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Design and synthesis of 4″,6″-unsaturated cyclic ADP-carbocyclic-ribose, a Ca^{2+}-mobilizing agent selectively active in T cells

Takashi Kudoh [a], Takashi Murayama [b], Minako Hashii [c], Haruhiro Higashida [c], Takashi Sakurai [b], Clarisse Maechling [d], Bernard Spiess [d], Karin Weber [e], Andreas H. Guse [e], Barry V.L. Potter [f], Mitsuhiro Arisawa [a], Akira Matsuda [a], Satoshi Shuto [a,*]

[a] Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan
[b] Department of Pharmacology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
[c] Department of Biophysical Genetics, Kanazawa University Graduate School of Medicine, Kanazawa 920-8640, Japan
[d] Institut Gilbert Laustriat, Département de Pharmacochimie de la Communication Cellulaire, UMR 7175, Faculté de Pharmacie, 74 route du Rhin, BP60024, F-67401 Illkirch, France
[e] The Calcium Signalling Group, University Medical Center Hamburg-Eppendorf, Center of Experimental Medicine, Institute of Biochemistry and Molecular Biology I: Cellular Signal Transduction, Martinistrasse 52, 20246 Hamburg, Germany
[f] Wolfson Laboratory of Medicinal Chemistry, Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, UK

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ABSTRACT

We previously developed cyclic ADP-carbocyclic-ribose (cADPcR, **3a**) as a stable mimic of cyclic ADP-ribose (cADPR, **1**), a Ca^{2+}-mobilizing second messenger. The unsaturated carbocyclic-ribose analogs of cADPR, i.e., 4″,6″-didehydro-cADPcR (**8a**) and its inosine congener 4″,6″-didehydro-cIDPcR (**8b**) were newly designed and successfully synthesized using the key intramolecular condensation reaction with *S*-phenyl phosphorothioate-type substrates. The Ca^{2+}-mobilizing potency of the compounds was examined in sea urchin egg homogenates, NG108-15 neuronal cells, and permeabilized Jurkat T-lymphocytes, which may indicate that 4″,6″-didehydro-cADPcR is the first cADPR analog selectively active in T cells. Acid–base behavior and conformation of **8a** were also investigated and compared with those of cADPcR.

1. Introduction

Considerable attention has been focused on cyclic ADP-ribose (cADPR, **1**, Fig. 1), a naturally occurring metabolite of NAD^+ originally reported by Lee and co-workers.[1] cADPR has been shown to mobilize intracellular Ca^{2+} in various cells, such as sea urchin eggs, pancreatic β-cells, smooth muscle, cardiac muscle, T-lymphocytes, and cerebellar neurons and therefore, in recent years, cADPR has been recognized as a general mediator involved in Ca^{2+} signaling.[2] A most recent property of cADPR shown using CD38 knockout mice is that it is critical for social behavior by regulating oxytocin secretion.[3]

Synthesis of cADPR analogs has been extensively investigated,[4–8] since these can be used in investigating the mechanism of cADPR-mediated Ca^{2+} signaling pathways and are also expected to be lead structures for the development of potential drug candidates, due to the important physiological roles of cADPR.[2] Because of the unique 18-membered pyrophosphate structure and instability of cADPR, chemical synthesis of cADPR and its analogs has proved to be rather difficult[4a,b,6a] and consequently, cADPR analogs have been synthesized predominantly by enzymatic and chemo-enzymatic methods using ADP-ribosyl cyclase-catalyzed cyclization under mild conditions.[5] For example, 8-NH_2-cADPR (**2**), which is a potent antagonist of cADPR, was enzymatically synthesized and effectively used in biological studies.[2,5a] However, the analogs obtained by these methods are limited due to the substrate-specificity of the ADP-ribosyl cyclase.[4a,b,5]

Therefore, in recent years, methods for the chemical synthesis of cADPR analogs have been extensively studied to develop useful cADPR analogs, which could not be prepared by enzymatic and chemo-enzymatic methods.[6–8] In the synthesis of cADPR and its analogs, construction of the large 18-membered ring structure is the key step, and we have developed an efficient method for forming the 18-membered ring employing *S*-phenyl phosphorothioate-type substrates.[6]

cADPR is in a zwitterionic form with a positive charge around the N(1)–C(6)–N^6 moiety (pK_a=8.3), making the molecule unstable, since the charged adenine moiety attached to the anomeric carbon of the N1-linked ribose can be an efficient leaving group. Accordingly, cADPR is readily hydrolyzed at the unstable N1-ribosyl linkage of its adenine moiety to produce ADP-ribose (ADPR), even in neutral aqueous solution.[9] Under physiological conditions, cADPR

* Corresponding author. Fax: +81 11 7063769.
E-mail address: shu@pharm.hokudai.ac.jp (S. Shuto).

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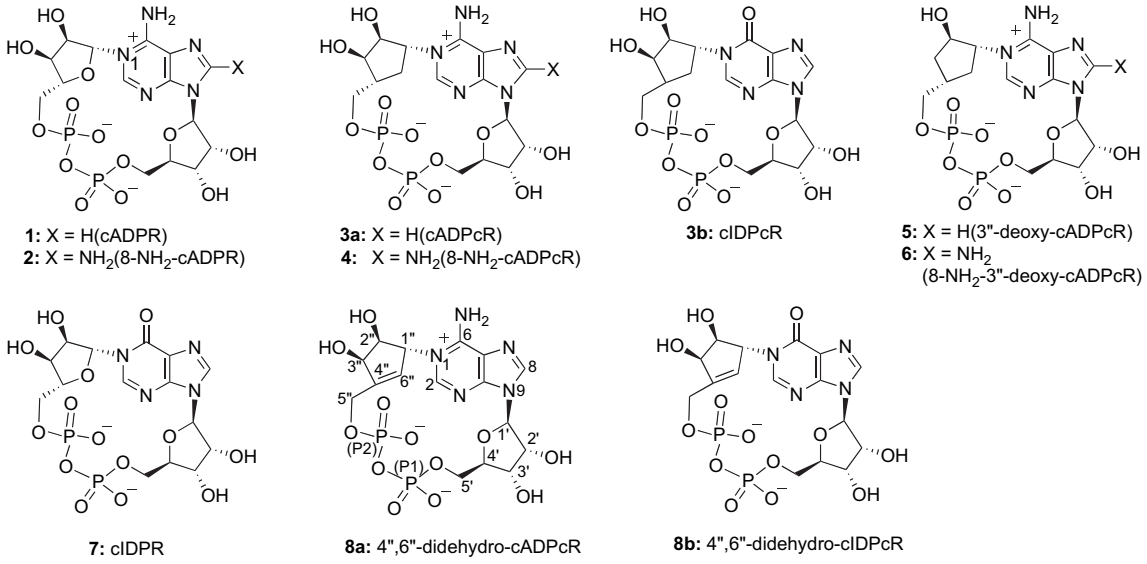


Figure 1. cADPR (**1**) and its analogues.

is also hydrolyzed at the N1-ribosyl linkage by cADPR hydrolase to give the inactive ADPR.[9] Therefore, we designed cyclic ADP-carbocyclic-ribose (cADPcR, **3a**) as a stable mimic of cADPR, in which the oxygen atom in the N1-ribose ring of cADPR is replaced by a methylene group. Using the chemical synthetic method described above, we accomplished the total synthesis of cADPcR,[6d] to show that it is actually resistant to both enzymatic and chemical hydrolysis due to its stable N1-alkyl linkage instead of the unstable N1-glycosidic linkage of cADPR, and that it, like cADPR, effectively mobilizes intracellular Ca^{2+} in sea urchin eggs.[6d]

Further structure–activity relationship (SAR) studies employing cADPcR as the lead structure with evaluation in the sea urchin egg system have shown that the N1-ribose moiety may be critically important for Ca^{2+}-mobilizing potency and also agonist/antagonist switching of cADPR activity.[7] For example, deletion of the 3″-hydroxyl of cADPcR resulted in providing the most potent Ca^{2+}-mobilizing compound 3″-deoxy-cADPcR (**5**) in the series;[7c] only a subtle change, i.e., '–O–' into '–CH_2–', brought about a dramatic switch from a highly potent antagonist 8-NH_2-cADPR (**2**) to an almost equipotent agonist 8-NH_2-cADPcR (**4**);[7a] and also, when the 3″-hydroxyl was removed from 8-NH_2-cADPcR, it gave a borderline compound 8-NH_2-3″-deoxy-cADPcR (**6**), a potent partial agonist with a remarkably low EC_{50} value.[7e]

Our attention has been focused on developing cell-type selective cADPR analogs, which can be useful as biological tools and/or potential drug leads. Thus, we found that although cADPcR is more potent than cADPR in neuronal cells,[7b] it is almost inactive in T cells, while cADPR works effectively as a Ca^{2+}-mobilizing second messenger in the same T cell system.[6e,7c] These are very important findings indicating that the target proteins and/or the mechanism of action of cADPR in T cells and neuronal cells are different.

As described above, synthetic studies of compounds employing cADPcR as the lead structure have provided a series of biologically and chemically stable mimics of cADPR and disclosed SAR of cADPR analogs. In the course of these studies, we newly designed and synthesized the unsaturated carbocyclic-ribose analog of cADPR, i.e., 4″,6″-didehydro-cADPcR (**8a**) and its inosine congener 4″,6″-didehydro-cIDPcR (**8b**). Biological evaluation in several systems demonstrated that 4″,6″-didehydro-cADPcR is the first cADPR analog selectively active in T cells. Acid–base behavior and conformation of **8a** were also investigated and compared with those of cADPcR. In this paper, we describe these results in detail.[10]

2. Results and discussion

2.1. Design of the target compounds

Carbocyclic nucleosides have been known as effective mimics of natural nucleosides.[11] An antibiotic aristeromycin (Fig. 2) is a naturally occurring mimic of adenosine, in which the ribose of adenosine is replaced by a carbocyclic-ribose, and has antiviral and antitumor activities, due to its competitive inhibition to adenosine against *S*-adenosylhomocysteine (AdoHcy) hydrolase.[12,13] A cADPR analog, the adenosine moiety of which was replaced with aristeromycin, has been reported as a hydrolysis resistant cADPR mimic.[5h]

Another nucleosidic antibiotic neplanocin A (Fig. 2), the structure of which corresponds to the 4′,6′-unsaturated derivative of aristeromycin, is one of the most potent inhibitor of AdoHcy hydrolase known so far.[12,14] Because of the remarkable inhibitory effect on the enzyme, neplanocin A has significant antiviral and also antitumor effects, clearly surpassing aristeromycin. Therefore, an unsaturated carbocyclic-ribose may mimic the ribose more precisely than the corresponding saturated congener.

Similar improvements in pharmacological potency of compounds by replacing a saturated tetrahydrofuran or thiophene ring with the corresponding unsaturated cyclopentene congener have also been observed, for example, in studies of carba-analogs of prostanoids[15a] or β-lactam antibiotic carbapenems.[15b] In these compounds with a cyclopentene ring, the π-electrons of the unsaturated bond might effectively mimic the unpaired electrons on oxygen or a sulfur atom in the ring.

