Molecular Recognition of Adenophostin, a Very Potent Ca^{2+} Inducer, at the D-*myo*-Inositol 1,4,5-Trisphosphate Receptor‡

Hitoshi Hotoda,*,∥ Kazuhiro Murayama,∥ Shuichi Miyamoto,§,∥ Yoriko Iwata,∥ Masaaki Takahashi,⊥ Yumi Kawase,⊥ Kazuhiko Tanzawa,⊥ and Masakatsu Kaneko∥

Exploratory Chemistry Research and Biological Research Laboratories, Sankyo Co., Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan

Received January 19, 1999; Revised Manuscript Received March 31, 1999

ABSTRACT: The recognition mode of adenophostin A at the D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)-P_3] receptor was investigated. Comparison of conformations of Ins(1,4,5)P_3 and adenophostin A by using the combination of NMR and molecular mechanics (MM) calculations demonstrated that adenophostin A adopted a moderately extended conformation regarding the distance between the 2′-phosphoryl group and the 3″,4″-bisphosphate motif, as suggested previously [Wilcox, R. A. et al. (1995) *Mol. Pharmacol. 47*, 1204−1211]. Based on the nuclear Overhauser effect (NOE) observed between 3′-H and 1″-H and on MM calculations, the molecular shape of adenophostin A proved to be an extended form at least in solution, in contrast to Wilcox's compactly folded, preliminary hairpin model. GlcdR(2,3′,4′)P_3, an adenophostin analogue without adenine moiety, was found to be less potent than adenophostin A and almost equipotent to Ins(1,4,5)P_3. We propose the possibility that (i) the optimal spatial arrangement of the three phosphoryl groups and/or (ii) the interaction of the adenine moiety of adenophostin A with the putative binding site, if it exists in the vicinity of the Ins(1,4,5)P_3-binding site, might account for the exceptional potency of adenophostin A.

It is well-recognized that the activation of phosphoinositidase C via stimulation of an extracellular G-protein-coupled receptor results in the release of D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3,[1] Figure 1] as a second messenger. Ins(1,4,5)P_3 binds to the receptor protein that contains both an Ins(1,4,5)P_3 recognition site and a Ca^{2+} channel to mobilize nonmitochondrial intracellular Ca^{2+} in a wide variety of cell types (*1*). Efforts have been made to understand the mode of recognition of Ins(1,4,5)P_3 at the Ins(1,4,5)P_3 receptor [Ins(1,4,5)P_3R] (*2*,*3*) and to obtain an antagonist or a partial agonist designed rationally from Ins-(1,4,5)P_3 (*4*−*6*).

We isolated very potent Ins(1,4,5)P_3R agonists, adenophostins A and B, from the culture broth of *Penicillium brevicompactum* SANK 11991 and SANK 12177 (*7*−*9*). The structures of adenophostins A and B are distinct from that of Ins(1,4,5)P_3 except for the presence of three phosphoryl groups. The Ins(1,4,5)P_3R-binding activity and Ca^{2+}-mobilizing activity of adenophostin A, as well as adenophostin B,

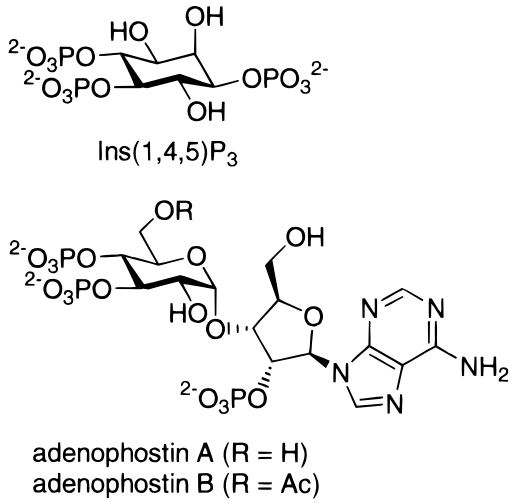


FIGURE 1: Ins(1,4,5)P_3 and adenophostins A and B.

are approximately 100 times more potent than those of the natural ligand, Ins(1,4,5)P_3 (*8*). Moreover, adenophostins are resistant to Ins(1,4,5)P_3-metabolizing enzymes, Ins(1,4,5)P_3 5-phosphatase and Ins(1,4,5)P_3 3-kinase, explaining their prolonged activity (*8*).

Because it has the highest known affinity for the Ins(1,4,5)-P_3R and it has a unique structure, adenophostin has received much attention since its discovery. DeLisle et al. reported that adenophostin A was able to stimulate the influx of Ca^{2+} across the plasma membrane without inevitably emptying the Ins(1,4,5)P_3-sensitive intracellular Ca^{2+} stores (*10*). Recently, Sato et al. confirmed that Ca^{2+} oscillations, lasting for at least 3 h as observed at fertilization, were produced

‡ This paper is part 3 of the series, IP_3 Receptor−Ligand.

* Corresponding author. E-mail: hotoda@shina.sankyo.co.jp. Fax: +81-3-5436-8570.

§ To whom the correspondence regarding molecular modeling should be addressed. E-mail: miya@shina.sankyo.co.jp.

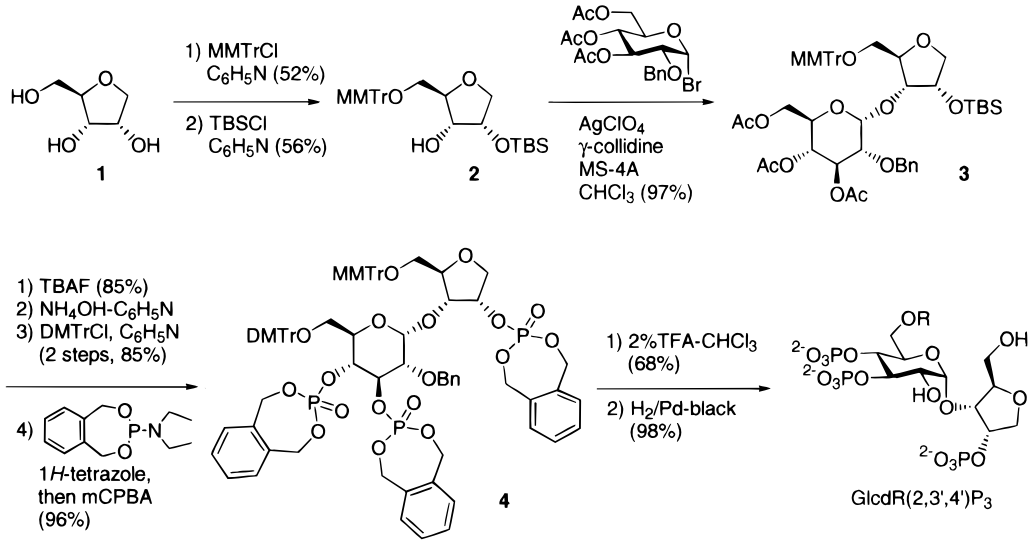
∥ Exploratory Chemistry Research Laboratories.

