at the University of Bath Mass Spectrometry Service using +ve and −ve fast atom bombardment (FAB) with *m*-nitrobenzyl alcohol as the matrix. High resolution accurate mass spectra were recorded at the University of Bath Mass Spectrometry Service. Optical rotations were measured at ambient temperature using an Optical Activity Ltd AA-10 polarimeter in a cell volume of 1 cm^3 or 5 cm^3 and specific rotation are given in 10^{-1} deg cm^3 g^{-1}. Fluorescence measurements were made on a Perkin Elmer LS 50B fluorimeter. UV spectra were recorded using a Perkin Elmer Lambda EZ201 spectrometer. HPLC analysis was carried out on a Hewlett-Packard series chromatograph with a strong anion-exchange resin (MP1 AG, column size 3 × 150 mm). A linear gradient of 0–50% 150 mM TFA was used as eluent at 1 cm^3 min^{-1}

over 60 min, with the UV detector set at 254 nm. Synthetic phosphates were assayed using an adaptation of the Briggs phosphate test.[69]

Molecular modelling experiments

Initially, force field modelling and stochastic conformational searching with MOE (2003.2) did not find global minimum structures that could easily be reconciled with the NOE and ^{1}H NMR data. *Ab initio* modelling with Gaussian 98-RevA.6, running on a SGI Octane2, IRIX 6.54 was attempted with HF/3–21G*, B3LYP/3–21G* and CPCM/B3LYP/6–31+G(d) but failed to find satisfactory minima structures with proton steric clashes in accordance with the NOE data. Modelling the phosphate anion moieties with SCRF continuum models may result in an energetic conflict between electrostatic repulsions between phosphate anion and the tendency of SCRF envelopes to minimise the volume of solvent cavities.

Favourable results were obtained when explicit water molecules were included. Using MOE, Linux RedHat10, the structures were drawn and dihedral angles adjusted to reflect the NOE and ^{1}H NMR data. A 6 Å spherical water envelope was then placed around the solutes with the water soak facility in MOE. The solutes were fixed and the water molecules minimised with MMFF94 with non bonding cut-off adjusted to 12 Å. Once complete, the structures were minimised using Mopac2002, implemented in Cache-Pro6.11 running windows98rev2, on a Desktop (Dell) PC without constraints. The semi-empirical method PM5 was used as implemented in Mopac2002. Extra keywords were used in Mopac2002 to enable reasonable convergence, PRECISE, LET DDMIN = 0.00 GEO-OK, GRAD, GNORM = 0.1. Cosmo solvation was not used and the clusters were simply treated as gas phase entities. Minimised solute structures are shown with explicit solvent molecules hidden.

Stable transfection of DT40 cells with rat Ins(1,4,5)P_3R1

The open reading frame of rat Ins(1,4,5)P_3R1 was amplified by PCR from the expression vector pCMVI-9-Ins(1,4,5)P_3R1[70] using the following primers: 5′–AGGAATTCG-CCACCATG-TCTGACAAAATG-3′ and 5′–CCGGTACCG-AATTCTTA-GGCTGGCTGCTGT-3′ and cloned as an EcoRI fragment into pcDNA3 (Invitrogen). The chicken β-actin hybrid promoter[71] was excised from the vector pAneo[72] and cloned in place of the CMV promoter upstream of the Ins(1,4,5)P_3R1 open reading frame to create the construct pcDNA3-Ins(1,4,5)P_3R1. DT40 cells in which the genes for all three endogenous Ins(1,4,5)P_3R subtypes have been deleted (DT40/InsP_3R–KO)[73] were stably transfected by electroporation with linearized pcDNA3-Ins(1,4,5)P_3R1 using a Gene Pulser apparatus (Bio-Rad Laboratories) at 330 V, 500 μF with 5 μg DNA/10^6 cells. Clonal isolation was carried out in the presence of 2 mg ml^{-1} G-418 and positive clones were amplified and screened for the presence of rat Ins(1,4,5)P_3R1 by western blotting using an anti-peptide antiserum[74] corresponding to the C-terminal 15-residues of rat Ins(1,4,5)P_3R1.