In nucleosides, conformation around the glycoside linkage is one of the determinants of their biological activities, and they generally prefer a conformation that avoids steric repulsion

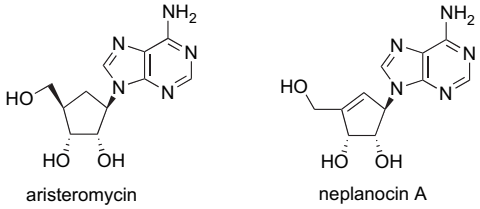


Figure 2. Naturally occurring carbocyclic adenine nucleosides.

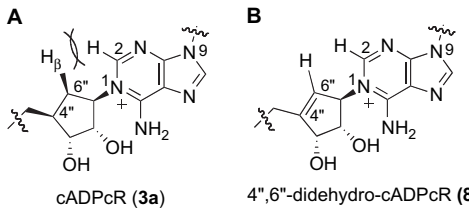


Figure 3. Possible steric effect between the carbocyclic and adenine moieties: A, in cADPcR (**3a**); B, in 4″,6″-didehydro-cADPcR (**8a**).

between the nucleobase and sugar moieties.[16] Therefore, in cADPR and its analogs, the most stable conformation can be that in which steric repulsion between the adenine moiety and the N1- and N9-ribose moieties, particularly between the adenine H-8 and the N9-ribose H-2′ and also between the adenine H-2 and the N1-ribose H-2″, is minimal. It should be noted that, in cADPcR, the sp^3-hydrogens on the tetrahedral C6″, especially the H-6″β, which is absent in cADPR, can be sterically repulsive to the adenine H-2 (Fig. 3A), which might make its conformation different from that of cADPR at least to some extent. When the carbocyclic-ribose of the N1-moiety is replaced with the corresponding 4,6-unsaturated one, a sterically repulsive sp^3-hydrogen on the C6″ is absent, as shown in Figure 3B. Therefore, we thought that, in 4″,6″-didehydro-cADPcR (**8a**), three-dimensional positioning of the N1-unsaturated carbocyclic-ribose and the adenine moieties might be more analogous to that in cADPR.

Another difference between cADPR and cADPcR is the pK_a value for protonation at the N^6-position. The pK_a value of cADPcR is somewhat higher compared with that of cADPR,[6d] which might affect features important for binding to target proteins. Introduction of an unsaturated bond into the carbocyclic-ribose of cADPcR might lower the pK_a and accordingly, the pK_a of 4″,6″-didihydro-cADPcR might be more similar to that of cADPR.

Taking these results and considerations into account, we decided to synthesize the 4″,6″-unsaturated analog **8a** (4″,6″-didehydro-cADPcR) of cADPcR as a new target and investigate its Ca^{2+}-mobilizing effect.

We previously synthesized cyclic IDP-carbocyclic-ribose (**3b**),[6a,c] the inosine congener of cADPcR, and showed that it was inactive in sea urchin eggs and in neuronal cells.[6d,7b] We also planned to synthesize and evaluate the 4″,6″-unsaturated analog **8b** of cIDPcR, since cyclic inosine diphosphate ribose (**7**, cIDPR) and its derivatives synthesized recently have been disclosed to have potent Ca^{2+}-mobilizing effects.[5g,8b–e]

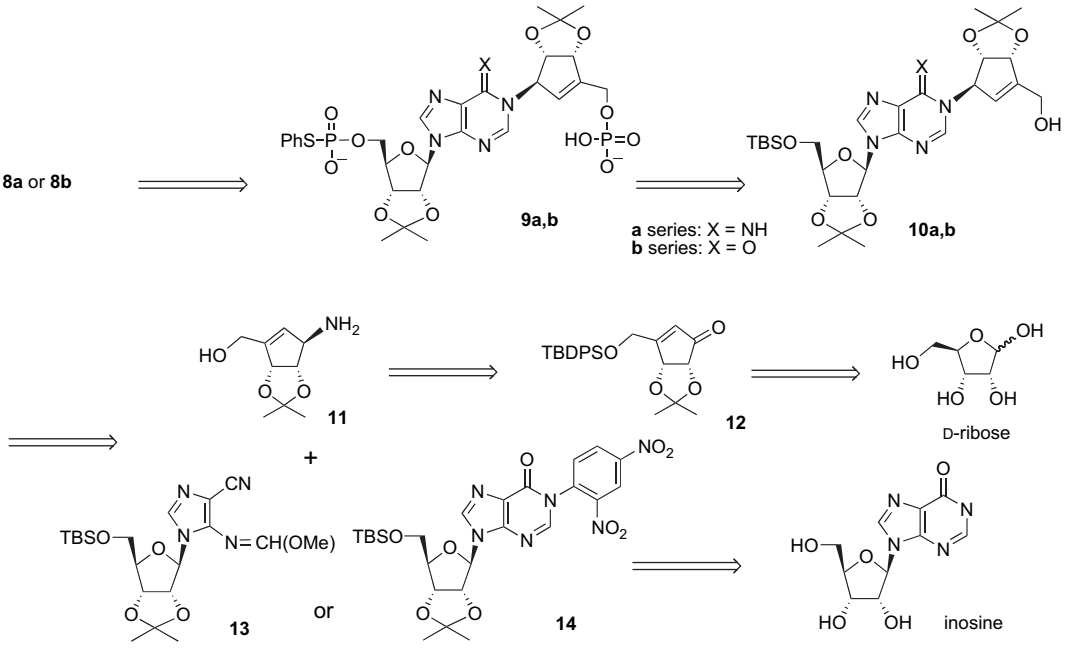
2.2. Synthetic plan

We planned to synthesize the target 4″,6″-didehydro-cADPcR (**8a**) and its inosine congener **8b** by a route, as summarized in Scheme 1, in which intramolecular condensation reaction with a *S*-phenyl phosphorothioate-type substrate forming the 18-membered pyrophosphate structure was employed as the key step. This method was developed by us[6] and has been effectively used by other groups[8] in the synthesis of a variety of cADPR analogs.

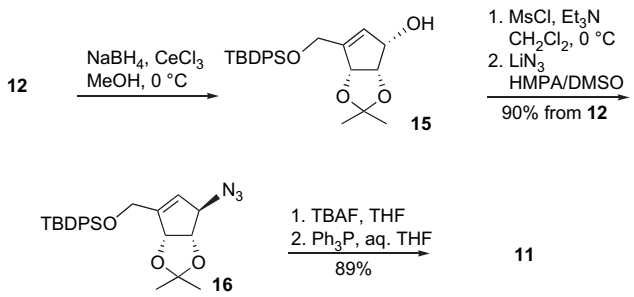
Thus, treatment of *S*-phenyl phosphorothioate-type substrates **9a** or **9b** having the N1-4″,6″-unsaturated carbocyclic-ribose with $AgNO_3$/MS 3 Å as a promoter[6b–d] was expected to form the desired cyclized products, subsequent acidic treatment of which for deprotection would furnish the target **8a** or **8b**. The *S*-phenyl phosphorothioate-type substrates **9a** and **9b** could be converted from the N1-unsaturated carbocyclic-ribosyl adenosine derivative **10a** and its inosine congener **10b**, which would be constructed by condensation between the optically active cyclopentenyl amine **11** with known nucleoside **13**[6d,17a] or **14**,[6c,17b] readily prepared from inosine. The amine **11** would be prepared from **12**, which can be synthesized from D-ribose by the procedure recently developed by Jeong and co-workers.[18]

2.3. Synthesis

The chiral cyclopentenyl amine **11** was prepared from a known chiral cyclopentenone **12**[18]as shown in Scheme 2.[19] 1,2-Reduction of the enone system of **12** with $NaBH_4/CeCl_3$ stereoselectively gave the allylic α-alcohol **15**, of which mesylation and subsequent treatment with LiN_3 in HMPA/DMSO afforded the β-azide **16.** Removal of the TBDPS group of **16**, followed by reduction of the azido group with Ph_3P in aqueous THF gave the desired amine **11**.



Scheme 1. Synthetic plan for the target compounds **8a** and **8b**.



Scheme 2. Synthesis of the chiral cyclopentenyl amine **11**.

4″,6″-Didehydro-cADPcR (**8a**) and -cIDPcR (**8b**) were successfully synthesized from the chiral cyclopentenyl amine **11** as shown in Scheme 3. The 4″,6″-didehydro-N1-carbocyclic-ribosyl adenosine derivative **10a** was obtained in 58% yield by the treatment of a mixture of the amine **11** and the imidazole nucleoside **13** with K_2CO_3 in MeOH at room temperature. When the amine **11** was heated with inosine derivative **14** in DMF at 50 °C and then the resulting mixture was treated with K_2CO_3, it produced the corresponding N1-substituted inosine derivative **10b** in 35% yield. The 5″-hydroxy group of **10a** or **10b** was protected with a dimethoxytrityl (DMTr) group and the 5′-*O*-TBS group of the product was removed with TBAF to give **17a** or **17b**, respectively. Treatment of **17a** or **17b** with an *S*,*S*′-diphenylphosphorodithioate (PSS)/2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl)/pyridine system[20] gave the 5′-bis(phenylthio)phosphate **18a** or **18b**, respectively. After removal of 5″-*O*-DMTr group of **18a** or **18b** with aqueous AcOH, a phosphoryl group was introduced at the resulting 5″-primary hydroxyl by Yoshikawa's method with $POCl_3/(EtO)_3PO$,[21] followed by treatment of the product with H_3PO_2 and Et_3N[22] in the presence of *N*-methylmaleimide (NMM) in pyridine,[6d] to afford the corresponding *S*-phenyl phosphorothioate **9a** or **9b**, respectively. Although the yield of this step was low, probably due to the unstable allylic phosphate system in the reaction product, the substrates **9a** and **9b** for the key intramolecular condensation reaction[23] were obtained.

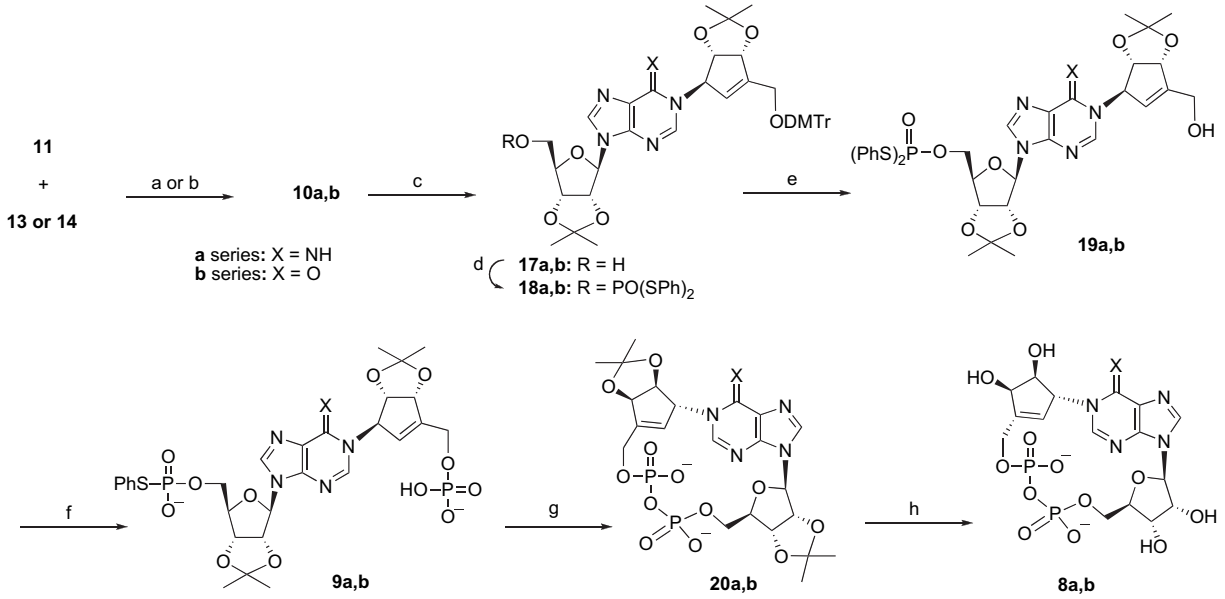
When a solution of **9a** in pyridine was added slowly to a mixture of a large excess of $AgNO_3$ and Et_3N in the presence of MS 3 Å in pyridine at room temperature,[6] the desired cyclization product **20a** was obtained in 65% yield. Similarly, the other substrate **9b** was condensed to give the cyclization product **20b** in 57% yield. Finally, removal of the isopropylidene groups of **20a** and **20b** with aqueous HCO_2H, followed by treatment with aqueous ammonia furnished 4″,6″-didehydro-cADPcR (**8a**) and its inosine congener 4″,6″-didehydro-cIDPcR (**8b**), respectively.

