Design, Synthesis, and Chemical and Biological Properties of Cyclic ADP-4-Thioribose as a Stable Equivalent of Cyclic ADP-Ribose

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Here we describe the successful synthesis of cyclic ADP-4-thioribose (cADPtR, 3), designed as a stable mimic of cyclic ADP-ribose (cADPR, 1), a Ca²⁺-mobilizing second messenger, in which the key N1-β-thioribosyladenosine structure was stereoselectively constructed by condensation between the imidazole nucleoside derivative 8 and the 4-thioribosylamine 7 via equilibrium in 7 between the α-anomer (7α) and the β-anomer (7β) during the reaction course. cADPtR is, unlike cADPR, chemically and biologically stable, while it effectively mobilizes intracellular Ca²⁺ like cADPR in various biological systems, such as sea urchin homogenate, NG108-15 neuronal cells, and Jurkat T-lymphocytes. Thus, cADPtR is a stable equivalent of cADPR, which can be useful as a biological tool for investigating cADPR-mediated Ca²⁺-mobilizing pathways.

Keywords: Cyclic ADP-Ribose, cADPR, Second Messenger, Ca²⁺-Release.

INTRODUCTION

Cyclic ADP-ribose (cADPR, 1, Fig. 1), a metabolite of NAD⁺ (nicotinamide adenine dinucleotide), was originally isolated from sea urchin by Lee and co-workers (Clapper et al., 1987). cADPR mobilizes intracellular Ca²⁺ in various mammalian cells, such as, pancreatic β-cells, smooth muscle, cardiac muscle, T-lymphocytes, and cerebellar neurons, and therefore, cADPR is now recognized as a general mediator of intracellular Ca²⁺ signaling (Galione, 1993; Lee, 1997; Galione et al., 1998; Guse, 1999; Higashida et al., 2001; Lee, 2002; Guse, 2004; Zhang and Li, 2006; Jin et al., 2007; Venturi et al., 2010; Lee, 2012).

Analogs of cADPR have been extensively designed and synthesized, since they are potentially useful for investigating the mechanism of cADPR-mediated Ca²⁺ release (Walseth and Lee, 1993; Lee et al., 1993; Zhang and Sih, 1995; Ashamu et al., 1995; Wagner et al., 2003, 2005; Moreau et al., 2006, 2012; Galeone et al., 2002; Huang et al., 2002; Gu et al., 2004; Xu et al., 2006; Swarbrick and Potter, 2012; Yu et al., 2012; Shuto et al., 1998; Fukuoka et al., 2000; Shuto et al., 2001; Guse et al., 2002; Shuto et al., 2003; Kudoh et al., 2005; Hashii et al., 2005; Kudoh et al., 2008; Zhang et al., 1999; Shuto and Matsuda, 2004; Guse, 2004; Potter and Walseth, 2004). cADPR analogs are also expected to be lead structures for...
the development of potential drug candidates, due to the important physiological roles of cADPR (Galiane, 1993; Lee, 1997; Galiane 1998; Guse, 1999; Higashida et al., 2001; Lee, 2002; Guse, 2004; Zhang, 2006; Jin et al., 2007; Venturi et al., 2010; Lee, 2012). cADPR analogs have been synthesized predominantly by enzymatic and chemo-enzymatic methods using ADP-ribosyl cyclase-catalyzed cyclization under mild conditions (Walseth and Lee, 1993; Lee et al. 1993; Zhang and Sih, 1995; Ashamu et al., 1995; Wagner et al., 2003, 2005; Moreau et al., 2006, 2012). The analogs obtained by these methods, however, are limited due to the substrate-specificity of the ADP-ribosyl cyclase (Zhang et al., 1999; Shuto and Mat-suda, 2004; Guse, 2004; Potter and Walseth, 2004).