⊥ Biological Research Laboratories.

[1] Abbreviations: Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; Ins-(1,4,5)P_3R, D-*myo*-inositol 1,4,5-trisphosphate receptor; GlcdR(2,3′,4′)-P_3, 3-*O*-(α-D-glucosyl)-1-deoxy-D-ribose 2,3′,4′-*O*-trisphosphate; GlcA-(3″,4″)P_2, 3-*O*-(α-D-glucosyl)-adenosine 3″,4″-*O*-bisphosphate; GlcaE-(2,3′,4′)P_3, (2*S*,3*R*)-3-*O*-(α-D-glucosyl)-1,4-anhydroerythritol 2,3′,4′-*O*-trisphosphate; 6″-Ac-GlcI(2′,3″,4″)P_3, 3′-*O*-(6-*O*-acetyl-α-D-glucosyl)-inosine 2′,3″,4″-*O*-trisphosphate.

10.1021/bi990114r CCC: $18.00
Published on Web 06/29/1999

Scheme 1: Synthetic Route for GlcdR(2,3′,4′)P_3



by a single injection of adenophostin B into oocytes, demonstrating the possible usefulness of adenophostins for parthenogenetic oocyte activation in the biotechnology of animal reproduction (*11*).

The total synthesis of adenophostin A has been previously done by us (*12*, *13*) and by van Boom's group (*14*, *15*). Several analogues of adenophostin were synthesized (*16*−*26*) and tested for Ca^{2+}-mobilizing activity, demonstrating the importance of the adenosine moiety of adenophostin for the activity (*16*−*23*).

In the present paper, we report the synthesis, the Ins(1,4,5)-P_3R-binding activity, and the Ca^{2+}-mobilizing activity of an adenophostin analogue lacking the adenine moiety as well as a comparison of conformations of Ins(1,4,5)P_3 and adenophostin A elucidated using the combination of NMR and molecular mechanics (MM) calculations. The importance of the spatial arrangement of the three phosphoryl groups and the putative interaction of the adenine moiety of adenophostin in the vicinity of the Ins(1,4,5)P_3-binding site on the Ins(1,4,5)P_3R are discussed.

MATERIALS AND METHODS

Materials. Adenophostins A and B were isolated from the culture broth of *P. brevicompactum* SANK11991 (*7*). Ins-(1,4,5)P_3 was purchased from Calbiochem. [^{3}H]Ins(1,4,5)P_3 and $^{45}CaCl_2$ were from DuPont-New England Nuclear. 3-*O*-(α-D-Glucosyl)-1-deoxy-D-ribose 2,3′,4′-*O*-trisphosphate [GlcdR(2,3′,4′)P_3] was synthesized (Scheme 1) by the modification of the previous procedure for the total synthesis of adenophostin A (*12*). Briefly, 1-deoxy-D-ribofuranose **1** (*27*) was suitably protected to give **2** followed by glycosylation with 2-*O*-benzyl-3,4,6-tri-*O*-acetyl-α-D-glucosyl bromide (*28*) under the influence of $AgClO_4$ and γ-collidine. After manipulations of the protecting groups of the resulting α-linked dimer **3** and subsequent phosphorylation, the resulting compound **4** was deprotected to give the desired GlcdR(2,3′,4′)P_3. ^{1}H NMR (D_2O, external TMS) δ 5.06 (d, J = 3.4 Hz, 1H), 4.23 (quartet, J = 8.8 Hz, 1H), 4.09 (dd, J = 6.8 and 4.9 Hz, 1H), 3.95−3.40 (m, 11H); IR (KBr) 3194, 1687, 1460, 1403, 1151, 1086, 1041, 977, 938 cm^{-1}; HRMS (FAB) m/z 534.9991 $(M - H)^-$ $\Delta_{ppm} = -5.2$. Full experimental details for the synthesis of GlcdR(2,3′,4′)P_3 are given in Supporting Information. Recently, Matsuda's group synthesized the same compound as GlcdR(2,3′,4′)P_3, and its affinity to porcine cerebellar Ins(1,4,5)P_3R was determined by us to be 25 nM (IC_{50}) (*29*).

Ins(1,4,5)P_3 Receptor-Binding Assay. The Ins(1,4,5)P_3R was purified from rat cerebellum using heparin-agarose and concanavalin A-Sepharose (*30*). The [^{3}H]Ins(1,4,5)P_3-binding assay was performed with the poly(ethylene glycol) precipitation method (*31*), in which 1 μg of the purified receptor was incubated in 100 μL of 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10 nM [^{3}H]Ins(1,4,5)-P_3, and GlcdR(2,3′,4′)P_3 or unlabeled Ins(1,4,5)P_3 for 10 min at 4 °C. Nonspecific binding was measured in the presence of 1 μM cold Ins(1,4,5)P_3.

Measurement of $^{45}Ca^{2+}$ Release. For the cell-free study, rat cerebellar microsomes were prepared (*32*) and suspended at 0.5 mg of protein/mL in 10 mM HEPES−KOH, pH 7.2, 120 mM KCl, 1 mM dithiothreitol, 2 mM $MgCl_2$, 1 μg/mL oligomycin, and 50 μM $CaCl_2$ (containing 2 μCi/mL $^{45}Ca^{2+}$). EGTA was added to buffer-free Ca^{2+} to 100 nM (*33*). Ca^{2+} uptake was initiated by the addition of 1 mM ATP and an ATP-regenerating system (10 mM phosphocreatine and 10 units/mL creatine kinase). The reaction mixture was incubated at 30 °C, and 100 μL aliquots were removed to determine intravesicular Ca^{2+} content by a filtration assay.

Measurement of ^{1}H NMR Spectra. For the preparation of the NMR sample, adenophostin A was dissolved at 10 mM in 50 mM phosphate buffer in ^2H_2O at p^{2}H 6.8 (isotope effect of 0.4 was added to the pH meter reading). 1D-^{1}H NMR spectrum was measured at 298 K on the JNM GSX-400 spectrometer (400 MHz). NOESY spectrum was obtained with a mixing time of 800 ms at 298 K on the Bruker AMX-360 spectrometer (360 MHz).