Cell culture

DT40/Ins(1,4,5)P_3R–KO cells stably expressing recombinant rat InsP_3R1 (DT40/Ins(1,4,5)P_3R1 cells) were cultured in suspension in RPMI 1640 medium supplemented with foetal bovine serum (10%), L-glutamine (2 mM), 2-mercaptoethanol (50 μM) and heat-inactivated chicken serum (1%). Cells were incubated in a humidified atmosphere (95% O_2; 5% CO_2 at 37 °C) and passaged every 2–3 days when they had reached a density of *ca.* 2 × 10^6 cells ml^{-1}.

Measurement of Ca^{2+} release from permeabilized cells

The effects of Ins(1,4,5)P_3 on intracellular Ca^{2+} stores were measured using a low-affinity Ca^{2+}-indicator trapped within the intracellular stores of permeabilized cells. DT40/Ins(1,4,5)P_3R1 cells were harvested by centrifugation (650 × *g*; 2 min) and re-suspended (2–3 × 10^7 cells ml^{-1}) in Hepes-buffered saline (HBS: 135 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 11.6 mM Hepes, 11.5 mM D-glucose, pH 7.3) supplemented with Mag-fluo-4AM (20 μM), Pluronic F-127 (0.05%) and bovine serum albumin (1 mg ml^{-1}). After 1 h at 20 °C in the dark, the Mag-fluo-4-loaded cells were harvested (650 × *g*; 2 min) and re-suspended (*ca.* 2 × 10^6 cells ml^{-1}) in Ca^{2+}-free cytosolic-like medium (CLM: 140 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM Pipes, pH 7.0). The cells were permeabilized by incubation with saponin (10 μg ml^{-1}, 4 min at 37 °C), harvested (650 × *g*; 2 min) and resuspended in Mg^{2+}-free CLM (140 mM KCl, 20 mM NaCl, 1 mM EGTA, 375 μM $CaCl_2$ (*ca.* 200 nM free [Ca^{2+}]), 20 mM Pipes, pH 7.0). The permeabilized cells (with Mag-fluo-4 trapped within the lumen of the ER) were then attached to 96-well plates (*ca.* 8 × 10^5 cells per well) coated with poly-L-lysine (0.01%) and centrifuged onto the plate (300 × *g*; 2 min). Immediately before an experiment, the cells were washed twice in Mg^{2+}-free CLM to remove cytosolic Mag-fluo-4 and the plates were then mounted in a Flexstation fluorescence plate reader (Molecular Devices, Sunnyvale, CA), which allows automated additions to the sample wells while recording fluorescence. Mag-fluo-4 fluorescence was monitored by excitation at 485 nm with emission detected at 520 nm. Active Ca^{2+} uptake into the ER was initiated by addition of Mg^{2+}-ATP (1.5 mM) and after 150 s, when the stores had loaded to a steady-state Ca^{2+} content, Ins(1,4,5)P_3 was added. The amount of Ca^{2+} released by Ins(1,4,5)P_3 was expressed as a fraction of the total Ca^{2+} content of the ER as assessed by addition of 1 μM ionomycin. Data are presented as means ± s.e. means from *n* independent experiments, each performed in triplicate.

Concentration–effect relationships were fitted to four-parameter logistic equations using non-linear curve-fitting procedures (GraphPad Prism, San Diego, CA).