2.4. Ca^{2+}-mobilizing activity in sea urchin egg homogenate

The Ca^{2+}-mobilizing ability of 4″,6″-didehydro-cADPcR (**8a**) and its inosine congener **8b** in sea urchin egg homogenate was determined fluorometrically by monitoring Ca^{2+} with fura-2,[24] and the results were compared with those of cADPR (**1**) and cADPcR (**3a**) (Fig. 4). 4″,6″-Didehydro-cADPcR (**8a**) showed slight Ca^{2+} release above 10 μM (Fig. 4A). The EC_{50} value of **8a** was ~190 μM (assuming that it is a full agonist), which is over 2000 times larger than that of cADPcR (EC_{50}=79 nM). Ca^{2+} release by 1 μM cADPcR after treatment with **8a** was not affected up to 3 μM and consistently reduced at 10 and 30 μM, indicating that **8a** does not have antagonistic activity (Fig. 4B). Thus, **8a** is only slightly active for cADPR-induced Ca^{2+} mobilization in sea urchin egg homogenate. The inosine congener **8b** did not show any agonistic and antagonistic activity on cADPR-induced Ca^{2+} release.

2.5. Ca^{2+}-mobilizing activity in neuronal cells

The effect of 4″,6″-didehydro-cADPcR (**8a**) and its inosine congener **8b** on the Ca^{2+}-mobilizing activity was also studied in neuronal cells, using the combined patch voltage-clamp and Ca^{2+}-monitoring technique.[7b] The membrane of NG108-15 mouse neuroblastoma x rat glioma hybrid cell was sealed with a patch pipette, disrupted to infuse the compound, and then voltage-clamped. The two analogs **8a** and **8b** did not evoke any apparent changes in $[Ca^{2+}]_i$ by themselves at the resting membrane potential of around −40 mV, as previously observed for cADPcR.[7b,c] We next examined the effect of **8a** and **8b** on depolarization-induced $[Ca^{2+}]_i$ elevation using an identical protocol for testing cADPcR (Fig. 5). Membrane depolarization from −40 to −20 mV in control cells evoked a slight $[Ca^{2+}]_i$ increase with an average peak value of 167±18%



Scheme 3. Synthesis of the target compounds **8a** and **8b**. Reagents and conditions: (a) K_2CO_3, MeOH, rt, 58% (**10a**); (b) (1) DMF, 50 °C, (2) K_2CO_3, DMF, 50 °C, 35% (**10b**); (c) (1) DMTrCl, pyridine, rt, (2) TBAF, THF, AcOH, rt, quant. (**17a**), 61% (**17b**); (d) PSS, TPSCl, pyridine, rt, 74% (**18a**), 71% (**18b**); (e) aq 60% AcOH, rt, 80% (**19a**), 79% (**19b**); (f) (1) $POCl_3$, $(EtO)_3PO$, 0 °C, (2) H_3PO_2, Et_3N, NMM, pyridine, 0 °C–rt, 28% (**9a**), 36% (**9b**); (g) $AgNO_3$, MS 3 Å, Et_3N, pyridine, rt, 65% (**20a**), 57% (**20b**); (h) aq HCO_2H, rt, (2) aq NH_3, 86% (**8a**), 82% (**8b**).

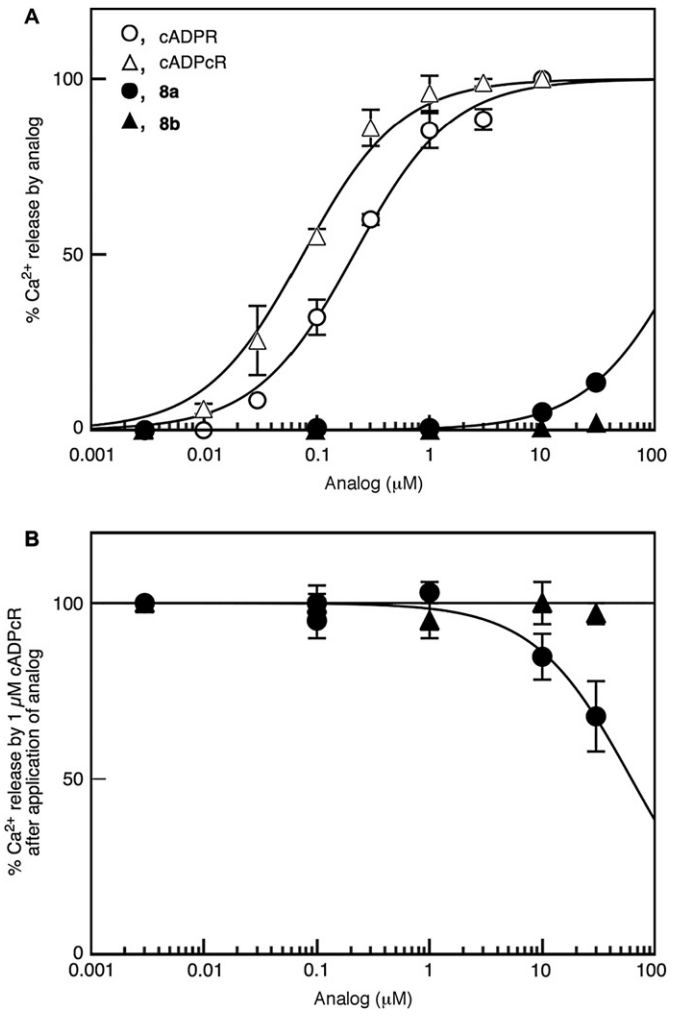


Figure 4. Ca^{2+}-mobilizing activity of 4″,6″-didehydro-cADPcR (**8a**) and its inosine congener **8b** in sea urchin egg homogenate. The activity was measured fluorometrically with fura-2. (A) Dose-dependent Ca^{2+} release by the compounds. The Ca^{2+} release by each compound was normalized by Ca^{2+} release with 10 μM cADPR. (B) Evaluation of antagonistic activity. Ca^{2+} release by 1 μM cADPcR was measured after application of indicated concentrations of the compounds. Reduction in the cADPcR-induced Ca^{2+} release by **8a** corresponds to its agonistic activity, indicating that it has no antagonistic activity. Data are mean±SEM (n=3).

(mean±SEM; n=4) of the pre-depolarization level, resulting from activated voltage-activated Ca^{2+} channels (VACCs) and subsequent Ca^{2+} release by Ca^{2+}-induced Ca^{2+} release (CICR) mechanism. Injection of **8a** at 10 μM, which could induce a near maximum response and discriminate the difference in efficiency, showed slight and statistically non-significant potentiating effect on the depolarization-induced $[Ca^{2+}]_i$ rise. The average $[Ca^{2+}]_i$ level was 207±22% (n=9) at the initial peak. The inosine congener **8b** showed no effect on depolarization-induced $[Ca^{2+}]_i$ increases (Fig. 5A and B). On the other hand, injection of 10 μM of cADPcR facilitated depolarization-induced $[Ca^{2+}]_i$ increases: the peak $[Ca^{2+}]_i$ level was 295±30% (n=4) of the pre-depolarization level, which was equal or more potent than that of natural cADPR, as previously described.[7b,c] Therefore, our results indicate that **8a** does not possess apparent Ca^{2+}-mobilizing activity in NG108-15 neuronal cells, in contrast to cADPcR as the potent ligand.

2.6. Ca^{2+}-mobilizing activity in T cells

The Ca^{2+}-mobilizing effect of **8a** and **8b** was evaluated also in permeabilized Jurkat T cells. Both cADPR (**1**) and cIDPR (**7**) released Ca^{2+} from intracellular stores with similar potency.[5g] While cADPcR (**3a**) as compared to cADPR (**1**) was only a weak agonist for Ca^{2+} release in permeabilized Jurkat T cells,[6e] 4″,6″-didehydro-cADPcR (**8a**) was much more potent (Fig. 6A). At 100 μM 4″,6″-didehydro-cADPcR induced a robust response while almost no effect of cADPcR was obtained (Fig. 6A). Interestingly, at 500 μM 4″,6″-didehydro-cADPcR a significantly higher amplitude was observed as compared to cADPR (Fig. 6A). Though cIDPR showed a concentration–response curve very similar to cADPR,[5g] its carbocyclic inosine congener cIDPcR (**3b**) was not active up to 500 μM (Fig. 6A).

In the next series of experiments, the potential antagonistic effect of cADPcR, cIDPcR, 4″,6″-didehydro-cADPcR, and 4″,6″-didehydro-cIDPcR on Ca^{2+} release induced by a submaximal concentration of cADPR (30 μM) was analyzed. No statistically significant differences as compared to controls were observed up to 500 μM for all compounds (Fig. 6B).

2.7. NMR titration study

We next investigated the acid–base behavior of the ionizable groups for 4″,6″-didehydro-cADPcR (**8a**) and also for cADPcR (**3a**), since the ionizable groups, such as N^6-imino or the pyrophosphate group, may have a critical role in the binding of the compound with target biomolecules.

The ^1H and ^{31}P NMR titration experiments were done in the pH range 2.5–11.5 for cADPcR and 4″,6″-didehydro-cADPcR in ^2H_2O. Provided that the observed chemical shift variation for the phosphorus and for the proton resonances mainly depends on the electronic effects accompanying the protonation process, the protonation equilibrium constants could be derived from the chemical shift variations of the nuclei as a function of pH.[25] Both compounds show only one protonation equilibrium in the studied pH range, which corresponds to an imino base equilibrium on the adenine ring (Fig. 7).[4] The corresponding pK_a values determined are, respectively, 8.65 and 8.45 for cADPcR and 4″,6″-didehydro-cADPcR. No acidic pK_a value was evidenced showing that phosphate group protonation takes place at a pH lower than 2.5.

Therefore, while the ionizable groups of cADPcR and of 4″,6″-didehydro-cADPcR behave similarly in the pH range 2.5–11.5, introduction of an unsaturated bond into the carbocyclic-ribose of cADPcR (8.65) lowered the pK_a, as we expected. The pK_a of 4″,6″-didehydro-cADPcR (8.45) was rather similar to that of cADPR (8.3).[26]

Difference in chemical shift values, $\Delta\delta=\delta_p-\delta_d$ (ppm), between fully protonated, δ_p, and fully deprotonated, δ_d, state is shown in Table 1. The adenine ring protons H2 and H8, close to the imine group, are good reporter nuclei for the protonation, and consequently show relatively large chemical shift variation, in almost the same extent for both compounds.