Molecular Modeling for Ins(1,4,5)P_3 and Adenophostin A. All calculations were run on IRIS workstations. Geometry optimizations in quantum mechanics calculations were performed using the semiempirical molecular orbital method AM1 of the MOPAC program. Single-point ab initio calculations were then carried out using the program SPARTAN with a STO-3G basis set to determine the

electrostatic potential charges. As for molecular mechanics calculations to find stable molecular conformations, systematic conformational searches were performed using the grid scan technique followed by energy minimizations with the adopted basis Newton−Raphson method, within the software package QUANTA/CHARMm. All calculations on Ins(1,4,5)P_3 and adenophostin A were carried out on the structures in which every phosphoryl group takes the monoanion form, leading the net charge of each molecule equal to −3. A distance-dependent dielectric of 4r was employed in the molecular mechanics calculations.

RESULTS

Binding of GlcdR(2,3′,4′)P_3 to Rat Cerebellar Ins(1,4,5)P_3 Receptor. The effect of GlcdR(2,3′,4′)P_3 on [^{3}H]Ins(1,4,5)P_3 binding to the Ins(1,4,5)P_3R purified from rat cerebellum was tested. As reported previously (*8*), adenophostins A and B inhibit [^{3}H]Ins(1,4,5)P_3 binding more potently than Ins(1,4,5)P_3: the IC_{50} values for adenophostin A, adenophostin B, and Ins(1,4,5)P_3 are 1.3, 1.3, and 23 nM, respectively. The K_i values for adenophostins A and B are both calculated to be 0.18 nM, while that of Ins(1,4,5)P_3 is 15 nM. Thus, the affinity of adenophostins for the Ins(1,4,5)P_3R is approximately 100-fold higher than that of Ins(1,4,5)P_3. In the present study, the affinity of GlcdR(2,3′,4′)P_3, the adenophostin analogue lacking the adenine moiety, was roughly the same as that of Ins(1,4,5)P_3: the IC_{50} value for GlcdR(2,3′,4′)P_3 was 10 nM, while that of adenophostin A as a control was 1.7 nM.

Ca^{2+}-Releasing Activity of GlcdR(2,3′,4′)P_3. The Ca^{2+}-releasing activity of GlcdR(2,3′,4′)P_3 from rat cerebellar microsomes was assessed. As reported previously (*8*), the ED_{50} values for adenophostin A, adenophostin B, and Ins(1,4,5)P_3 are 1.4, 1.5, and 170 nM, respectively, in our assay system, demonstrating that adenophostins are roughly 100-fold more potent than Ins(1,4,5)P_3. In the present study, GlcdR(2,3′,4′)P_3 was found to be less potent than adenophostin but still more potent than Ins(1,4,5)P_3: the ED_{50} value for GlcdR(2,3′,4′)P_3 was 28 nM, while that of adenophostin A as a control was 3.3 nM.

^{1}H NMR Analysis of Adenophostin A. ^{1}H NMR of adenophostin A (10 mM) was measured in 50 mM phosphate buffer (p^2H 6.8) at 25 °C to confirm the conformation of adenophostin A in solution. The proton−proton coupling constants for the ribose moiety of adenophostin A were as follows: $^3J_{1′,2′}$, $^3J_{2′,3′}$, and $^3J_{3′,4′}$ were 6.6, 5.1, and 2.9 Hz, respectively. In general, the $^3J_{1′,2′}$ value of the ribose moiety reflects the ratio of C2′-*endo* form to C3′-*endo* form, which is calculated by the following equation: [C2′-*endo*] (%) = $[^3J_{1′,2′}/(^3J_{1′,2′} + {}^3J_{3′,4′})] \times 100$, where $^3J_{1′,2′} + {}^3J_{3′,4′}$ is nearly 10 Hz (*34*). Thus, the [C2′-*endo*] (%) of adenophostin A is calculated to be nearly 70%, suggesting that adenophostin A prefers the type S conformer. As to the glucose moiety of adenophostin A, the proton−proton coupling constants were as follows: $^3J_{1″,2″}$, $^3J_{2″,3″}$, $^3J_{3″,4″}$, and $^3J_{4″,5″}$ were 3.3, 10.3, 9.5, and 9.5 Hz, respectively, indicating the chair conformation with two equatorial phosphoryl groups. Next, the NOESY spectrum of adenophostin A was measured. The typical NOEs across the glycosyl bonds are shown in Figure 2. The NOEs between 8-H and 1′-H and between 8-H and 2′-H demonstrate that the adenine moiety of adenophostin A prefers syn (nearly close to but not high anti) orientation in solution. The NOE between 3′-H and 1″-H indicates that the spatial arrangement of the two sugar moieties, the ribose and the glucose of adenophostin A, with respect to a couple of crucial torsion angles around this α-glycosyl linkage is as depicted in Figure 2.

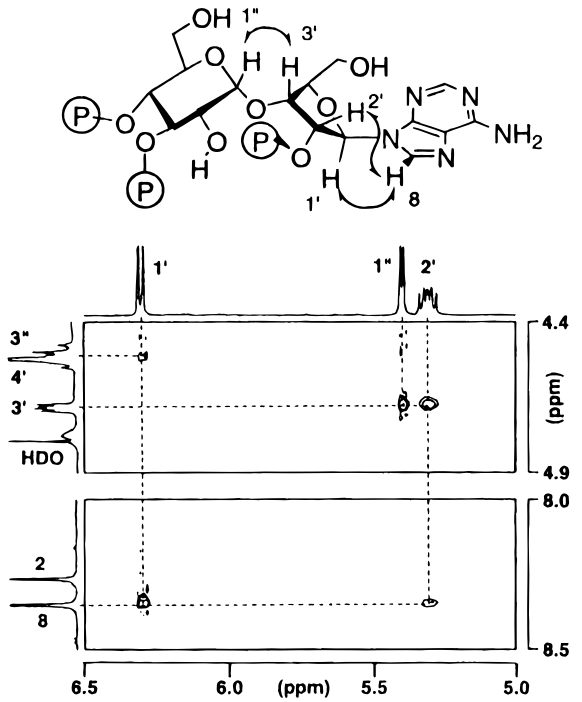


FIGURE 2: NOEs across two glycosyl linkages of adenophostin A (10 mM) dissolved in 50 mM phosphate buffer in ^2H_2O at p^2H 6.8. The NOESY spectrum was measured at 360 MHz with a mixing time of 800 ms at 298 K.