2″,5′,6″-Tri-*O*-benzyl-3′-*O*-*α*-D-glucopyranosyl adenosine (7). A mixture of 2′,3″,4″-tri-*O*-acetyl-2″,5′,6″-tri-*O*-benzyl-3′-*O*-*α*-D-glucopyranosyl-6-chloro-9-β-D-ribofuranosidopurine **6** (44 mg, 52.1 μmol) in ethanolic ammonia (3 cm^3) was heated at 60 °C in a pressure tube for 72 h. The mixture was concentrated under reduced pressure and was purified by radial band chromatography (RBC) (ethyl acetate → ethyl acetate–methanol 14 : 1) to give the title compound **7** (33 mg, 92%). mp 93–97 °C (from ethanol) (lit.[16] mp 95–115 °C); $[\alpha]_D^{22}$ 5.7 (*c* = 1.06, $CHCl_3$) (lit.[16] $[\alpha]_D^{25}$ −2.3 (*c* 2.6, $CHCl_3$)); δ_H(270 MHz; $CDCl_3$; Me_4Si) 3.43 (1 H, dd, $J_{1'',2''}$ 3.5, $J_{2'',3''}$ 9.9, H-2″), 3.52–3.78 (5 H, m, H-5′$_A$, H-5′$_B$, H-4″, H-6″$_A$, H-6″$_B$), 3.86–3.96 (1 H, m, H-5″), 4.09 (1 H, t, $J_{2'',3''} = J_{3'',4''}$ 9.9, H-3″), 4.24–4.30 (1 H, m, H-3′), 4.35–4.40 (1 H, m, H-4′), 4.45–4.56 (4 H, m, 2 × OCH_2Ar), 4.59–4.83 (3 H, m, OCH_2Ar, H-2′), 4.90 (1H, d, $J_{1'',2''}$ 3.5, H-1″), 6.10 (1 H, d, $J_{1',2'}$ 6.7, H-1′), 6.34 (2 H, s, NH_2), 7.21–7.39 (15 H, m, ArH), 8.05, 8.26 (2 H, 2 s, H-2, H-8); δ_C(100.5 MHz; $CDCl_3$; Me_4Si) 69.85 (C-6″), 70.19 (C-5′ and C-4″), 72.58 (C-5″), 72.98 (C-3″), 73.90, 73.93, 74.30 (3 × OCH_2Ar), 75.93 (C-2′), 79.64 (C-2″), 80.28 (C-3′), 83.21 (C-4′), 87.97 (C-1′), 99.77 (C-1″), 119.47 (C-5), 127.84, 127.97, 128.17, 128.54, 128.61, 128.78, 128.83, 128.96 (Ar*C*H), 137.04, 137.65, 138.17 (3 × *ipso*-C of Bn ring), 139.04 (C-8), 149.62 (C-4), 152.90 (C-2), 155.68 (C-6); *m*/*z* (FAB) 700.1 [(M + H)$^+$, 70%]; (mass calcd for $C_{37}H_{42}N_5O_9$ (M + H)$^+$, 700.29826; found 700.29749.

2″,5′,6″-Tri-*O*-benzyl-2′,3″,4″-tris(dibenzyloxyphosphoryl)-3′-*O*-*α*-D-glucopyranosyl adenosine (8). A mixture of the triol **7** (382 mg, 0.55 mmol), bis(benzyloxy) (diisopropylamino)-phosphine (631 mg, 1.83 mmol) and imidazolium triflate[39] (393 mg, 1.82 mmol) in anhydrous DCM (4 cm^3) was stirred at room temperature under N_2 for 90 min. TLC (ethyl acetate–hexane, 7 : 3) indicated the complete conversion to the trisphosphite so water (2 drops) was added and the reaction was cooled to −78 °C. *m*CPBA (331 mg, 1.93 mmol) was

added, followed after 10 min by 10% aq. Na_2SO_3 (10 cm^3) and ethyl acetate (20 cm^3). The reaction was allowed to attain room temperature with the organic phase being washed consecutively with sat. $NaHCO_3$ (20 cm^3) and brine (20 cm^3), dried ($MgSO_4$) and concentrated under reduced pressure. Purification by RBC ($CHCl_3$-acetone 4 : 1 → 6:4) furnished the fully protected trisphosphate **8** (431 mg, 53%). δ_H(270 MHz; $CDCl_3$; Me_4Si) 3.50–3.70 (5 H, m, H-5′$_A$, H-5′$_B$, H-2″, H-6″$_A$, H-6″$_B$), 3.79–3.88 (1 H, m, H-5″), 4.12 (1 H, AB, J_{AB} 7.3, 0.5 × OCH_2Ar) 4.26–4.86 (14 H, m, 5.5 × OCH_2Ar, H-3′, H-4′, H-4″), 4.87–5.06 (8 H, m, 3.5 ×; OCH_2Ar, H-3″), 5.32 (1H, d, $J_{1'',2''}$ 3.7, H-1″), 5.56–5.66 (1 H, m, H-2′), 5.70 (2 H, br s, NH_2), 6.34 (1 H, d, $J_{1',2'}$ 6.6, H-1′), 6.94–7.41 (45 H, m, ArH), 7.90, 8.25 (2 H, 2 s, H-8, H-2); δ_C(100.5 MHz; $CDCl_3$; Me_4Si) 68.64 (C-6″), 69.44–70.41 (C-5′, 6 × POCH_2Ar with C–P coupling), 70.16 (C-5″), 71.91, 73.66, 73.90 (3 × OCH_2Ar), 74.10 (C-4″), 74.68 (C-3′), 77.04 (C-2″), 77.55 (C-2′ with C–P coupling), 78.39 (C-3″ with C–P coupling), 82.72 (C-4′), 85.89 (C-1′), 95.67 (C-1″), 119.98 (C-5), 127.15, 127.81, 127.89, 127.93, 127.96, 128.04, 128.16, 128.56, 128.49, 128.51, 128.62, 128.65, 128.69, 128.74, 128.80 (ArCH), 135.23–136.37 (6 × *ipso*-C of benzylphospho ring with C–P coupling) 137.52, 137.77, 138.18 (3 × *ipso*-C of Bn ring), 139.64 (C-8), 150.25 (C-4), 153.11 (C-2), 155.33 (C-6); δ_P(161.8 MHz; $CDCl_3$; ^1H decoupled) −0.13, −0.86, −0.01 (3 s); *m/z* (FAB) 1480.4 [$(M + H)^+$, 90%]; mass calcd for $C_{79}H_{81}N_5O_{18}P_3$ $(M + H)^+$, 1480.47895; found 1480.47537.