In recent years, methods for the chemical synthesis of cADPR analogs have been studied to develop useful cADPR analogs (Galeone et al., 2002; Huang et al., 2002; Gu et al., 2004; Xu et al., 2006; Swarbrick et al., 2012; Yan et al., 2012; Shuto et al., 1998; Fukuoka et al., 2000; Shuto et al., 2001; Guse et al., 2002; Shuto et al., 2003; Kudoh et al., 2005; Hashii et al., 2005; Kudoh et al., 2007; Zhang et al., 1999; Shuto et al., 2001). Although cADPR is replaced by a methylene group. cADPRc is both chemically and biologically stable, and effectively mobilizes intracellular Ca$^{2+}$ in sea urchin eggs (Shuto et al., 2001). Although cADPRc is more potent than cADPR in neuronal cells (Hashii et al., 2005), it is almost inactive in T cells, whereas natural cADPR effectively mobilizes Ca$^{2+}$ in both neuronal cells and T cells (Guse et al., 2002; Kudoh et al., 2005).

Figure 1. cADPR (1), cADPRc (2), cADPRR (3) and cIDPR (4).

Although intensive studies of the signaling pathway that uses cADPR are still needed because of its biological importance, the biological as well as chemical instability of cADPR limits further studies of its physiological role. Therefore, stable analogs of cADPR exhibiting Ca$^{2+}$-mobilizing activity in various cells including T cells are required. Thus, we newly designed and synthesized a 4-thioribose-containing analog of cADPR, i.e., cyclic ADP-4-thioribose (cADPcR, 3), in which the N1-ribose of cADPR is replaced by a 4-thioribose. Evaluations in several biological systems revealed that cADPRc is a stable equivalent of cADPR, which mobilizes intracellular Ca$^{2+}$ not only in sea urchin eggs and neuronal cells, but also in T cells. Here we report the detailed results of these studies on cADPRc.

RESULTS AND DISCUSSION

Design of cADPRc

cADPR is in an equilibrium between the N$^\circ$-protonated amino-form and the N$^\circ$-deprotonated imino-form, as shown in Figure 2 (Lee et al., 1989; Kim et al., 1993; Lee et al., 1994; Gu and Sih, 1994; Wada et al., 2005; Wada and Pope, 2001; Graham et al., 2004). One difference between cADPR and cADPRc is their $pK_a$ values for protonation at the N$^\circ$-position. The $pK_a$ of cADPRc (8.9) (Guse et al., 2002) is somewhat higher than that of TPR (8.3) (Kim et al., 1994), which might affect its interaction with target proteins. Under physiological conditions, cADPR is in a mixture of the protonated amino form and the deprotonated imino form, where most of cADPRc should be present in the amino-form due to its relatively higher $pK_a$. If cADPR binds to the target protein in T cells in the imino-form, activity of cADPRc would be decreased. In recent years, cADPR analogs with a hypoxanthine base instead of an adenine base, such as cyclic IDP-ribose (cIDPR, 4, Fig. 1), were synthesized,
and these hypoxanthine-type analogs also mobilized Ca\(^{2+}\) in T cells, while they were almost inactive in other biological systems (Moreau et al., 2006; Huang et al., 2002; Gu et al., 2004; Xu et al., 2006). These findings support the idea of an active imino-form of cADPR in T cells, because the C6-O\(^2\) moiety might work as a bioisostere of the plane C6-N\(^0\) moiety of the imino-form of cADPR, due to their analogous unsaturated \(\pi\)-electronic structural features.

Another difference between cADPR and cADPtR might be their three-dimensional structures. 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 between the nucleobase and sugar moieties (Saenger, 1983). Therefore, in cADPR and its analogs, the most stable conformation can be one with minimal steric repulsion between the adenine moiety and both of the N1- and N9-ribose moieties. It should be noted that, in cADPtR, the hydrogens on the tetrahedral sp\(^3\)-C6\(^{-}\), particularly the H6\(^{-}\)-\(\beta\), which is absent in cADPR, are rather sterically repulsive to the adenine H2 (Fig. 3). Accordingly, the stable conformation of cADPtR would differ from that of cADPR due to the steric effects (Kudoh et al., 2008).