In the N9-linked ribose ring, only H1′, the nearest from the protonable group, shows a significant variation in chemical shift values, and the curves for both compounds are almost superimposed.

The phosphorus nuclei of P1, the phosphate group linked to the N9-ribose moiety, and P2, the phosphate linked to the N1-carboxyribose moiety, are differently affected by protonation in cADPcR and 4″,6″-didehydro-cADPcR (Fig. 8A). Both phosphate groups of 4″,6″-didehydro-cADPcR are quite equally sensitive to the protonation process, whereas only phosphate P2, linked to the carbocyclic-ribose ring, shows significant chemical shift variation during protonation (Fig. 8A).

Finally, in case of the N1-linked carbocyclic-ribose ring, one can see in Table 1 clear differences in cADPcR and 4″,6″-didehydro-cADPcR data. The most interesting nucleus is the H1″ proton, which shows a negative chemical shift variation during protonation in the case of cADPcR and a positive chemical shift variation in case of the didehydro analog **8a** (Fig. 8B). Such a wrongway shift, as for

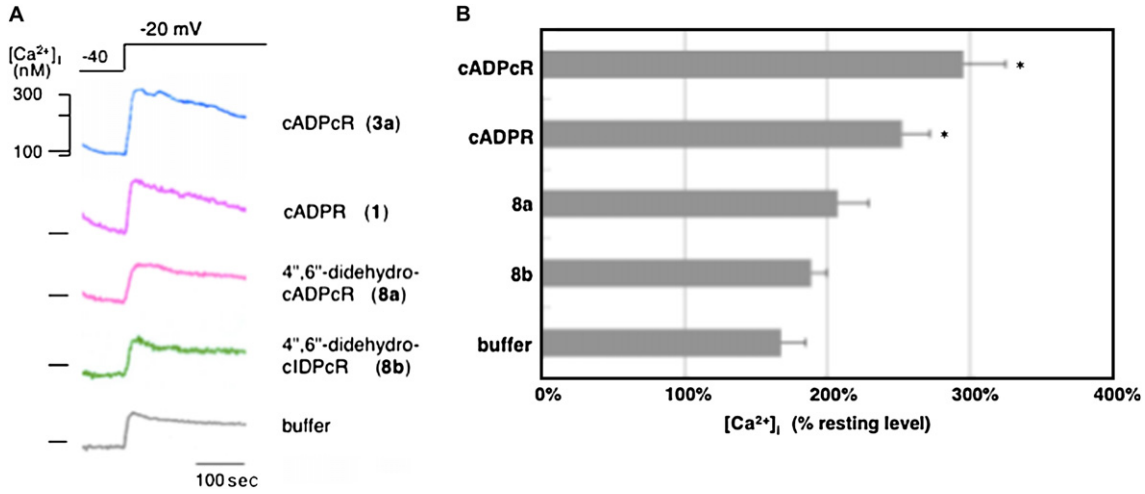


Figure 5. Effects of cADPR, cADPcR, 4″,6″-didehydro-cADPcR (**8a**), and its inosine congener **8b** on membrane depolarization-evoked $[Ca^{2+}]_i$ increases in patch-clamped NG108-15 cells. (A) Traces show $[Ca^{2+}]_i$ changes in fura-2 loaded cells infused with 10 mM of cADPcR, cADPR, **8a**, **8b** or without compound (buffer). At about 100 s before the beginning of each trace, cell membranes were ruptured with pipettes filled with these test compounds. Then, the membranes were voltage-clamped at −40 mV, followed by depolarization to −20 mV at the indicated point. (B) $[Ca^{2+}]_i$ levels were plotted at the peak after depolarization to −20 mV and are given as percentages of that at −40 mV. Each bar is mean±SEM of 4–9 experiments. *Values in cells pretreated with cADPcR or cADPR were significantly higher than those in cells without compounds at $p<0.05$.

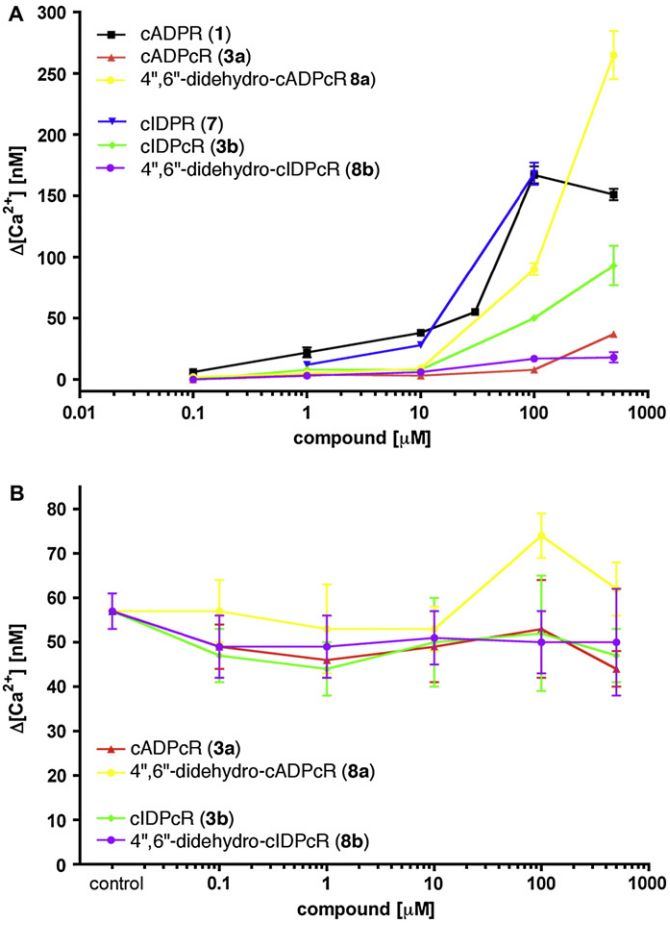


Figure 6. Effects of cADPR and its analogues in permeabilized Jurkat T cells. Jurkat T cells were permeabilized by saponin. Ca^{2+} stores were refilled by addition of ATP and ATP-regenerating system. $[Ca^{2+}]$ was determined fluorometrically using fura-2/free acid. (A) Compounds were added at the concentrations indicated. Data are differences between the Ca^{2+} concentration upon addition and before addition of compound. Mean±SEM (n=3–6). (B) Compounds were added at the concentrations indicated prior to addition of cADPR (30 mM). Data represent the Ca^{2+} release by cADPR (30 mM) and are differences between the Ca^{2+} concentration upon addition and before addition of cADPR. Mean±SEM (n=3–6).

cADPcR, may be explained by a conformational change, already observed, for instance, when a phosphate group approaches the proton,[27] or by a modification in ring currents during protonation.[28]

No unexpected effect is seen in H2″ titration profile, this proton is much more affected by the protonation in cADPcR than in 4″,6″-didehydro-cADPcR. Almost the same profile, but in a lesser extent, is seen for H6″ nuclei.

2.8. Conformational analysis

The three-dimensional structure of biologically active compounds in aqueous solution is very important from the viewpoint of bioactive conformation. The conformations of 4″,6″-didehydro-cADPcR (**8a**) were constructed by molecular dynamics calculations with a simulated annealing method based on the NOE constraints of the intramolecular proton pairs measured in D_2O.[7c]

In Figure 9, the structure of **8a** obtained is shown with those of cADPR (**1**) and cADPcR (**3a**) by the same method.[7c] The calculated structures of cADPR (Fig. 9A) and cADPcR (Fig. 9B) were analogous, each adopting a 2′-*endo* form in the N9-ribose moiety and a *syn*-form around the N9-glycosyl linkage. However, their three-dimensional arrangement of the N1-ribose moiety and the adenine ring is somewhat different. In cADPcR, the adenine H-2 is located above the center of the N1-carbocyclic-ribose ring due probably to its repulsion to the H-6″β, where the H-2 is located just above the ring-oxygen of the N1-ribose in cADPR.

In 4″,6″-didehydro-cADPcR (**8a**), arrangement of the N1-ribose moiety and the adenine ring is inverted compared with those of cADPR and cADPcR, in which the rather bulky 6-NH_2 group is located just above the plane sp^2-C6, and the H2 is near to the H1″ of the carbocyclic-ribose. This may be because a sterically repulsive β-hydrogen on the sp^3-C6 in cADPcR is absent in 4″,6″-didehydro-cADPcR. In addition, the flat C1″–C6″–C4″–C5″-structure might distort the conformation of the large 18-membered ring. As a result, its entire three-dimensional structure of the molecule is

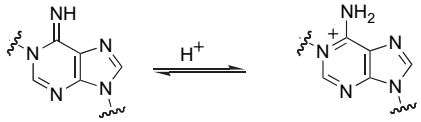


Figure 7. Imino base equilibrium on the adenine moiety.

Table 1
Difference in chemical shift values, $\Delta\delta=\delta_p-\delta_d$ (ppm), between fully protonated, δ_p, and fully deprotonated, δ_d, state

	Adenine ring protons		N9-ribose protons						N1-carboxylic-ribose protons						Pyrophosphate	
	H2	H8	H1′	H2′	H3′	H4′	H5′a	H5′b	H1″	H2″	H3″	H4″	H5″	H6″a	H6″b	P1	P2
cADPcR (**3a**)	0.50	0.40	0.16	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.40	0.32	0.13	<0.1	0.19	0.21	0.1	−0.32
4″,6″-Didehydro-cADPcR (**8a**)	0.58	0.40	0.15	<0.1	—	<0.1	<0.1	<0.1	0.10	<0.1	0.13	—	<0.1	<0.1		−0.31	−0.23

Significant values are greater than 0.1 ppm.

significantly different from that of cADPR or cADPcR, which can be the reason why 4″,6″-didehydro-cADPcR is almost inactive in sea urchin egg and neuronal cell systems.

2.9. Discussion

As described above, the 4″,6″-unsaturated analogs **8a** and **8b** have been successfully synthesized, which, concomitant with the previous examples, clearly demonstrates that the strategy employing a *S*-phenyl phosphorothioate-type substrate in the key intramolecular condensation reaction forming the pyrophosphate linkage is very effective in total syntheses of cADPR-related compounds.

Considering the highly potent biological activity of neplanocin A, the 4′,6′-unsaturated carbocyclic adenosine analog, we designed and synthesized 4″,6″-didehydro-cADPcR (**8a**). However, 4″,6″-didehydro-cADPcR is shown to be almost inactive in sea urchin homogenate and also in neuronal cell systems. The conformational analysis study suggested that the entire three-dimensional structure of 4″,6″-didehydro-cADPcR is significantly different from that of cADPR and cADPcR, which might be the reason why 4″,6″-didehydro-cADPcR has only slight Ca^{2+}-mobilizing activity in the sea urchin and neuronal cell systems.