Molecular Modeling for Ins(1,4,5)P_3 and Adenophostin A. A conformational analysis of Ins(1,4,5)P_3 and adenophostin A was performed in order to elucidate the stable conformations and the geometrical features of both molecules. In the initial conformation of Ins(1,4,5)P_3, the inositol ring adopted a chair conformation in which all three phosphoryl groups are equatorial, as was determined by ^{1}H NMR (*35*). After a geometry optimization with the semiempirical method, a single-point ab initio calculation was carried out to obtain the electrostatic potential charge of each atom. By using the resulting partial charges, we carried out grid scans followed by energy minimizations with molecular mechanics in order to find stable conformations of Ins(1,4,5)P_3. The initial conformational search on $\tau_a-\tau_f$ (Figure 3) gave 989 stable conformers. On the basis of the most stable conformer thus obtained, a conformational search on $\eta_1-\eta_6$ was then performed to afford a final 303 stable conformers of Ins(1,4,5)P_3. The most stable conformer of Ins(1,4,5)P_3 is shown in Figure 4I.

As for the initial conformation of adenophostin A, the glucose moiety adopted the chair conformation with equatorial 3″- and 4″-phosphoryl groups as determined by ^{1}H NMR study described above. After a geometry optimization with the semiempirical molecular orbital method, the electrostatic potential charges of adenophostin A were determined by a single-point ab initio calculation. With the partial charges thus obtained, grid scans followed by energy minimizations were performed in order to find stable conformations of

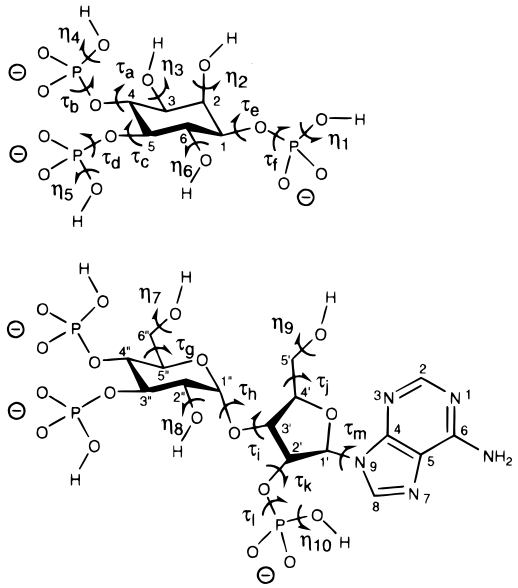


FIGURE 3: Torsion angles in MM calculations.

adenophostin A. Starting geometries with C2′-*endo* puckering as determined by ^{1}H NMR were first generated by changing the torsion angles of $\tau_g-\tau_m$ systematically, leaving the conformation of the 3″- and 4″-phosphoryl groups as it was. Full-energy minimization of them gave 365 stable conformers. While adenophostin A proved to prefer C2′-*endo* form by means of ^{1}H NMR, the puckering of the most stable conformer thus obtained was C3′-*endo*. The lowest-energy conformer among the conformers with C2′-*endo* puckering was found to be less stable by 2.3 kcal/mol than the above most stable conformer with C3′-*endo* puckering. The final conformational search on $\eta_7-\eta_{10}$ did not affect the above two conformers. Thus, the most stable conformer with C3′-*endo* puckering (conformer A; Figure 4A; Table 1) and the local minimum conformer with C2′-*endo* puckering (conformer B; Figure 4B; Table 1) were obtained.

The global shape of adenophostin A as well as the position of the 2′-phosphoryl group relative to the 3″,4″-bisphosphoryl group is strongly dependent on the torsional angles of τ_h and τ_i. The relationship between the conformational energy and these torsional angles was investigated in detail by a molecular mechanics calculation. By using the most stable conformer A, we carried out a conformational search in 30° increments for τ_h and τ_i to provide an energy contour map with respect to these torsional angles. As shown in Figure 5, a local minimum conformer C with the geometry corresponding to Wilcox's conformer (region c, $\tau_h = 317°$ and $\tau_i = 88°$) was found besides the most stable conformer A (region a, $\tau_h = 65°$ and $\tau_i = 85°$). While conformers A and B both take the extended shape with *syn*-N-glycoside, conformer C as well as Wilcox's conformer adopts a compactly folded hairpin shape with *anti*-N-glycoside (Figure 4, Table 1). When conformer B with C2′-*endo* puckering was used as the starting conformer, the map similar to that in Figure 5 was obtained (see Supporting Information).

DISCUSSION

Because it has the highest known affinity for the Ins(1,4,5)-P_3R and it has a unique structure, adenophostin has received much attention since its discovery (*7*−*9*). The structure of

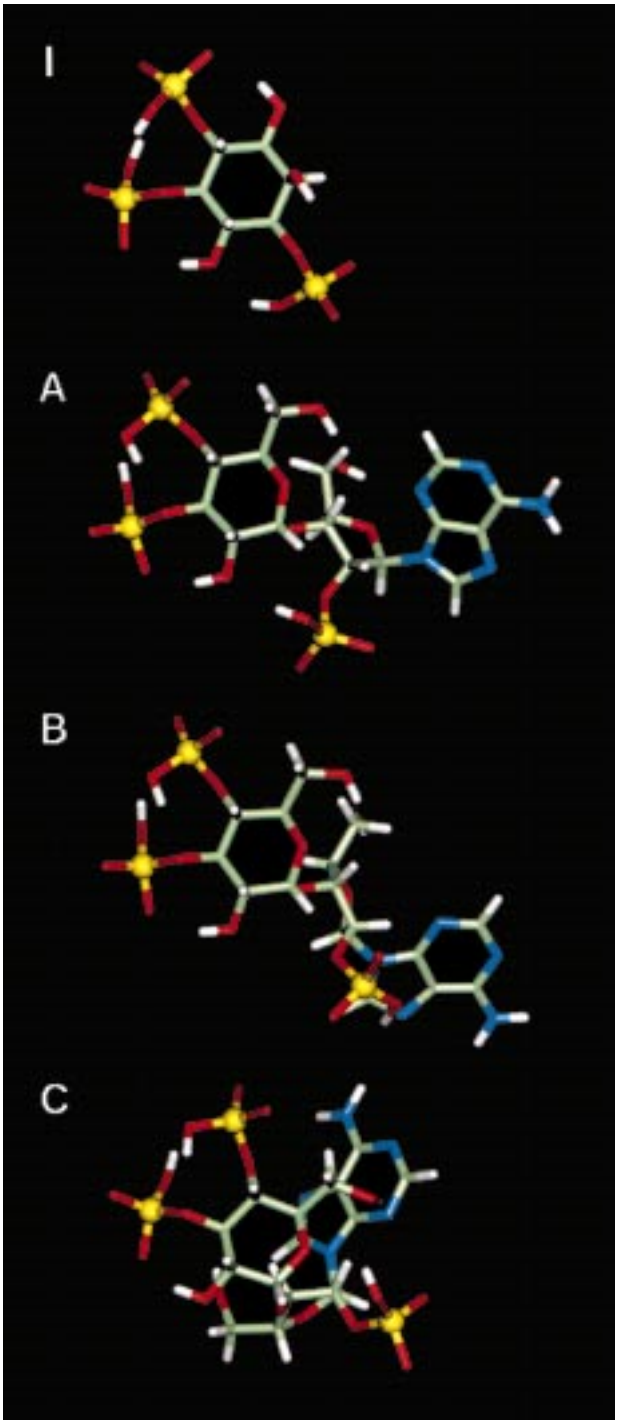


FIGURE 4: (I) Fully energy-minimized conformer for Ins(1,4,5)P_3; (A−C) adenophostin A: (A) the most stable conformer A with C3′-*endo* puckering, (B) the local minimum conformer B with C2′-*endo* puckering, and (C) the local minimum conformer C with the geometry corresponding to Wilcox's conformer.