Taking these considerations into account, we newly designed cADPtR, a 4-thioribose analog of cADPR. 4\(^{-}\)-Thionucleosides are recognized as useful bioisosteres of the natural nucleosides, which are extensively used in studies of medicinal chemistry and chemical biology (Reist et al., 1964; Bobek and Whistler, 1970; Dyson et al., 1991; Bellon et al., 1993; Yoshimura et al., 1997; Naka et al., 2000; Takahashi et al., 2009). The 4-thioribose in 4\(^{-}\)-thionucleosides does not only effectively mimics the ribose of nucleosides, but also the N-4-thioribosyl linkage is more stable than the corresponding N-ribosyl linkage against both chemical and enzymatic hydrolysis (Elzageheid et al., 1999; Toyohara et al., 2003). In addition, the \(pK_a\) value of cADPtR is expected to be similar to that of cADPR due to the electron-withdrawing property of sulfur atom. This type of \(pK_a\) adjustment of biologically active compounds by replacing a methylene with a sulfur has been reported previously (Ganelin and Owen, 1977). Furthermore, the conformation of cADPtR, particularly spatial positioning of the N1-thioribose and adenine moieties, would be similar to that of cADPR, because both sulfur and oxygen have a similar \(sp^3\) configuration bearing two non-bonding electron pairs. Thus, we expected that cADPtR might be a stable cADPR equivalent that is active in various cells including T cells.

**Synthetic Plan**

The retrosynthetic scheme for the target cADPtR (3) is shown in Figure 4. For the synthesis of cADPtR, construction of the 18-membered pyrophosphate structure is an important step. The structure was likely to be constructed using the intramolecular condensation reaction with an S-phenyl phosphorothioate-type substrate that we developed (Fukuoka et al., 2000; Shuto et al., 2001), which has been effectively used in the synthesis of a variety of cADPR analogs (Galeone et al., 2002; Huang et al., 2002; Gu et al., 2004; Xu et al., 2006; Swarbrick et al., 2012; Yu et al., 2012). Thus, treatment of the S-phenyl phosphorothioate-type substrates 5 having the N1-4-thioribosyladenosine structure with AgNO\(_3\)/MS3A as a promoter (Fukuoka et al., 2000; Shuto et al., 2001) would form the desired cyclization product, and subsequent deprotection would furnish the target cADPtR.

In this synthetic plan, we first had to achieve stereoselective construction of the N1-\(\beta\)-thioribosyladenosine structure. Although no 4-thioribose derivatives having an anomic amino function such as 7 have been reported to date, 4-thioblosylamine 7 is likely to be present as an anomic mixture, due to the electron-donating property of the hemiaminal ether nitrogen attaching at the anomeric position. We speculated that if the 4-thioblosylamine derivative 7 is indeed in equilibrium between the \(\alpha\)-anomer (7\(\alpha\)) and the \(\beta\)-anomer (7\(\beta\)), stereoselective construction of the N1-\(\beta\)-thioribosyladenosine structure might be
achieved. In the 4-thioribosylamine 7, the α-face would be rather sterically more hindered than the β-face due to its rigid 5,5-cis ring system. Thus, as shown in Figure 5, in the condensation reaction, the β-anomer 7β might preferentially react with the imidazole nucleoside 8, while the α-anomer 7α might not react due to the steric hindrance by the adjacent isopropylidene moiety. Thus, we expected that, in the reaction course, the relatively less reactive α-anomer would not undergo the condensation, but rather would be effectively converted into the more reactive β-anomer via the equilibrium to result in accumulation of the desired β-condensation product 6β.

**Synthesis**

The 4-thioribosylamine 7 was prepared from the known 1-deoxy-4-thioribose derivative 9 (Jeong et al., 2006), as shown in Scheme 1. While several procedures for synthesizing 4-thioribose derivatives have been reported (Reist et al., 1964; Bobek and Whistler, 1970; Dyson et al., 1991; Bellon et al., 1993; Yoshimura et al., 1997; Naka et al., 2000; Takahashi et al., 2009), we selected the procedure recently developed by Jeong and co-workers (Jeong et al., 2006), which is effective for large scale preparation of the 4-thio-1-deoxyribose derivative 9 having the desired 2,3-O-isopropylidene protecting group. Oxidation of 9 with m-CPBA and subsequent heating of the resulting sulfoxide product in Ac₂O to initiate the Pummerer rearrangement afforded the 1-acetoxy product 11 as an anomic mixture (α/β = 1:5). When the anomic mixture 11 was treated with TMSN₃ and SnCl₂ in CH₂Cl₂, the β-azide 12 was obtained stereoselectively in high yield, probably due to the steric demand of the reaction intermediate. Reduction of the azido group of 12 by catalytic hydrogenation, followed by removal of the O-acetyl group by heating it in MeOH gave the 4-thioribosylamine 7, which appeared to be an anomic mixture (α/β = 1:2) as we expected.