3′-*O*-α-D-Glucopyranosyl adenosine 2′,3″,4″-trisphosphate (adenophostin A) (2). A mixture of **8** (256 mg, 0.17 mmol) and moist 20% $Pd(OH)_2$ on carbon (*ca.* 300 mg) in methanol (10.5 cm^3), cyclohexene (5.5 cm^3) and milliQ water (0.75 cm^3) was heated at 80 °C for 4 h. The mixture was passed through a membrane filter to remove the catalyst washing well with MeOH and water. The filtrate was concentrated under reduced pressure with the resulting residue being purified on an MP1 AG ion-exchange column eluting with 0–100% 150 mM TFA. The relevant fractions (elution between 55–85%) were concentrated to give adenophostin A **2** (105 mg, 90%) as the free acid. δ_H(270 MHz; D_2O) 3.59–3.77 (6 H, m, H-2″, H-5″, H-5′$_A$, H-5′$_B$, H-6″$_A$, H-6″$_B$), 3.99 (1 H, q, $J_{3'',4''} = J_{4'',5''}$ 9.5, H-4″), 4.28 (1 H, m, $J_{3',4'}$ 3.3, H-4′), 4.39 (1 H, q, $J_{2'',3''} = J_{3'',4''}$ 9.5, H-3″) 4.50 (1 H, m, H-3′), 5.13 (1 H, d, J = 3.7 Hz, H-1″), 5.16 (1 H, m, H-2′), 6.20 (1 H, d, $J_{1',2'}$ 6.2, H-1′), 8.25, 8.35 (2 H, 2 s, H-8, H-2); δ_C(150.8 MHz; D_2O) 60.25 (C-6″), 61.10 (C-5′), 70.50 (C-2″), 71.61 (C-5″), 73.13 (C-4″), 73.99 (C-3′), 75.94 (C-2′ with C–P coupling), 78.14 (C-3″ with C–P coupling), 84.56 (C-4′), 87.24 (C-1′), 98.30 (C-1″), 119.08 (C-5), 143.50 (C-8), 144.62 (C-2), 148.39 (C-4), 150.03 (C-6); δ_P(161.8 MHz; D_2O; ^1H decoupled) 0.25, 0.81, 1.17 (3 s); *m/z* (FAB) 668.1 [$(M–H)^-$, 100%]; mass calcd for $C_{16}H_{27}N_5O_{18}P_3$ $(M + H)^+$, 670.05585; found 670.05635.