The titration studies suggest that significant conformational change of the N1-carbocyclic-ribose moiety may occur dependent on the adenine ring protonation in cADPcR, but not in 4″,6″-didehydro-cADPcR. Although similar protonation-dependent conformational changes have been observed in biologically active phosphate compounds, such as inositol trisphosphate or inositol tetrakisphosphate,[26] this is the first example disclosed in cADPR-related

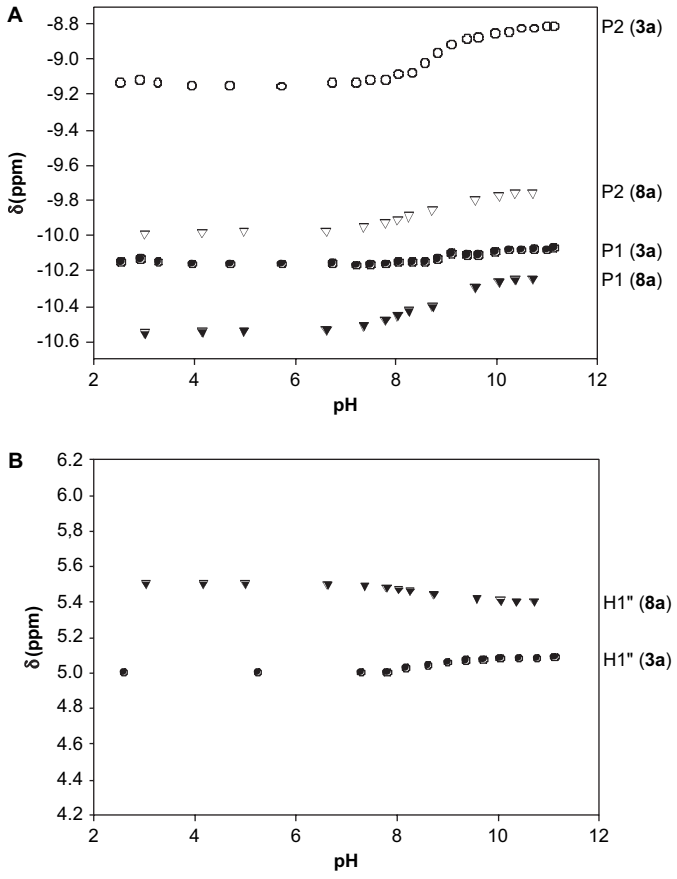


Figure 8. (A) ^{31}P NMR titration of cADPcR (**3a**) (circles) and 4″,6″-didehydro-cADPcR (**8a**). Full symbols stands for P1 (phosphate group linked to the N9-ribose moiety) and open symbols stands for P2 (phosphate group linked to the N1-carboxyribose moiety). (B) ^{1}H NMR titration of H1″ nucleus of cADPcR (**3a**) (circles) and 4″,6″-didehydro-cADPcR (**8a**) (triangles).

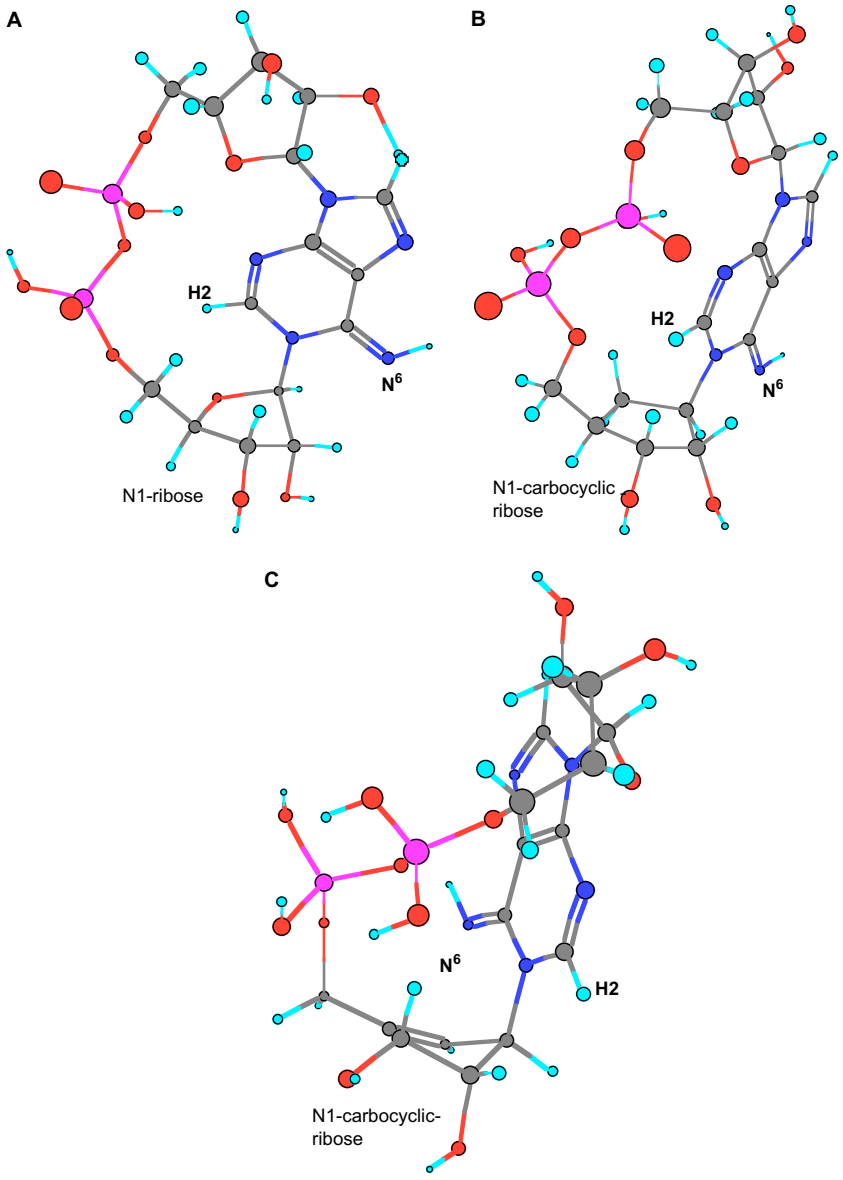


Figure 9. Calculated structures of cADPR (**1**, **A**), cADPcR (**3a**, **B**), and 4″,6″-didehydro-cADPcR (**8a**, **C**) by molecular dynamics calculations with a simulated annealing method based on the NOE data in D_2O.

compounds. This kind of conformational change might be important in the interaction between the target proteins and cADPR or its analogs, and the difference between cADPcR and 4″,6″-didehydro-cADPcR in the biological activity might be related to the protonation-dependent conformational behavior of the molecule.

We showed that 4″,6″-didehydro-cADPcR is active in T cells, but is almost inactive in sea urchin egg homogenate and also in neuronal cells, which may indicate some selectivity in T cells. cADPR analogs having selectivity between mammalian cell types, i.e., compounds selectively active in some mammalian cells but inactive in other mammalian cells would be useful tools to investigate the target biomolecules as well as the Ca^{2+}-mobilizing mechanisms. We recently identified cADPcR and its 3″-deoxy derivative **5** as the first kind of mammalian cell-type selective compound: these compounds are significantly active in neuronal cells but almost inactive in T cells. 4″,6″-Didehydro-cADPcR was identified as another example having selectivity between mammalian cell types. The cell-type selectivities of 4″,6″-didehydro-cADPcR and cADPcR are complementary, i.e., T cell-selective and neuronal cell-selective, although no other cell types have yet been studied. These results may indicate that the recognition mechanism of cADPR by the target proteins could be different in T cells and neuronal cells. However, on the other hand, our results may simply reflect the difference between the cell preparations used: while in the neuronal model compounds were infused by the patch pipette, permeabilized T cells were prepared by several wash steps during which cytosolic proteins are lost. Although cytosolic proteins also diffuse into patch pipettes, the loss of potentially important factors is slow and likely less complete.

It is interesting that some aspects of the SAR of compounds in neuronal cells are likely to be related to that in sea urchin eggs. For example, cADPcR and 3″-deoxy-cADPcR are significantly active both in sea urchin egg homogenate and neuronal cells, but inactive in T cells.[7c] On the contrary, 4″,6″-didehydro-cADPcR and -cIDPcR are inactive in sea urchin egg homogenate and neuronal cells, but active in T cells. On the other hand, the EC_{50} of cADPR in sea urchin egg homogenates is 79 nM, while active concentrations in mammalian cells, e.g., neuronal or T cells, are in the micromolar-range. Clear-cut conclusions require molecular identification of cADPR receptors. Recent data showing that cADPR, in addition to induction

of intracellular Ca^{2+} release via ryanodine receptors, may also be involved in the fine tuning of the plasma membrane Ca^{2+} and Na^{+} channel TRPM2.[30] Thus, this second cADPR target complicates the situation dramatically, especially when data are obtained in intact cells.[30]

3. Conclusion

The unsaturated carbocyclic-ribose analogs of cADPR, i.e., 4″,6″-didehydro-cADPcR (**8a**) and its inosine congener 4″,6″-didehydro-cIDPcR (**8b**), designed as novel stable mimics of cADPR, were successfully synthesized using the key intramolecular condensation reaction with the *S*-phenyl phosphorothioate-type substrates. Titration studies on the compounds suggest that significant conformational change might occur dependent on protonation, which might be important for the biological activity of cADPR and its analogs. Evaluation of Ca^{2+}-mobilizing potency using three different biological systems, i.e., sea urchin egg homogenates, NG108-15 neuronal cells, and permeabilized Jurkat T-lymphocytes may indicate that 4″,6″-didehydro-cADPcR is the first cADPR analog to be selectively active in T cells. Thus, this study may be an entry to developing T cell-selective cADPR analogs, which can be useful as biological tools and/or drug leads.

4. Experimental

4.1. General

Chemical shifts are reported in parts per million downfield from Me_4Si (^1H), MeCN (^{13}C) or H_3PO_4 (^{31}P). All of the ^1H NMR assignments described were in agreement with COSY spectra. Thin-layer chromatography was done on Merck coated plate $60F_{254}$. Silica gel chromatography was done on Merck silica gel 5715. Reactions were carried out under an argon atmosphere.

*4.1.1. (1R,2S,3R)-1-Azido-4-(tert-butyldiphenylsilyloxymethyl)-2,3-O-isopropylidene-4-cyclopenten-2,3-diol (**16**)*

A mixture of **12** (1.19 g, 2.82 mmol), $NaBH_4$ (160 mg, 4.23 mmol), and $CeCl_3\cdot 7H_2O$ (1.05 g, 2.82 mmol) in MeOH (30 mL) was stirred at 0 °C for 10 min, and then aqueous AcOH (1 M, 2 mL) was added. The mixture was partitioned between EtOAc and H_2O, and the organic layer was washed with brine, dried (Na_2SO_4), and evaporated. A mixture of the residue, MsCl (327 μL, 4.23 mmol), and Et_3N (1.18 mL, 8.46 mmol) in CH_2Cl_2 (30 mL) was stirred at 0 °C for 15 min, and then ice cooled water was added. The mixture was partitioned between EtOAc and ice cooled water, and the organic layer was washed with brine, dried (Na_2SO_4), and evaporated. A mixture of the residue, LiN_3 (276 mg, 5.64 mmol), and HMPA (245 μL, 1.41 mmol) in DMSO (30 mL) was stirred at room temperature for 36 h. The mixture was partitioned between EtOAc and H_2O, and the organic layer was washed with brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (SiO_2, 5% EtOAc in hexane) to give **16** (1.14 g, 90%) as a colorless oil. ^1H NMR ($CDCl_3$, 500 MHz) δ 7.38–7.70 (m, 10H), 5.87 (m, 1H), 5.06 (d, 1H, *J*=5.6 Hz), 4.61 (d, 1H, *J*=5.6 Hz), 4.38 (m, 3H), 1.33, 1.32 (each s, each 3H), 1.10 (s, 9H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 150.9, 135.5, 134.8, 133.2, 133.2, 129.8, 127.8, 122.2, 112.1, 84.3, 83.3, 69.8, 61.1, 27.3, 26.8, 26.0, 19.3; HRMS (FAB, positive) calcd for $C_{25}H_{32}N_3O_3Si$: 450.2213 (MH^+), found: 450.2224.