We next investigated the key condensation between the 4-thioribosylamine 7 (α/β = 1:2) and the imidazole nucleoside 8 under various conditions. Under basic reaction conditions, i.e., K₂CO₃/MeOH which was previously used to construct the N₁-carbocyclic-ribosyladenine structures (Shuto et al., 2001), the desired β-condensation product 6b was actually obtained stereoselectively in 50% yield. Under the conditions, however, the N⁶-(4-thioribosy) product 13 via a Dimroth rearrangement was also obtained in 28% yield. As a result, we found that when 7 was treated with 8 (2.1 eq) in MeOH at room temperature without any bases, the β-product 6b was obtained in 61% yield concomitant with 5% of the α-product 6a, where the 4-thioribosylamine 7 was recovered in 17% yield (Scheme 2). Thus, the desired β-product 6b was successfully obtained in 73% conversion yield from the
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Scheme 2. Stereoselective synthesis of N1-β-thioribosyladenosine derivative 6β.

4-thioribosylamine (α/β = 1:2) via the α/β-equilibrium, as we hypothesized. In the reaction conditions, the N6-thioribosyl product 13 (4%) was also obtained.

The N1-substituted structures of 6a and 6b was confirmed based on their HMBC spectra, in which correlation between the H2 of the adenine and the C1′′ of the 4-thioribose moiety was observed. The α- and β-stereochemistries of 6a and 6b were confirmed by NOE data (see Fig. 11).

Conversion of the N1-β-thioribosyl product 6b into the 5′-phenyl phosphorothioate-type substrate 5 was next investigated (Scheme 3). The 5′′-hydroxy group of 6b was protected with a dimethoxytrityl (DMTr) group, and then the 5′′-O-TBS group of the product 14 was removed with TBAF to give 15. Treatment of 15 with an S,S′′-diphenylphosphorodithioate (PSS)/2,4,6-trisopropylbenzenesulfonyl chloride (TPSCl)/pyridine system (Sekine et al., 1985; Sekine and Hata, 1993), followed by removal of the 5′′-O-DMTr group of the product 16 with aqueous AcOH gave the 5′′-bis-S-(phenyl)phosphorothioate 17.

We next examined phosphorylation of the 5′′-hydroxyl of 17. However, treatment of 17 under the usual conditions of Yoshikawa's phosphorylation procedure with POCl3/(EtO)3PO (Yoshikawa et al., 1969), which was effective in the previous synthesis of cADPR and its analogs, gave none of the desired phosphorylation product 18.

Difficulties in the phosphorylation of the 4-thioribose moiety could be assumed to occur, at least partially, because phosphorylation of the 5′-primary hydroxyl of 4′-thioribonucleoside derivatives is rather difficult compared to the cases with usual ribonucleosides (Hancox and Walker, 1996; Alexandova et al., 1996). Walker and co-workers reported that phosphorylation of 4′-thiothymidine by Yoshikawa’s procedure did not give the desired corresponding 5′′-O-phosphate product. They suggested that elimination of the dichlorophosphate moiety would occur in an intermediate A due to effective neighboring group participation by the nucleophilic ring sulfur to result in reproducing the starting material 4′-thiothymidine, via a thionium intermediate B and its subsequent hydrolysis, as shown in Scheme 4(a) (Hancox and Walker, 1996).

We thought that a zwitterionic phosphorylating reagent 19 (Scheme 4(b)), reported by Asseline and Thuong (Asseline and Thuong et al., 1988), might be effective for the phosphorylation of 17, because in the phosphorylation...
intermediate C, the leaving ability of the phosphate moiety would be decreased due to the negatively charged oxygen. Thus, when 17 was treated with 19 in pyridine at −30 °C and then the reaction was quenched with triethylammonium acetate (TEAA) buffer, a compound likely to be the desired phosphorylated 18 was detected as a main peak by HPLC analysis. The product, without purification, was further treated with H₃PO₂ and Et₃N in pyridine to remove the phenylthio group (Hata et al., 1987) affording the phosphorylation product 5 after purification by ion-exchange chromatography (Scheme 5).