***N*-2,3-Etheno-3′-*O*-α-D-glucopyranosyl adenosine 2′, 3″,4″-trisphosphate (4).** To a solution of **2** (11 mg, 16.4 μmol) in 50% aq. chloroacetaldehyde (1 cm^3) was added NaOAc until pH 4–4.5 was attained. The solution was stirred at 37 °C for 48 h and was washed with ethyl acetate (3 × 25 cm^3) and the aqueous layer was concentrated under reduced pressure. Purification using an MP1 AG ion-exchange column eluting with 0–100% 150 mM TFA (elution between 40–70%) gave the desired compound **4** (4.7 mg, 43%). Fluorescence (pH 7) (excitation) 296 nm, (emission) 412 nm; UV (H_2O)/nm λ_{max} 220 and 272; δ_H(270 MHz; D_2O) 3.68–3.94 (6 H, m, H-2″, H-5″, H-5′$_A$, H-5′$_B$, H-6″$_A$, H-6″$_B$), 4.11 (1 H, q, $J_{3'',4''} = J_{4'',5''}$ 9.1, H-4″), 4.43 (1 H, m, H-4′), 4.50 (1 H, q, $J_{2'',3''} = J_{3'',4''}$ 9.1, H-3″) 4.67 (1 H, m, $J_{3',4'}$ 3.3, H-3′), 5.24 (1 H, d, $J_{1'',2''}$ 3.2, H-1″), 5.39 (1 H, m, H-2′), 6.48 (1 H, d, $J_{1',2'}$ 5.7, H-1′), 8.22, 8.87 (2 H, 2 d, $J_{10,11}$ 2.2, H-10, H-11), 8.68, 9.35 (2 H, 2 s, H-8, H-2); δ_C(100.5 MHz; D_2O) 60.27 (C-6″), 61.07 (C-5′), 70.60 (C-2″), 71.70 (C-5″), 72.92 (C-4″), 73.83 (C-3′), 75.59 (C-2′ with C–P coupling), 77.88 (C-3″ with C–P coupling), 84.40 (C-4′), 87.67 (C-1′), 98.15 (C-1″), 114.38 (C-10). 119.08 (C-5), 121.88 (C-11), 137.76 (C-8, C-2), 143.05 (C-4), 144.59 (C-6); δ_P(109.2 MHz; D_2O; ^1H decoupled) −0.17, 0.39, 0.77 (3 s); *m/z* (FAB) 694.0 [$(M + H)^+$, 75%]; mass calcd for $C_{18}H_{27}N_5O_{18}P_3$ $(M + H)^+$, 694.05640; found 694.05368.

8-Bromo-3′-*O*-α-D-glucopyranosyl adenosine 2′, 3″,4″-trisphosphate (5). To a stirred solution of adenophostin A **2** (22 mg, 32.9 μmol) in 0.5 M sodium acetate buffer (1 cm^3, pH 4.3) was added a solution of Br_2 (23 mg, 0.14 mmol) in 0.5 M sodium acetate buffer (2 cm^3, pH 4.3) dropwise. The reaction mixture was stirred in the dark at room temperature for 60 h monitoring the pH occasionally and adding additional buffer if necessary. The solution was partitioned between $CHCl_3$ (5 cm^3) and water with the aqueous layer being treated with solid $NaHSO_3$ to remove excess Br_2. The aqueous layer was further extracted with $CHCl_3$ (3 × 5 cm^3) and concentrated under reduced pressure. Purification using an MP1 AG ion-exchange column eluting with 0–100% 150 mM TFA (elution between 40–70%) gave the desired compound **5** (9.6 mg, 41%). δ_H(400 MHz; D_2O) 3.58–3.78 (6 H, m, H-2″, H-5″, H-5′$_A$, H-5′$_B$, H-6″$_A$, H-6″$_B$), 3.83 (1 H, q, $J_{3'',4''} = J_{4'',5''}$ 9.4, H-4″), 4.32 (2 H, m, H-4′, H-3″), 4.53 (1 H, m, $J_{3',4'}$ 5.3, H-3′), 5.26 (1 H, d, $J_{1'',2''}$ 3.5, H-1″), 5.30 (1 H, m, H-2′), 6.16 (1 H, d, $J_{1',2'}$ 7.4, H-1′), 8.01 (1 H, s, H-2); δ_C(150.8 MHz; D_2O) 60.41 (C-6″), 62.00 (C-5′), 71.32 (C-2″), 72.16 (C-4″) 72.30 (C-5″), 73.67 (C-3′), 74.37 (C-2′ with C–P coupling), 77.187 (C-3″ with C–P coupling), 85.56 (C-4′), 89.46 (C-1′), 98.12 (C-1″), 119.89 (C-5), 128.86 (C-8), 149.75 (C-4), 152.39 (C-2), 154.68 (C-6); δ_P(161.8 MHz; D_2O; ^1H decoupled) 3.67 and 2.54 (2 × brs integrating as 1 : 2 respectively); *m/z* (FAB) 746.0, 748.0 [$(M–H)^-$, 45%]; mass calcd for $C_{16}H_{24}{}^{79}BrN_5O_{18}P_3$ $(M–H)^-$, 745.95126 and $C_{16}H_{24}{}^{81}BrN_5O_{18}P_3$ $(M–H)^-$, 747.94922; found 745.94998 and 747.94975.