*4.1.2. (1R,2S,3R)-1-Amino-4-hydroxymethyl-2,3-O-isopropylidenedioxy-4-cyclopentene (**11**)*

A mixture of **16** (1.49 g, 3.31 mmol) and TBAF (1.0 M in THF, 5.00 mL, 5.00 mmol) in THF (20 mL) was stirred at room temperature for 3 h, and then evaporated. The residue was purified by column chromatography (SiO_2, 25% EtOAc in hexane) to give the desilylated product (649 mg, 93%) as a colorless oil. A mixture of the desilylated product (649 mg, 3.00 mmol) and Ph_3P (1.58 g, 6.00 mmol) in THF/H_2O (1:1, 30 mL) was stirred at room temperature for 7 h. The reaction mixture was partitioned between EtOAc and H_2O, the organic layer was washed with H_2O, and the combined water layer was evaporated and lyophilized to give **11** (533 mg, 96%) as a white solid. ^1H NMR (DMSO-d_6, 500 MHz) δ 5.51 (m, 1H), 5.02 (d, 1H, *J*=5.6 Hz), 4.25 (d, 1H, *J*=5.6 Hz), 4.01 (d, 1H, *J*=15.6 Hz), 3.96 (d, 1H, *J*=15.6 Hz), 3.65 (m, 1H), 1.26, 1.23 (each s, each 3H); ^{13}C NMR (DMSO-d_6, 125 MHz) δ 145.6, 129.3, 110.1, 87.5, 83.2, 61.6, 58.1, 27.5, 25.9; HRMS (FAB, positive) calcd for $C_9H_{16}NO_3$: 186.1130 (MH^+), found: 186.1128.

*4.1.3. N-1-{(1R,2S,3R)-4-Hydroxymethyl-2,3-isopropylidenedioxycyclopent-4-en-1-yl}-5′-O-(tert-butyldimethylsilyl)-2′,3′-O-isopropylideneadenosine (**10a**)*

A mixture of **13** (39 mg, 90 μmol), **11** (17 mg, 90 μmol), and K_2CO_3 (1 mg, 7 μmol) in MeOH (1 mL) was stirred at room temperature for 28 h and then evaporated. The residue was partitioned between EtOAc and H_2O, and the organic layer was washed with brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (SiO_2, 5% MeOH in $CHCl_3$) to give **10a** (31 mg, 58%) as an oil. ^1H NMR ($CDCl_3$, 500 MHz) δ 7.80 (s, 1H), 7.62 (s, 1H), 6.00 (d, 1H, *J*=2.8 Hz), 5.91 (m, 1H), 5.73 (m, 1H), 5.22 (d, 1H, *J*=5.7 Hz), 5.04 (dd, 1H, *J*=2.8, 6.1 Hz), 4.86 (dd, 1H, *J*=6.1, 2.3 Hz), 4.59 (d, 1H, *J*=5.7 Hz), 4.46 (d, 1H, *J*=15.1 Hz), 4.40 (d, 1H, *J*=15.1 Hz), 4.38 (ddd, 1H, *J*=2.3, 3.7, 4.0 Hz), 3.82 (dd, 1H, *J*=3.7, 11.3 Hz), 3.77 (dd, 1H, *J*=4.0, 11.3 Hz), 1.59, 1.46, 1.36, 1.35 (each s, each 3H), 0.84 (s, 9H), 0.030, 0.024 (each s, each 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 154.4, 151.9, 144.3, 140.8, 136.7, 123.7, 122.9, 114.1, 112.6, 91.3, 86.9, 85.4, 84.7, 83.6, 81.3, 65.9, 63.5, 59.9, 27.4, 27.2, 25.8, 25.3, 18.0, −5.5, −5.6; HRMS (FAB, positive) calcd for $C_{28}H_{44}N_5O_7Si$: 590.3010 (MH^+), found: 590.3019; UV (MeOH) λ_{max}=260, 295 (sh) nm.

*4.1.4. N-1-{(1R,2S,3R)-4-Dimethoxytrityloxymethyl-2,3-isopropylidenedioxycyclopent-4-en-1-yl}-2′,3′-O-isopropylideneadenosine (**17a**)*

A mixture of **10a** (117 mg, 0.199 mmol) and DMTrCl (202 mg, 0.60 mmol) in pyridine (2 mL) was stirred at room temperature for 10 h. After addition of MeOH (2 mL), the resulting mixture was evaporated. The residue was partitioned between EtOAc and H_2O, and the organic layer was washed with brine, dried (Na_2SO_4), and evaporated. A mixture of the residue, TBAF (1.0 M in THF, 400 μL, 0.40 mmol), and AcOH (11 μL, 0.2 mmol) in THF (2 mL) was stirred at room temperature for 1 h and then evaporated. The residue was purified by column chromatography (SiO_2, 5% MeOH in EtOAc) to give **17a** (174 mg, quant.) as an oil. ^1H NMR ($CDCl_3$, 500 MHz) δ 7.66 (s, 1H), 7.63 (s, 1H), 6.84–7.47 (m, 13H), 6.00 (m, 1H), 5.97 (m, 1H), 5.78 (d, 1H, *J*=4.3 Hz), 5.05 (m, 3H), 4.55 (d, 1H, *J*=5.6 Hz), 4.48 (m, 1H), 4.01 (m, 1H), 3.92 (m, 1H), 3.82 (m, 1H), 3.79 (s, 6H), 3.75 (m, 1H), 1.64, 1.42, 1.37, 1.30 (each s, each 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 167.7, 158.6, 154.0, 151.7, 144.6, 144.5, 139.8, 138.1, 136.0, 135.7, 132.4, 130.8, 129.9, 129.9, 128.8, 128.0, 127.9, 126.9, 125.3, 121.4, 114.2, 113.2, 112.6, 93.9, 86.7, 85.7, 84.6, 84.0, 83.6, 81.4, 68.1, 66.1, 63.1, 61.3, 55.2, 38.7, 30.3, 28.9, 27.5, 26.2, 25.2, 23.7, 22.9; HRMS (FAB, positive) calcd for $C_{43}H_{48}N_5O_9$: 778.3452 (MH^+), found: 778.3458; UV (MeOH) λ_{max}=260, 295 (sh) nm.

*4.1.5. N-1-{(1R,2S,3R)-4-Dimethoxytrityloxymethyl-2,3-O-isopropylidenedioxycyclopent-4-en-1-yl}-5′-O-{bis(phenylthio)-phosphoryl}-2′,3′-O-isopropylideneadenosine (**18a**)*

After stirring a mixture of PSS (883 mg, 2.3 mmol) and TPSCl (631 mg, 2.1 mmol) in pyridine (8 mL) at room temperature for 1 h, **17a** (600 mg, 0.771 mmol) was added, and the resulting mixture was stirred at room temperature for further 2 h and then evaporated. The residue was partitioned between EtOAc and H_2O, and the

organic layer was washed with brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (SiO_2, 1% MeOH in $CHCl_3$) to give **18a** (591 mg, 74%) as a foam. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.68 (s, 1H), 7.56 (s, 1H), 6.82–7.46 (m, 23H), 5.99 (m, 2H), 5.95 (m, 1H), 5.19 (dd, 1H, J=2.1, 6.2 Hz), 4.97 (d, 1H, J=5.3 Hz), 4.94 (dd, 1H, $J_{3',2'}$=6.2, 2.7 Hz), 4.43 (m, 2H), 4.41 (d, 1H, J=5.3 Hz), 4.37 (m, 1H), 3.99 (d, 1H, J=14.5 Hz), 3.79 (s, 6H), 3.76 (d, 1H, J=14.5 Hz), 1.60, 1.40, 1.37, 1.27 (each s, each 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 158.6, 154.3, 151.1, 144.7, 144.6, 140.7, 137.4, 136.0, 135.8, 135.3, 135.2, 135.1, 135.1, 129.9, 129.7, 129.4, 128.6, 128.0, 127.9, 126.9, 125.8, 124.2, 122.0, 114.6, 113.2, 112.4, 90.9, 86.7, 84.7, 84.5, 83.7, 81.1, 65.7, 61.3, 55.2, 27.5, 27.1, 26.1, 25.3; ^{31}P NMR ($CDCl_3$, 202 MHz) δ 50.7 (s); HRMS (FAB, positive) calcd for $C_{55}H_{57}N_5O_{10}PS_2$: 1042.3284 ($MH^+$), found: 1042.3293; UV (MeOH) λ_{max}=260, 295 (sh) nm.

4.1.6. *N-1-{(1R,2S,3R)-4-Hydroxymethyl-2,3-isopropylidenedioxycyclopent-4-en-1-yl}-5′-O-{bis(phenylthio)-phosphoryl}-2′,3′-O-isopropylideneadenosine (**19a**)*

A solution of **18a** (551 mg, 0.529 mmol) in aqueous 60% AcOH (6 mL) was stirred at room temperature for 3 h and then evaporated. The residue was partitioned between EtOAc and aqueous saturated $NaHCO_3$, and the organic layer was washed with H_2O and brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (SiO_2, 10% MeOH in EtOAc) to give **19a** (314 mg, 80%) as an oil. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.69 (s, 1H), 7.63 (s, 1H), 7.28–7.49 (m, 10H), 5.99 (d, 1H, J=2.0 Hz), 5.87 (m, 1H), 5.72 (m, 1H), 5.17 (dd, 1H, J=2.0, 6.2 Hz), 5.12 (d, 1H, J=5.7 Hz), 4.94 (dd, 1H, J=6.2, 1.7 Hz), 4.46 (d, 1H, J=5.7 Hz), 4.37–4.44 (m, 4H), 4.33 (d, 1H, J=15.3 Hz), 1.59, 1.45, 1.35, 1.30 (each s, each 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 154.1, 152.4, 144.6, 140.8, 137.7, 135.3, 135.2, 135.1, 135.0, 129.7, 129.6, 129.4, 125.7, 125.7, 125.6, 125.6, 124.0, 122.4, 114.5, 112.5, 90.9, 85.0, 84.9, 84.5, 84.4, 83.4, 81.1, 66.4, 65.9, 59.7, 27.3, 27.1, 25.8, 25.2; ^{31}P NMR ($CDCl_3$, 202 MHz) δ 50.7 (s); HRMS (FAB, positive) calcd for $C_{34}H_{39}N_5O_8PS_2$: 740.1978 (MH^+), found: 740.1987; UV (MeOH) λ_{max}=259, 295 (sh) nm.