adenophostin is distinct from that of Ins(1,4,5)P_3 except for the presence of three phosphoryl groups; however, the Ins-(1,4,5)P_3R-binding activity and Ca^{2+}-mobilizing activity of adenophostin are approximately 100 times more potent than those of the natural ligand, Ins(1,4,5)P_3. As reported previously (*9*), GlcA(3″,4″)P_2 (Figure 6) lacking the 2′-phosphoryl group shows reduced affinity by three orders; however, it still retains weak Ca^{2+}-mobilizing activity, indicating that the 2′-phosphoryl group might not be crucial but rather have an enhancing effect like the 1-phosphoryl group of Ins(1,4,5)-

Table 1: Conformational Data for Ins(1,4,5)P_3 and Adenophostin A

compound		conformation[a] shape	τ_h	τ_i	ribose	*N*-glycoside	χ (τ_m)	distance between two phosphorus atoms (Å) 1−4 (2′-4″)[b]	1−5 (2′-3″)	4−5 (3″-4″)	ΔE[c] (kcal/mol)
Ins(1,4,5)P_3	our model	chair						8.1	6.9	4.1	
	Wilcox's model[d]	chair						8.0	7.3	5.5	
	crystal structure[e]	chair						8.2	7.2	4.3	
adenophostin A	conformer A	extended	65°	85°	C3′-*endo*	*syn*	57°	9.2	7.3	4.1	0
	conformer B	extended	59°	100°	C2′-*endo*	*syn*	56°	9.6	8.2	4.1	2.3
	conformer C	hairpin	317°	88°	C3′-*endo*	*anti*	176°	8.4	8.7	4.1	8.9
	Wilcox (MD)[d]	hairpin			C3′-*endo*	*anti*		6.7−10.7	7.5−11.5		

[a] Puckerings of the glucose of adenophostin models are all chair. [b] Numbers in parentheses are based on adenophostin A. [c] Conformational energies relative to the most stable conformer A. [d] See ref *18*. [e] Taken from the X-ray crystal structure of the complex of spectrin PH domain with Ins(1,4,5)P_3 (PDB code: 1btn). See ref *39*.

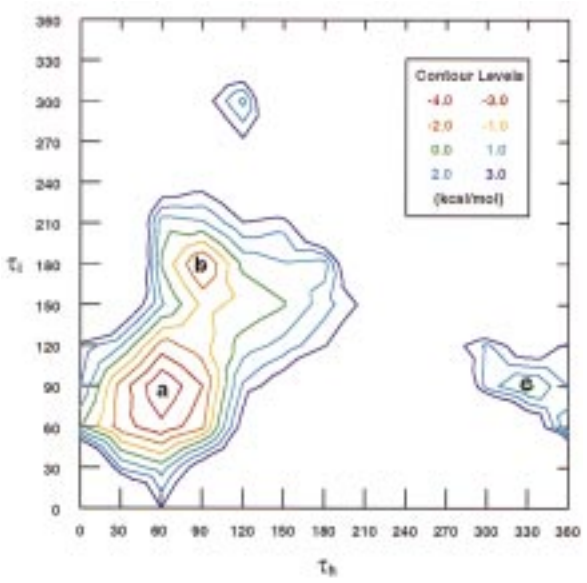


FIGURE 5: Conformational energy contour map for adenophostin A calculated starting from the most stable conformer A with respect to rotation around τ_h and τ_i; (a) the most stable conformer A; (b) a local minimum conformer (extended shape) with a relative conformational energy of 1.7 kcal/mol; and (c) conformer C with the geometry corresponding to Wilcox's conformer.

P_3 (*2*, *3*). Thus, it is easily presumed that the 3″,4″-bisphosphate/2″-hydroxyl motif of adenophostin mimics the crucial 4,5-bisphosphate/6-hydroxyl motif of Ins(1,4,5)P_3. Consequently, the 5″-position of adenophostin A can be superimposed with the 3-hydroxyl group of Ins(1,4,5)P_3, which is relatively unimportant for Ins(1,4,5)P_3R recognition (*3*). On the other hand, the Ins(1,4,5)P_3 analogues substituted at the 3-position with substituents with steric bulk exhibited progressively reduced affinity for Ins(1,4,5)P_3R (*36*−*38*). It might therefore seem odd that bulky structures at the 5″-positions of adenophostins A and B, CH_2OH and CH_2OAc, respectively, should be compatible with high potency. This problem has been solved by Potter's group using 6-deoxy-6-hydroxymethyl *scyllo*-Ins(1,2,4)P_3 (Figure 6) bearing an equatorial CH_2OH group at this position, demonstrating that the CH_2OH group at the 5″-position of adenophostin A should be tolerated by the Ins(1,4,5)P_3R (*19*).

Pioneering studies by Wilcox's group using computer-aided molecular modeling for adenophostin A demonstrated that the position of the 2′-phosphoryl group of adenophostin A relative to the 3″,4″-bisphosphate motif might be slightly more extended than the corresponding positions of Ins(1,4,5)-P_3, suggesting that the high potency of adenophostin A might due to the optimal positioning of the 2′-phosphoryl group (*18*). To investigate the validity of the hypothesis that the difference of spatial arrangement of the three phosphoryl groups between Ins(1,4,5)P_3 and adenophostin A accounted for the difference of potency, we carried out a conformation analysis and shape comparison of Ins(1,4,5)P_3 and adenophostin A using the combination of NMR study and molecular mechanics calculations.