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References

1 M. J. Berridge, *Nature*, 1993, **361**, 315–325.
2 M. J. Berridge, M. D. Bootman and P. Lipp, *Nature*, 1998, **395**, 645–648.
3 B. V. L. Potter and D. Lampe, *Angew. Chem., Int. Ed. Engl.*, 1995, **34**, 1933–1972.
4 A. M. Riley, A. J. Laude, C. W. Taylor and B. V. L. Potter, *Bioconjugate Chem.*, 2004, **15**, 278–289.
5 M. Takahashi, T. Kagasaki, T. Hosoya and S. Takahashi, *J. Antibiot.*, 1993, **46**, 1643–1647.
6 M. Takahashi, K. Tanzawa and S. Takahashi, *J. Biol. Chem.*, 1994, **269**, 369–372.
7 S. Takahashi, T. Kinoshita and M. Takahashi, *J. Antibiot.*, 1994, **47**, 95–100.
8 V. Correa, A. M. Riley, S. Shuto, G. Horne, E. P. Nerou, R. D. Marwood, B. V. L. Potter and C. W. Taylor, *Mol. Pharmacol.*, 2001, **59**, 1206–1215.
9 S. Shuto, K. Tatani, Y. Ueno and A. Matsuda, *J. Org. Chem.*, 1998, **63**, 8815–8824.
10 R. D. Marwood, S. Shuto, D. J. Jenkins and B. V. L. Potter, *Chem. Commun.*, 2000, 219–220.
11 J. Hirota, T. Michikawa, A. Miyawaki, M. Takahashi, K. Tanzawa, I. Okura, T. Furuichi and K. Mikoshiba, *FEBS Lett.*, 1995, **368**, 248–252.
12 L. Missiaen, J. B. Parys, I. Sienaert, K. Maes, K. Kunzelmann, M. Takahashi, K. Tanzawa and H. De Smedt, *J. Biol. Chem.*, 1998, **273**, 8983–8986.
13 S. A. Morris, E. P. Nerou, A. M. Riley, B. V. L. Potter and C. W. Taylor, *Biochem. J.*, 2002, **367**, 113–120.
14 R. D. Marwood, A. M. Riley, V. Correa, C. W. Taylor and B. V. L. Potter, *Bioorg. Med. Chem. Lett.*, 1999, **9**, 453–458.
15 R. D. Marwood, D. J. Jenkins, V. Correa, C. W. Taylor and B. V. L. Potter, *J. Med. Chem.*, 2000, **43**, 4278–4287.