4.1.7. *N-1-{(1R,2S,3R)-2,3-Isopropylidenedioxy-4-(phosphonoxymethyl)cyclopent-4-en-1-yl}-5′-O-(phenylthiophosphoryl)-2′,3′-O-isopropylideneadenosine (**9a**)*

A mixture of $POCl_3$ (93 μL, 1.0 mmol) and **19a** (72 mg, 98 μmol) in $PO(OEt)_3$ (1.0 mL) was stirred at 0 °C for 5 h. After addition of aqueous saturated $NaHCO_3$ (3 mL), the resulting mixture was stirred at 0 °C for 10 min. To the mixture were added triethylammonium acetate (TEAA) buffer (2.0 M, pH 7.0, 1 mL) and H_2O (5 mL), and the resulting solution was applied to a C_{18} reversed phase column (1.1×17 cm). The column was developed using a linear gradient of 0–66% MeCN in TEAA buffer (0.1 M, pH 7.0, 400 mL). Appropriate fractions were evaporated and excess TEAA was removed by C_{18} reversed phase column chromatography (1.1×17 cm, eluted with 80% aqueous MeCN). Appropriate fractions were evaporated and the residue was co-evaporated with pyridine (2.0 mL×3). A mixture of the residue, *N*-methylmaleimide (43 mg, 0.39 mmol), H_3PO_2 (40 μL, 0.78 mmol), and Et_3N (54 μL, 0.39 mmol) was stirred at 0 °C for 5 h under shading. After addition of TEAA buffer (1.0 M, pH 7.0, 2 mL), the resulting mixture was evaporated. The residue was partitioned between EtOAc and H_2O, and the aqueous layer was evaporated. A solution of the residue in H_2O (5 mL) was applied to a C_{18} reversed phase column (1.1×17 cm), and the column was developed using a linear gradient of 0–35% MeCN in TEAA buffer (0.1 M, pH 7.0, 400 mL). Appropriate fractions were evaporated and excess TEAA was removed by C_{18} reversed phase column chromatography (1.1×17 cm, eluted with 60% aqueous MeCN). Appropriate fractions were evaporated and the residue was lyophilized to give **9a** (23 mg, 28%) as a triethylammonium salt. ^{1}H NMR (D_2O, 500 MHz) δ 8.42 (s, 1H), 8.41 (s, 1H), 7.17–7.29 (m, 5H), 6.34 (d, 1H, J=2.0 Hz), 6.08 (m, 1H), 5.62 (m, 1H), 5.48 (d, 1H, J=5.8 Hz), 5.43 (dd, 1H, J=2.0, 5.9 Hz), 4.99 (dd, 1H, J=5.9, 1.3 Hz), 4.72 (m, 1H), 4.67 (m, 3H), 4.20 (m, 1H), 4.13 (m, 1H), 3.19 (q, 6H, J=7.3 Hz), 1.63, 1.55, 1.43, 1.40 (each s, each 3H), 1.27 (t, 9H, J=7.3 Hz); ^{13}C NMR (D_2O, 125 MHz) δ 151.5, 151.4, 150.9, 146.9, 144.6, 144.2, 133.0, 133.0, 129.9, 129.5, 128.3, 122.3, 119.6, 115.1, 114.9, 92.0, 86.7, 86.6, 84.5, 84.4, 83.8, 81.9, 70.6, 66.3, 62.0, 47.1, 26.6, 26.4, 25.3, 24.7, 8.7; ^{31}P NMR (D_2O, 202 MHz) δ 16.7 (s), 1.7 (s); HRMS (FAB, negative) calcd for $C_{28}H_{34}N_5O_{12}P_2S$: 726.1405 [$(M-H)^-$], found: 726.1392; UV ($H_2O$) λ_{max} 261 nm.

4.1.8. *4″,6″-Didehydro-cyclic ADP-carbocyclic-ribose diacetonide (**20a**)*

To a mixture of $AgNO_3$ (127 mg, 0.75 mmol), Et_3N (105 μL, 0.75 mmol), and MS 3 Å (200 mg) in pyridine (35 mL), a solution of **9a** (30 mg, 36 μmol) in pyridine (35 mL) was added slowly over 15 h, using a syringe-pump, at room temperature under shading. The MS 3 Å was filtered off with Celite and washed with H_2O. To the combined filtrate and washings was added TEAA buffer (2.0 M, pH 7.0, 2 mL), and the resulting solution was evaporated. The residue was partitioned between EtOAc and H_2O, and the aqueous layer was evaporated. A solution of the residue in H_2O (5 mL) was applied to a C_{18} reverse phase column (1.1×17 cm), and the column was developed using a linear gradient of 0–30% MeCN in TEAA buffer (0.1 M, pH 7.0, 400 mL). Appropriate fractions were evaporated and excess TEAA was removed by C_{18} reverse phase column chromatography (1.1×17 cm, eluted with 50% aqueous MeCN). Appropriate fractions were evaporated and the residue was lyophilized to give **20a** (17 mg, 65%) as a triethylammonium salt. ^{1}H NMR (D_2O, 500 MHz) δ 8.71 (s, 1H), 8.43 (s, 1H), 6.39 (m, 1H), 6.31 (m, 1H), 5.73 (m, 2H), 5.60 (d, 1H, J=5.2 Hz), 5.54 (m, 1H), 4.96 (m, 1H), 4.81 (d, 1H, J=5.2 Hz), 4.58 (m, 1H), 4.52 (m, 1H), 4.06 (m, 1H), 3.86 (m, 1H), 3.21 (q, 6H, J=7.3 Hz), 1.65, 1.49, 1.46, 1.42 (each s, each 3H), 1.29 (t, 9H, J=7.3 Hz); ^{13}C NMR (D_2O, 125 MHz) δ 151.3, 150.9, 147.4, 145.3, 144.5, 126.8, 119.6, 115.2, 114.1, 91.7, 86.7, 84.7, 82.9, 82.5, 81.1, 68.7, 64.2, 62.2, 47.1, 26.8, 26.4, 25.8, 24.7, 8.7; ^{31}P NMR (D_2O, 202 MHz) δ −10.5 (d, J=15.3 Hz), −10.6 (d, J=15.3 Hz); HRMS (FAB, negative) calcd for $C_{22}H_{28}N_5O_{12}P_2$: 616.1215 [$(M-H)^-$], found: 616.1188; UV (H_2O) λ_{max} 260 nm.

4.1.9. *4″,6″-Didehydro-cyclic ADP-carbocyclic-ribose (**8a**)*

A solution of **20a** (9.0 mg, 13 μmol) in aqueous 60% HCO_2H (1 mL) was stirred at room temperature for 4 h and then evaporated. After the residue had been co-evaporated with H_2O (2 mL×3), aqueous 28% NH_3 (1 mL) was added. The mixture was stirred at room temperature for 4 h and then evaporated. After the residue had been co-evaporated with H_2O (2 mL×3), the resulting residue was dissolved in TEAB buffer (0.1 M, pH 7.0, 100 μL), and the solution was lyophilized to give **8a** (6.0 mg, 86%) as a triethylammonium salt. ^{1}H NMR (D_2O, 500 MHz, K^+ salt) δ 8.63 (s, 1H), 8.49 (s, 1H), 6.31 (m, 1H), 6.07 (d, 1H, J=5.7 Hz), 5.44 (m, 1H), 5.29 (m, 1H), 4.91 (m, 2H), 4.67 (m, 1H), 4.62 (m, 2H), 4.34 (m, 2H), 4.07 (m, 1H); ^{13}C NMR (D_2O, 125 MHz) δ 153.1, 151.6, 147.4, 146.0, 143.3, 124.4, 121.0, 91.3, 85.0, 74.1, 73.6, 73.2, 71.0, 69.0, 64.7, 61.7; ^{31}P NMR (D_2O, 202 MHz) δ −10.0 (d, J=15.3 Hz), −10.6 (d, J=15.3 Hz); NOE correlations H-2–H-1″, H-2–H-2″, H-2–H-6″, H-2–H-3″, H-8–H-1′, H-1″–H-6″, H-1″–H-5″a, H-1′–H-2′, H-1′–H-4′, H-2′–H-3′, H-2′–H-4′, H-6″–H-3″, H-5′a–H-4′, H-5′a–H-5′b, H-4′–H-5′b, H-5″a–H-5″b, H-2″–H-3″; HRMS (FAB, negative) calcd for $C_{16}H_{20}N_5O_{12}P_2$: 536.0589 [$(M-H)^-$], found: 536.0577; UV (H_2O) λ_{max} 260 nm.

4.1.10. *N-1-{(1R,2S,3R)-4-Hydroxymethyl-2,3-isopropylidenedioxycyclopent-4-en-1-yl}-5′-O-(tert-butyldimethylsilyl)-2′,3′-O-isopropylideneinosine (**10b**)*

A mixture of **14** (511 mg, 0.868 mmol) and **11** (241 mg, 1.30 mmol) in DMF (400 μL) was stirred at 50 °C for 45 h. The

reaction mixture was diluted with EtOAc and washed with H_2O. The water layer was evaporated and co-evaporated with toluene (3 mL×3) to recover **11**. The organic layer was washed with brine, dried (Na_2SO_4), and evaporated. A mixture of the residue and K_2CO_3 (240 mg, 1.74 mmol) in DMF (9 mL) was stirred at 50 °C for 30 min. The reaction mixture was diluted with EtOAc and washed with H_2O and brine, dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (SiO_2, 75% EtOAc in hexane) to give **10b** (179 mg, 35%) as a brown foam. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.01 (s, 1H), 7.87 (s, 1H), 6.09 (d, 1H, J=2.6 Hz), 5.64 (m, 2H), 5.33 (d, 1H, J=5.7 Hz), 5.03 (dd, 1H, J=2.6, 6.0 Hz), 4.88 (dd, 1H, J=6.0, 2.3 Hz), 4.69 (d, 1H, J=5.7 Hz), 4.51 (m, 2H), 4.41 (m, 3H), 1.61, 1.45, 1.38, 1.33 (each s, each 3H), 0.85 (s, 9H), 0.062, 0.036 (each s, each 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 156.3, 151.9, 146.8, 145.4, 138.5, 123.7, 122.9, 114.2, 112.5, 91.4, 87.0, 85.5, 84.4, 84.3, 81.3, 63.5, 62.8, 59.9, 27.3, 27.2, 25.9, 25.3, 18.3, −5.5, −5.6; HRMS (FAB, positive) calcd for $C_{28}H_{43}N_4O_8Si$: 591.2850 (MH^+), found: 591.2862; UV (MeOH) λ_{max} 245 nm.

4.1.11. *N-1-{(1R,2S,3R)-4-Dimethoxytrityloxymethyl-2,3-isopropylidenedioxycyclopent-4-en-1-yl}-2′,3′-O-isopropylideneinosine (**17b**)*

Compound **17b** (oil, 158 mg, 61%) was obtained from **10b** (195 mg, 0.330 mmol) as described for the synthesis of **17a**. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.00 (s, 1H), 7.86 (s, 1H), 6.83–7.48 (m, 13H), 5.93 (d, 1H, J=4.1 Hz), 5.90 (m, 1H), 5.83 (m, 1H), 5.17 (d, 1H, J=5.6 Hz), 5.09 (m, 1H), 5.07 (m, 1H), 4.61 (d, 1H, J=5.6 Hz), 4.50 (m, 1H), 4.00 (m, 1H), 3.96 (m, 1H), 3.82 (m, 1H), 3.78 (s, 6H), 3.75 (m, 1H), 1.64, 1.39, 1.36, 1.27 (each s, each 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.0, 158.5, 156.0, 151.4, 146.2, 145.1, 144.6, 139.7, 135.9, 135.7, 132.8, 132.0, 132.0, 131.9, 131.9, 129.9, 129.8, 128.5, 128.4, 127.9, 127.8, 126.8, 125.7, 121.4, 114.2, 113.2, 112.5, 93.2, 86.6, 86.2, 84.6, 84.1, 81.5, 66.3, 62.9, 61.2, 60.3, 55.1, 27.4, 26.0, 25.2, 21.0, 14.1; HRMS (FAB, positive) calcd for $C_{43}H_{47}N_4O_{10}$: 779.3292 (MH^+), found: 779.3284; UV (MeOH) λ_{max} 272, 265 nm.