As to the spatial arrangement of three phosphoryl groups, the result of our calculation for Ins(1,4,5)P_3 has good agreement with the X-ray crystal structure of the complex of Ins(1,4,5)P_3 with spectrin PH domain (*39*), especially for the distance between two phosphorus atoms at the 4- and 5-positions (Table 1). The proton−proton vicinal coupling constants for the glucose moiety of adenophostin A were found to be consistent with those of the corresponding moiety of Ins(1,4,5)P_3 obtained by Cerdan et al. (*35*), demonstrating that the glucose moiety adopted the chair conformation with two equatorial phosphoryl groups. In contrast to Ins(1,4,5)-P_3, adenophostin A has a relatively flexible skeleton and the spatial arrangement of the 2′-phosphoryl group is strongly dependent on the rotation around τ_h and τ_i (Figures 3 and 5). The NOE observed between 3′-H and 1″-H demonstrates that these two protons are spatially arranged in the vicinity in solution. The most stable conformer A (extended shape, $\tau_h = 65°$ and $\tau_i = 85°$), formulated using molecular mechanics, is well consistent with the above NOE result. The proton−proton vicinal couplings for the ribose ring of adenophostin A indicated that the ribose ring preferred the C2′-*endo* form. Unfortunately, however, the puckering of the ribose of the most stable conformer A obtained by calculations was C3′-*endo*. Because of rapid equilibration between the C3′-*endo* (N) form and C2′-*endo* (S) form, it is difficult to determine the actual puckering form of the ribose of adenophostin A bound to the Ins(1,4,5)P_3R. Consequently, we speculate that conformer B, which is consistent with the NOE observed between 3′-H and 1″-H (extended shape, $\tau_h = 59°$, $\tau_i = 100°$) and has the C2′-*endo* puckering of the ribose, is the most likely conformation of adenophostin A at least in solution (Figure 4B, Table 1). Moreover, the torsion angles at the N-glycosyl linkages for the conformers A and B were both syn (τ_m (χ) = 57° and 56°, respectively), indicating good agreement with the results of NOEs observed between 8-H and 1′-H and between 8-H and 2′-H. The comparison of the fully energy-minimized structure of Ins-(1,4,5)P_3 and the most likely conformer B of adenophostin

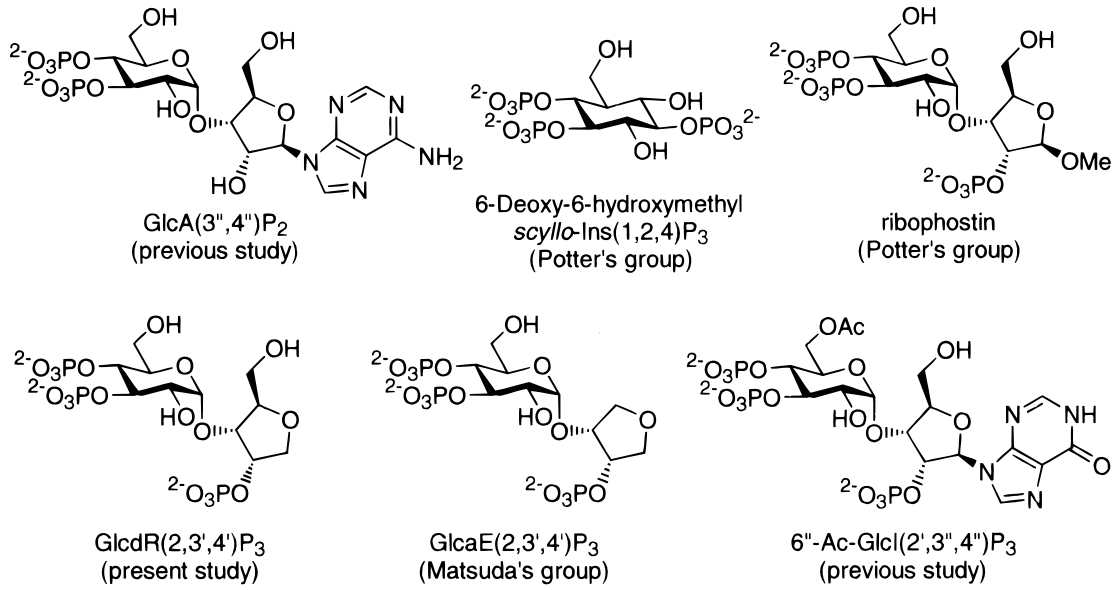


FIGURE 6: Adenophostin analogues.

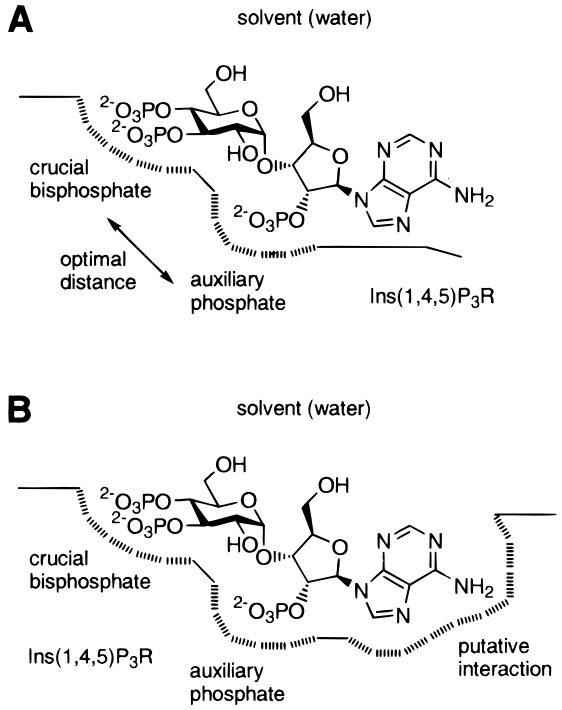


FIGURE 7: Putative mode of recognition of adenophostin A at Ins(1,4,5)P_3R.

A demonstrates that adenophostin A takes moderately extended structure with respect to the distance between the 2′-phosphoryl group and the 3″,4″-bisphosphate motif (Table 1) as suggested by Wilcox's group (*18*). The result indicates the possibility that the relative position of the 2′-phosphoryl group of adenophostin A is optimal for the recognition at Ins(1,4,5)P_3R (Figure 7A).