16 R. D. Marwood, A. M. Riley, D. J. Jenkins and B. V. L. Potter, *J. Chem. Soc., Perkin Trans. 1*, 2000, 1935–1947.
17 R. D. Marwood, V. Correa, C. W. Taylor and B. V. L. Potter, *Tetrahedron: Asymmetry*, 2000, **11**, 397–403.
18 H. J. Rosenberg, A. M. Riley, V. Correa, C. W. Taylor and B. V. L. Potter, *Carbohydr. Res.*, 2000, **329**, 7–16.
19 H. J. Rosenberg, A. M. Riley, R. D. Marwood, V. Correa, C. W. Taylor and B. V. L. Potter, *Carbohydr. Res.*, 2001, **332**, 53–66.
20 H. J. Rosenberg, A. M. Riley, A. J. Laude, C. W. Taylor and B. V. L. Potter, *J. Med. Chem.*, 2003, **46**, 4860–4871.
21 A. M. Riley and B. V. L. Potter, *Tetrahedron Lett.*, 1999, **40**, 2213–2216.
22 A. M. Riley, V. Correa, M. F. Mahon, C. W. Taylor and B. V. L. Potter, *J. Med. Chem.*, 2001, **44**, 2108–2117.
23 H. Abe, S. Shuto and A. Matsuda, *J. Org. Chem.*, 2000, **65**, 4315–4325.
24 S. Shuto, G. Horne, R. D. Marwood and B. V. L. Potter, *Chem. Eur. J.*, 2001, **7**, 4937–4946.
25 M. de Kort, A. D. Regenbogen, H. S. Overkleeft, R. A. John Challiss, Y. Iwata, S. Miyamoto, G. A. van der Marel and J. H. van Boom, *Tetrahedron*, 2000, **56**, 5915–5928.
26 M. de Kort, V. Correa, A. R. Valentijn, G. A. van der Marel, B. V. L. Potter, C. W. Taylor and J. H. van Boom, *J. Med. Chem.*, 2000, **43**, 3295–3303.
27 M. De Kort, A. R. Valentijn, G. A. Van der Marel and J. H. Van Boom, *Tetrahedron Lett.*, 1997, **38**, 7629–7632.
28 M. De Kort, J. Luijendijk, G. A. Van der Marel and J. H. Van Boom, *Eur. J. Org. Chem.*, 2000, 3085–3092.
29 N. C. R. Van Straten, G. A. Van Der Marel and J. H. Van Boom, *Tetrahedron Lett.*, 1996, **37**, 3599–3602.
30 N. C. R. Van Straten, N. M. A. J. Kriek, Z. A. C. Cziria, G. A. Van Der Marel and J. H. Van Boom, *Tetrahedron*, 1997, **53**, 6539–6554.
31 N. C. R. Van Straten, G. A. Van Der Marel and J. H. Van Boom, *Tetrahedron*, 1997, **53**, 6523–6538.
32 N. C. R. Van Straten, G. A. Van Der Marel and J. H. Van Boom, *Tetrahedron*, 1997, **53**, 6509–6522.
33 H. Hotoda, K. Murayama, S. Miyamoto, Y. Iwata, M. Takahashi, Y. Kawase, K. Tanzawa and M. Kaneko, *Biochemistry*, 1999, **38**, 9234–9241.
34 C. T. Murphy, A. M. Riley, C. J. Lindley, D. J. Jenkins, J. Westwick and B. V. L. Potter, *Mol. Pharmacol.*, 1997, **52**, 741–748.
35 I. Bosanac, J. R. Alattia, T. K. Mal, J. Chan, S. Talarico, F. K. Tong, K. I. Tong, F. Yoshikawa, T. Furuichi, M. Iwai, T. Michikawa, K. Mikoshiba and M. Ikura, *Nature*, 2002, **420**, 696–700.
36 J. A. Secrist III, J. R. Barrio, N. J. Leonard and G. Weber, *Biochemistry*, 1972, **11**, 3499–3506.
37 J. R. Barrio, J. A. Secrist III and N. J. Leonard, *Proc. Natl. Acad. Sci. U. S. A.*, 1972, **69**, 2039–2042.
38 E. W. van Tilburg, J. V. Kunzel, M. de Groote and A. P. Ijzerman, *J. Med. Chem.*, 2002, **45**, 420–429.
39 Y. Hayakawa and M. Kataoka, *J. Am. Chem. Soc.*, 1998, **120**, 12395–12401.
40 S. Hanessian, T. J. Liak and B. Vanasse, *Synthesis-Stuttgart*, 1981, 396–397.
41 H. Hotoda, M. Takahashi, K. Tanzawa, T. Takahashi and M. Kaneko, *Tetrahedron Lett.*, 1995, **36**, 5037–5040.
42 J. R. Barrio, J. A. Secrist III and N. J. Leonard, *Biochem. Biophys. Res. Commun.*, 1972, **46**, 597–604.
43 J. A. Secrist III, J. R. Barrio and N. J. Leonard, *Science*, 1972, **175**, 646–647.