4.1.12. *N-1-{(1R,2S,3R)-4-Dimethoxytrityloxymethyl-2,3-isopropylidenedioxycyclopent-4-en-1-yl}-5′-O-{bis(phenylthio)-phosphoryl}-2′,3′-O-isopropylideneinosine (**18b**)*

Compound **18b** (oil, 149 mg, 71%) was obtained from **17b** (158 mg, 0.203 mmol) as described for the synthesis of **18a**. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.84 (s, 1H), 7.79 (s, 1H), 6.82–7.68 (m, 23H), 6.06 (d, 1H, J=2.5 Hz), 5.88 (m, 1H), 5.82 (m, 1H), 5.15 (dd, 1H, J=2.5, 6.3 Hz), 5.10 (d, 1H, J=5.6 Hz), 4.96 (dd, 1H, J=6.3, 3.1 Hz), 4.52 (d, 1H, J=5.6 Hz), 4.46 (m, 1H), 4.39 (m, 2H), 3.99 (d, 1H, J=15.5 Hz), 3.78 (s, 6H), 3.77 (d, 1H, J=15.5 Hz), 1.62, 1.40, 1.37, 1.30 (each s, each 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.1, 158.6, 158.5, 156.1, 150.9, 146.6, 145.4, 144.7, 139.0, 136.0, 135.8, 135.3, 135.2, 135.1, 135.0, 132.1, 132.1, 132.0, 131.9, 131.9, 129.9, 129.9, 129.7, 129.5, 129.4, 128.5, 128.4, 128.0, 127.9, 126.9, 125.7, 125.3, 121.9, 114.8, 113.2, 112.3, 90.8, 86.7, 84.8, 84.6, 84.4, 84.1, 80.9, 66.0, 61.2, 60.3, 55.2, 27.4, 27.1, 26.0, 25.3, 21.0, 14.2; ^{31}P NMR ($CDCl_3$, 202 MHz) δ 50.7 (s); HRMS (FAB, positive) calcd for $C_{55}H_{56}N_4O_{11}PS_2$: 1043.3125 ($MH^+$), found: 1043.3132.

4.1.13. *N-1-{(1R,2S,3R)-4-Hydroxymethyl-2,3-isopropylidene-dioxycyclopent-4-en-1-yl}-5′-O-{bis(phenylthio)-phospholyl}-2′,3′-O-isopropylideneinosine (**19b**)*

Compound **19b** (oil, 84 mg, 79%) was obtained from **18b** (149 mg, 0.143 mmol) as described for the synthesis of **19a**. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.86 (s, 1H), 7.83 (s, 1H), 7.27–7.67 (m, 10H), 6.05 (d, 1H, J=2.5 Hz), 5.65 (m, 1H), 5.62 (m, 1H), 5.26 (d, 1H, J=5.7 Hz), 5.13 (dd, 1H, J=2.5, 6.3 Hz), 4.95 (dd, 1H, J=6.3, 3.1 Hz), 4.59 (d, 1H, J=5.7 Hz), 4.45 (m, 1H), 4.40 (m, 4H), 1.61, 1.43, 1.36, 1.30 (each s, each 3H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 156.2, 152.2, 146.7, 145.6, 139.2, 135.3, 135.3, 135.1, 135.1, 129.8, 129.5, 128.5, 128.4, 125.7, 125.6, 125.3, 122.2, 114.7, 112.4, 90.8, 84.8, 84.7, 84.5, 84.4, 84.1, 81.0, 66.8, 66.2, 60.3, 59.9, 27.3, 27.1, 25.7, 25.3; ^{31}P NMR ($CDCl_3$, 202 MHz) δ 50.8 (s); HRMS (FAB, positive) calcd for $C_{34}H_{38}N_4O_{19}S_2$: 741.1818 (MH^+), found: 741.1831.

4.1.14. *N-1-{(1R,2S,3R)-2,3-Isopropylidenedioxy-4-(phosphonoxymethyl)cyclopent-4-en-1-yl}-5′-O-{(phenylthio)phospholyl}-2′,3′-O-isopropylideneinosine (**9b**)*

Compound **9b** (oil, 26 mg, 36%) was obtained from **19b** (84 mg, 0.11 mmol) as described for the synthesis of **9a**. ^{1}H NMR (D_2O, 500 MHz) δ 8.22 (s, 1H), 8.14 (s, 1H), 7.08–7.22 (m, 5H), 6.30 (m, 1H), 5.91 (m, 1H), 5.59 (m, 1H), 5.49 (d, 1H, J=5.9 Hz), 5.36 (d, 1H, J=5.7 Hz), 5.10 (d, 1H, J=5.9 Hz), 4.67 (m, 1H), 4.64 (m, 2H), 4.38 (d, 1H, J=5.7 Hz), 4.29 (m, 1H), 4.10 (m, 1H), 3.18 (q, 12H, J=7.3 Hz), 1.64, 1.51, 1.42, 1.39 (each s, each 3H), 1.26 (t, 18H, J=7.3 Hz); ^{13}C NMR (D_2O, 125 MHz) δ 157.9, 149.2, 149.1, 147.7, 146.6, 141.4, 132.8, 132.8, 129.9, 129.4, 128.2, 124.2, 115.2, 113.6, 91.2, 86.5, 86.4, 84.9, 84.2, 83.8, 81.8, 67.0, 66.3, 62.2, 47.1, 26.6, 26.4, 25.3, 24.7, 8.7; ^{31}P NMR (D_2O, 202 MHz) δ 17.4 (s), 1.0 (s); HRMS (FAB, negative) calcd for $C_{28}H_{33}N_4O_{13}P_2S$: 727.1246 [$(M-H)^-$], found: 727.1230; UV ($H_2O$) λ_{max} 244 nm.

4.1.15. *4″,6″-Didehydro-cyclic IDP-carbocyclic-ribose diacetonide (**20b**)*

Compound **20b** (13 mg, 57%) was obtained from **9b** (amorphous solid, 26 mg, 28 μmol) as described for the synthesis of **20a**. ^{1}H NMR (D_2O, 500 MHz) δ 8.45 (s, 1H), 8.19 (s, 1H), 6.30 (m, 1H), 6.22 (m, 1H), 5.72 (d, 1H, J=6.1 Hz), 5.61 (m, 1H), 5.55 (dd, 1H, J=6.1, 3.0 Hz), 5.48 (d, 1H, J=5.2 Hz), 4.93 (m, 1H), 4.60 (d, 1H, J=5.2 Hz), 4.53 (m, 1H), 4.46 (m, 1H), 3.98 (m, 1H), 3.81 (m, 1H), 3.19 (q, 12H, J=7.3 Hz), 1.62, 1.46, 1.45, 1.38 (each s, each 3H), 1.27 (t, 18H, J=7.3 Hz); ^{13}C NMR (D_2O, 125 MHz) δ 158.8, 149.5, 148.1, 146.1, 142.6, 128.4, 124.6, 115.0, 113.3, 91.5, 86.7, 84.7, 83.8, 82.4, 81.5, 65.3, 64.3, 62.4, 47.1, 26.8, 26.4, 25.7, 24.7, 8.7; ^{31}P NMR (D_2O, 202 MHz) δ −10.5 (d, J=15.3 Hz), −10.7 (d, J=15.3 Hz); HRMS (FAB, negative) calcd for $C_{22}H_{27}N_4O_{13}P_2$: 617.1055 [$(M-H)^-$], found: 617.1049; UV (H_2O) λ_{max} 251 nm.

4.1.16. *4″,6″-Didehydro-cyclic IDP-carbocyclic-ribose (**8b**)*

Compound **8b** (amorphous solid, 10 mg, 82%) was obtained from **20b** (13 mg, 16 μmol) as described for the synthesis of **8a**. ^{1}H NMR (D_2O, 500 MHz) δ 8.35 (s, 1H), 8.19 (s, 1H), 6.22 (m, 1H), 5.99 (d, 1H, J=6.2 Hz), 5.41 (m, 1H), 5.32 (dd, 1H, J=6.2, 4.8 Hz), 4.88 (m, 1H), 4.81 (d, 1H, J=4.0 Hz), 4.59–4.71 (m, 3H), 4.32 (m, 1H), 4.14 (d, 1H, J=4.0 Hz), 3.99 (m, 1H), 3.19 (m, 6H), 1.27 (m, 9H); ^{13}C NMR (D_2O, 125 MHz) δ 159.1, 151.5, 148.5, 144.9, 143.4, 126.3, 125.1, 91.1, 85.1, 75.4, 73.3, 73.1, 71.2, 66.0, 64.9, 61.9, 47.1, 8.7; ^{31}P NMR (D_2O, 202 MHz) δ −9.9 (d, J=15.3 Hz), −10.5 (d, J=15.3 Hz); HRMS (FAB, negative) calcd for $C_{16}H_{19}N_4O_{13}P_2$: 537.0429 [$(M-H)^-$], found: 537.0403; UV (H_2O) λ_{max} 251 nm.

4.2. Biological evaluations with sea urchin egg homogenate, neuronal cells, and T cells

These bioassays were carried out as reported previously.[7c]

4.2.1. *NMR titrations*

NMR titrations were carried out as previously reported.[29] The experiments were performed in two steps in which 0.50 cm^3 of the same initial solution of studied compounds of 3.0×10^{-3} mol dm^{-3} in ^2H_2O was successively subjected to potentiometric and NMR titrations. It should be noted that the glass electrode was calibrated in a concentration scale and the measurements done in ^2H_2O, so that here pH means the cologarithm of the concentration of $^2H^+$. The processing of the pH measurements allowed the total concentration of the ligand and the acid as well as the macroscopic

protonation constants to be determined. The HypNMR software[25] was used to check protonation constants. The ^{1}H NMR titration was performed on a Bruker DPX-500 Fourier transform spectrometer operating at 500.13 MHz. Spectra were acquired with water presaturation over a spectral width of 10 ppm using a 3 s relaxation delay and a $\pi/2$ pulse. 16 K data points were sampled with a corresponding 1.14 s acquisition time. The spectra had a digital resolution of 0.31 Hz per point. The temperature was controlled at 310.0±0.5 K. The proton resonances were assigned by performing proton–proton (double quantum filtered gradient accelerated COSY) and phosphorus–proton 2D correlation experiments at acidic and basic pHs (2 and 11.5, respectively), thus allowing the titration curves to be unambiguously characterized. ^{1}H, ^{13}C HSQC were used for the full attribution of the two phosphorus nuclei. Knowing non ambiguously ^{1}H chemical shift, it was easy to sort ^{31}P shift with HMQC ^{1}H, ^{31}P experiences using two different delay of evolution, one corresponding to 40 Hz and the other 5 Hz coupling.

4.2.2. Calculations

Calculations were carried out using the molecular-modeling software package SYBYL ver. 6.5 (Tripos, Inc.) on a Silicon Graphics O2 computer as reported previously.[7c]

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2008.07.068.

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