Wilcox et al. (*18*) presented a representative conformation of adenophostin A obtained from a molecular dynamics simulation. This conformation, which is compactly folded, is essentially distinct from our model, the extended conformer B, with respect to the torsion angle of τ_h. This observation indicates that Wilcox's conformer seems inconsistent with our NOE data observed between 3′-H and 1″-H. Additionally, while the N-glycosyl linkage of our conformer B is syn, that of Wilcox's conformer is anti, indicating the inconsistency with our NOE data observed between 8-H and 1′-H and between 8-H and 2′-H. The conformational energy contour map regarding τ_h and τ_i of adenophostin A suggested the existence of the compactly folded conformer like Wilcox's conformer in region c of Figure 5. Thus, the conformer in region c with *anti*-N-glycoside was modeled. After energy minimization, the local minimum conformer C with hairpin shape was obtained. The conformer C with the geometry corresponding to Wilcox's conformer has a relative conformational energy of 8.9 kcal/mol, indicating that conformer C is less stable by 6.6 kcal/mol than conformer B. In Wilcox's preliminary model as well as conformer C, moreover, two primary hydroxyl groups at the 5′- and 6″-positions adopt an opposing direction. The hydroxyl groups at these positions are presumably unimportant because adenophostin analogues bearing substituents at these positions retain high potency.[2] Thus, we presume it to be natural that the two relatively unimportant primary hydroxyl groups at 5′- and 6″-positions both take the same direction toward the solvent, and the crucial 3″,4″-bisphosphate/2″-hydroxyl motif and the important 2′-phosphate make an array to occupy the same side of the molecule, as found for conformers A and B but not C, to interact with the Ins(1,4,5)P_3R (Figure 7).

The major difference that distinguishes adenophostin from Ins(1,4,5)P_3 analogues is the presence of the adenine moiety. "Ribophostin", having a 1-β-MeO group in place of adenine moiety of adenophostin A, has been reported by Potter's group (*20*) (Figure 6). Their preliminary biological evaluation of ribophostin using permeabilized hepatocytes revealed a Ca^{2+}-mobilizing potency very close to that of Ins(1,4,5)P_3. To elucidate the effect of the adenine moiety of adenophostin A, we examined GlcdR(2,3′,4′)P_3 (Figure 6) having no substituent at the 1-position of the ribose ring. GlcdR(2,3′,4′)-P_3 was found to be a full agonist at Ins(1,4,5)P_3R of rat cerebellar microsomes. The Ca^{2+}-mobilizing activity of GlcdR(2,3′,4′)P_3 was much lower than that of adenophostin

[2] Hotoda, H., Murayama, K., Takahashi, M., Kawase, Y., Tanzawa, K., and Kaneko, M. (to be published elsewhere).

and roughly equal to that of Ins(1,4,5)P_3. Recently, Matsuda's group reported that GlcaE(2,3′,4′)P_3 (Figure 6), lacking both the adenine moiety and the 5′-CH_2OH group of adenophostin A, proved to be equipotent to Ins(1,4,5)P_3 using porcine cerebellar microsomes (*23*).[3] Since the above analogues lacking the adenine moiety are all less potent than adenophostin A and almost equipotent to Ins(1,4,5)P_3, the adenine moiety of adenophostin A appears to be an important factor that accounts for the high potency of adenophostin A. For instance, the adenine moiety of adenophostin A might possibly interact in the vicinity of Ins(1,4,5)P_3-binding site on Ins(1,4,5)P_3R (Figure 7B). Since the inosine-type analogue of adenophostin B, 6″-Ac-GluI(2′,3″,4″)P_3 (Figure 6), has been reported to retain high potency against Ins(1,4,5)P_3R (*9*), the 6-NH_2 group of adenophostin A is not involved in the putative interaction between adenine moiety and Ins(1,4,5)P_3R. To identify the putative adenine-binding site, if it exists in the vicinity of Ins(1,4,5)P_3-binding site, one should examine an adenophostin analogue having a photoaffinity probe such as 2- or 8-azido-adenine group (*40*) in place of the adenine group. A benzophenone group, which has been successfully employed for the Ins(1,4,5)P_3 derivatives by Prestwich's group (*41*, *42*), may be useful as a photoaffinity probe if the putative interaction is a hydrophobic one. There still remains an alternative possibility explaining the effect of the adenine moiety. In general, both 1-deoxyribose and 1-β-MeO-ribose are known to strongly prefer the C3′-*endo* form (*43*). Actually, the puckering of the ribose ring of ribophostin is the C3′-*endo* form ($^3J_{1,2} \cong 0$ Hz) (*20*), while adenophostin A prefers the C2′-*endo* form (conformer B), which is more extended with respect to the distance between the 2′-phosphoryl group and the 3″,4″-bisphosphate motif than the corresponding C3′-*endo* form (conformer A) (Table 1). Accordingly, the effect of the adenine moiety might be to direct the puckering propensity toward the C2′-*endo* form with highly extended phosphoryl groups; this might be optimal for the interaction at Ins(1,4,5)P_3R.

In conclusion, we propose two possibilities that could explain the exceptionally high potency of adenophostin A. The first one is that adenophostin A takes a moderately extended structure that might be optimal for the recognition at Ins(1,4,5)P_3R with respect to the distance between the 2′-phosphoryl group and the 3″,4″-bisphosphate motif. The second one is that the adenine moiety of adenophostin A directly interacts in the vicinity of the Ins(1,4,5)P_3-binding site on the Ins(1,4,5)P_3R, resulting in high affinity and strong Ca^{2+}-mobilizing activity. From the viewpoint of the effect of the adenine moiety, there is an alternative possibility that the adenine moiety might direct the puckering propensity toward the C2′-*endo* form with highly extended phosphoryl groups; this might be optimal for the interaction at Ins(1,4,5)-P_3R as discussed in the former case.

It was found that adenophostin A adopted extended conformation with C2′-*endo* puckering of the ribose and syn orientation at the N-glycosyl linkage in solution. We could propose a novel model for the conformation of adenophostin A by the combination of NMR study and molecular mechanics calculations.

[3] Very recently, Potter's group also reported the synthesis of GlcaE-(2,3′,4′)P_3. See ref *44*.

ACKNOWLEDGMENT

We thank Drs. Toshiyuki Akiyama, Toshio Takatsu, and Hayao Matsuhashi for their support in the NMR study.