44 R. Khazanchi, P. L. Yu and F. Johnson, *J. Org. Chem.*, 1993, **58**, 2552–2556.
45 D. Flaherty, P. Balse, K. Li, B. M. Moore and M. B. Doughty, *Nucleosides Nucleotides*, 1995, **14**, 65–76.
46 B. E. Haley, *Biochemistry*, 1975, **14**, 3852–3857.
47 R. A. Long, R. K. Robins and L. B. Townsend, *J. Org. Chem.*, 1967, **32**, 2751–2756.
48 K. Muneyama, R. J. Bauer, D. A. Shuman, R. K. Robins and L. N. Simon, *Biochemistry*, 1971, **10**, 2390–2395.
49 M. Ikehara and S. Uesugi, *Chem. Pharm. Bull.*, 1969, **17**, 348–354.
50 W. A. Wlassoff, M. I. Dobrikov, I. V. Safronov, R. Y. Dudko, V. S. Bogachev, V. V. Kandaurova, G. V. Shishkin, G. M. Dymshits and O. I. Lavrik, *Bioconjugate Chem.*, 1995, **6**, 352–360.
51 A. E. V. Haschemeyer and A. Rich, *J. Med. Biol.*, 1967, **27**, 369–384.
52 D. B. Davies and S. S. Danyluk, *Biochemistry*, 13, pp. 4417–4434.
53 S. S. Tavale and H. M. Sobell, *J. Mol. Biol.*, 1970, **48**, 109–123.
54 L. Mao, Y. Wang, Y. Liu and X. Hu, *J. Am. Chem. Soc.*, 2003, **125**, 14216–14217.
55 L. Mao, Y. Wang, Y. Liu and X. Hu, *J. Mol. Biol.*, 2004, **336**, 787–807.
56 J. Rahuel, C. Garcia-Echeverria, P. Furet, A. Strauss, G. Caravatti, H. Fretz, J. Schoepfer and B. Gay, *J. Mol. Biol.*, 1998, **279**, 1013–1022.
57 N. Zacharias and D. A. Dougherty, *Trends Pharmacol. Sci.*, 2002, **23**, 281–287.
58 B. Ahvazi, R. Coulombe, M. Delarge, M. Vedadi, L. Zhang, E. Meighen and A. Vrielink, *Biochem. J.*, 2000, **349**, 853–861.
59 K. Nakajima, A. Yamashita, H. Akama, T. Nakatsu, H. Kato, T. Hashimoto, J. Oda and Y. Yamada, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 4876–4881.
60 M. J. Adams, G. H. Ellis, S. Gover, C. E. Naylor and C. Phillips, *Structure*, 1994, **2**, 651–668.
61 J. S. Marchant, M. D. Beecroft, A. M. Riley, D. J. Jenkins, R. D. Marwood, C. W. Taylor and B. V. L. Potter, *Biochemistry*, 1997, **36**, 12780–12790.
62 J. C. Ma and D. A. Dougherty, *Chem. Rev.*, 1997, **97**, 1303–1324.
63 S. Mecozzi, A. P. West Jr. and D. A. Dougherty, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 10566–10571.
64 M. A. Abdallah, J. F. Biellmann, B. Nordstroem and C. I. Braenden, *Eur. J. Biochem.*, 1975, **50**, 475–481.
65 K. W. Olsen, R. M. Garavito, M. N. Sabesan and M. G. Rossmann, *J. Mol. Biol.*, 1976, **107**, 571–576.
66 H. M. Ke, Y. P. Zhang and W. N. Lipscomb, *Proc. Natl. Acad. Sci. U. S. A.*, 1990, **87**, 5243–5247.
67 D. D. Leonidas, R. Shapiro, L. I. Irons, N. Russo and K. R. Acharya, *Biochemistry*, 1999, **38**, 10287–10297.
68 D. D. Leonidas, R. Shapiro, L. I. Irons, N. Russo and K. R. Acharya, *Biochemistry*, 1997, **36**, 5578–5588.
69 D. A. Sawyer and B. V. L. Potter, *J. Chem. Soc., Perkin Trans. 1*, 1992, 923–932.
70 G. A. Mignery, C. L. Newton, B. T. Archer and T. C. Sudhof, *J. Biol. Chem.*, 265, pp. 12679–12685.
71 J. Miyazaki, S. Takaki, K. Araki, F. Tashiro, A. Tominaga, K. Takatsu and K. Yamamura, *Gene*, 79, pp. 269–277.
72 H. Sugawara, M. Kurosaki, M. Takata and T. Kurosaki, *EMBO J.*, 16, pp. 3078–3088.
73 H. Sugawara, M. Kurosaki, M. Takata and T. Kurosaki, *EMBO J.*, 16, pp. 3078–3088.
74 T. J. Cardy, D. Traynor and C. W. Taylor, *Biochem. J.*, 328 (Pt 3), pp. 785–793.