SUPPORTING INFORMATION AVAILABLE

Full experimental procedures for the synthesis of GlcdR-(2,3′,4′)P_3, 1D-^{1}H NMR spectrum (Figure 1), and NOESY spectrum (Figure 2) for adenophostin A, and conformational energy contour map starting from conformer B (Figure 3). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Berridge, M. J. (1993) *Nature 361*, 315−325.
2. Lu, P.-J., Gou, D.-M., Shieh, W.-R., and Chen, C.-S. (1994) *Biochemistry 33*, 11586−11597.
3. Potter, B. V. L., and Lampe, D. (1995) *Angew. Chem., Int. Ed. Engl. 34*, 1933−1972.
4. Dreef, C. E., Schiebler, W., van der Marel, G. A., and van Boom, J. H. (1991) *Tetrahedron Lett. 32*, 6021−6024.
5. Dreef, C. E., Jansze, J.-P., Elie, C. J. J., van der Marel, G. A., and van Boom, J. H. (1992) *Carbohydr. Res. 234*, 37−50.
6. Safrany, S. T., Wilcox, R. A., Liu, C., Dubreuil, D., Potter, B. V. L., and Nahorski, S. R. (1993) *Mol. Pharmacol. 43*, 499−503.
7. Takahashi, M., Kagasaki, T., Hosoya, T., and Takahashi, S. (1993) *J. Antibiot. 46*, 1643−1647.
8. Takahashi, M., Tanzawa, K., and Takahashi, S. (1994) *J. Biol. Chem. 269*, 369−372.
9. Takahashi, S., Kinoshita, T., and Takahashi, M. (1994) *J. Antibiot. 47*, 95−100.
10. DeLisle, S., Marksberry, E. W., Bonnett, C., Jenkins, D. J., Potter, B. V. L., Takahashi, M., and Tanzawa, K. (1997) *J. Biol. Chem. 272*, 9956−9961.
11. Sato, Y., Miyazaki, S., Shikano, T., Mitsuhashi, N., Takeuchi, H., Mikoshiba, K., and Kuwabara, Y. (1998) *Biol. Reprod. 58*, 867−873.
12. Hotoda, H., Takahashi, M., Tanzawa, K., Takahashi, S., and Kaneko, M. (1995) *Tetrahedron Lett. 36*, 5037−5040.
13. Hotoda, H., Takahashi, M., Tanzawa, K., Takahashi, S., Miyamoto, S., and Kaneko, M. (1995) *Nucleic Acids Symp. Ser. 34*, 163−164.
14. Van Straten, N. C. R., van der Marel, G. A., and van Boom, J. H. (1996) *Tetrahedron Lett. 37*, 3599−3602.
15. Van Straten, N. C. R., van der Marel, G. A., and van Boom, J. H. (1997) *Tetrahedron 53*, 6509−6522.
16. Jenkins, D. J., and Potter, B. V. L. (1995) *J. Chem. Soc., Chem. Commun.* 1169.
17. Jenkins, D. J., and Potter, B. V. L. (1996) *Carbohydr. Res. 287*, 169−182.
18. Wilcox, R. A., Erneux, C., Primrose, W. U., Gigg, R., and Nahorski, S. R. (1995) *Mol. Pharmacol. 47*, 1204−1211.
19. Riley, A. M., Murphy, C. T., Lindley, C. J., Westwick, J., and Potter, B. V. L. (1996) *Bioorg. Med. Chem. Lett. 6*, 2197−2200.
20. Jenkins, D. J., Marwood, R. D., and Potter, B. V. L. (1997) *Chem. Commun.*, 449−450.
21. Murphy, C. T., Riley, A. M., Lindley, C. J., Jenkins, D. J., Westwick, J., and Potter, B. V. L. (1997) *Mol. Pharmacol. 52*, 741−748.
22. Marchant, J. S., Beecroft, M. D., Riley, A. M., Jenkins, D. J., Marwood, R. D., Taylor, C. W., and Potter, B. V. L. (1997) *Biochemistry 36*, 12780−12790.
23. Tatani, K., Shuto, S., Ueno, Y., and Matsuda, A. (1998) *Tetrahedron Lett. 39*, 5065−5068.
24. De Kort, M., Valentijn, A. R. P. M., van der Marel, G. A., and van Boom, J. H. (1997) *Tetrahedron Lett. 38*, 7629−7632.

25. Van Straten, N. C. R., van der Marel, G. A., and van Boom, J. H. (1997) *Tetrahedron 53*, 6523−6538.
26. Van Straten, N. C. R., Kriek N. M. A. J., Cziria, Z. A. C., van der Marel, G. A., and van Boom, J. H. (1997) *Tetrahedron 53*, 6539−6554.
27. Plavec, J., Tong, W., and Chattopadhyaya, J. (1993) *J. Am. Chem. Soc. 115*, 9734−9746.
28. Brennan, S., and Finan, P. A. (1970) *J. Chem. Soc. C*, 1742−1744.
29. Shuto, S., Tatani, K., Ueno, Y., and Matsuda, A. (1998) *J. Org. Chem. 63*, 8815−8824.
30. Supattapone, P., Worley, P. F., Baraban, J. M., and Snyder, S. M. (1988) *J. Biol. Chem. 263*, 1530−1534.
31. Maeda, N., Niinobe, M., and Mikoshiba, K. (1990) *EMBO J. 9*, 61−67.
32. Supattapone, P., Danoff, S. K., Theibert, A. B., Joseph, S. K., and Snyder, S. H. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8747−8750.
33. Ghosh, T. K., Eis, P. S., Mullaney, J. M., Ebert, C. L., and Gill, D. L. (1988) *J. Biol. Chem. 263*, 11075−11079.
34. Altona, C., and Sandaralingam, M. (1973) *J. Am. Chem. Soc. 95*, 2333−2344.
35. Cerdan, S., Hansen, C. A., Johanson, R., Inubushi, T., and Williamson, J. R. (1986) *J. Biol. Chem. 261*, 14676−14680.
36. Liu, C., and Potter, B. V. L. (1994) *Tetrahedron Lett. 35*, 8457−8460.
37. Wilcox, R. A., Challiss, R. A. J., Traynor, J. R., Fauq, A. H., Ognyanov, V. I., Kozikowski, A. P., and Nahorski, S. R. (1994) *J. Biol. Chem. 269*, 26815−26821.
38. Hirata, M., Watanabe, Y., Kanematsu, T., Ozaki, S., and Koga, T. (1995) *Biochim. Biophys. Acta 1244*, 404−410.
39. Ferguson, K. M., Lemmon, M. A., Sigler, P. B., and Schlessinger, J. (1995) *Nat. Struct. Biol. 2*, 715−718.
40. Suhadolnik, R. J., Kariko, K., Sobol, R. W., Jr., Li, S. W., and Reichenbach, N. L. (1988) *Biochemistry 27*, 8840−8846.
41. Dorman, G., and Prestwich, G. D. (1994) *Biochemistry 33*, 5661−5673.
42. Prestwich, G. D., Dorman, G., Elliott, J. T., Marecak, D. M., and Chaudhary, A. (1997) *Photochem. Photobiol. 65*, 222−234.
43. Gerlt, J. A., and Youngblood, A. V. (1980) *J. Am. Chem. Soc. 102*, 7433−7438.
44. Marwood, R. D., Riley, A. M., Correa, V., Taylor, C. W., and Potter, B. V. L. (1999) *Bioorg. Med. Chem. Lett. 9*, 453−458.

BI990114R