With the S-phenyl phosphorothioate 5 in hand, we next investigated the intramolecular cyclization reaction. When a solution of 5 in pyridine was slowly added to a mixture of a large excess of AgNO₃ and Et₃N in the presence of MS3A in pyridine at room temperature (Fukuoka et al., 2000; Shuto et al., 2001), the desired cyclization product 19 was obtained in 76% yield. Finally, removal of the isopropylidene groups of 19 with aqueous HCO₂H produced the target cADPtR (3) in 49% yield (Scheme 5).

**Scheme 4.** Hydrolysis of 5′-dichlorophosphate B via intermediate A (a) and zwitterionic phosphorylating reagent 19 (b).

**Scheme 5.** Synthesis of cADPtR (3).

**Stability of cADPtR**

cADPtR (3) appeared to be rather chemically stable, since the final acidic removal of the isopropylidene groups of 20 was successful to produce cADPtR, as described above (Scheme 5).

The biological stability of cADPtR was investigated with a rat brain microsomal extract that contains cADPR degradation enzymes (Lee and Aarhus, 1993). We first treated cADPR with the extract at 37 °C to result in its rapid degradation. As expected, under the same conditions, cADPtR was almost completely resistant to degradation in the extract. After 120 min treatment, approximately 90% of cADPR disappeared, whereas most of cADPtR remained intact, indicating that cADPtR is stable under physiological conditions (see Fig. 12).

**pKₐ Value of cADPtR**

The pKₐ value of cADPtR (3) was determined using a previously reported method (Kim et al., 1993). This method is based on the pH-dependent UV spectral change between 285 nm and 300 nm area of cADPR or its analogs, due to the protonation and proton-dissociation at the N₆ position of the adenine ring. As a result, the pKₐ of cADPtR was determined to be 8.0, which was similar to that of cADPR (pKₐ = 8.3) (Kim et al., 1993) and about 1 unit lower than that of cADPcR (pKₐ = 8.9) (Guse et al., 2002).

**Ca²⁺-Mobilizing Activity in Sea Urchin Egg Homogenate**

We tested the Ca²⁺-mobilizing ability of cADPtR (3) as well as cADPR (1) and cADPcR (2) by fluorometrically monitoring Ca²⁺ with H. pulcherrimus sea urchin egg homogenate (Fig. 6) (Shiwa et al., 2002). cADPR released Ca²⁺ from the homogenate in a concentration-dependent manner with an EC₅₀ value of 214 nM, and cADPcR showed more potent activity (EC₅₀ = 54 nM) than cADPR, where the maximal Ca²⁺-mobilizing activity of cADPcR was almost equal to that of cADPR. These results are consistent with previous reports that cADPcR potently activates Ca²⁺ release in sea urchin eggs of A. crassispina or L. pictus (Shuto et al., 2001; Kudoh et al., 2008). As shown in Figure 6, cADPtR exhibited highly potent Ca²⁺-mobilizing ability (EC₅₀ = 36 nM), which was about 7-fold more potent than the natural second messenger cADPR and even more potent than cADPcR.

**Ca²⁺-Mobilizing Activity in Neuronal Cells**

The effect of cADPtR (3) on cytosolic Ca²⁺ mobilization in NG108-15 neuronal cells was studied in permeabilized conditions using digitonin as the detergent (Higashida et al., 1990; Amina et al., 2010). We first verified that extracellular application of digitonin itself did
not cause an increase in \([\text{Ca}^{2+}]\), in the conditions in which the plasma membrane was permeabilized with 250 nM of digitonin for nearly 5 min (Figs. 7(b) and (c)). We also confirmed that application of 100 mM cADPR (1), used as a positive control, caused a gradual and sustained Ca\(^{2+}\) release in the digitonin-permeabilized conditions, which allowed nucleotides to enter into the cytoplasm (Fig. 8(c)). When cADPR was applied extracellularly to intact NG108-15 cells, no increase in \([\text{Ca}^{2+}]\) was detected (data not shown). Figure 7(a) shows a representative field of cells with \([\text{Ca}^{2+}]\), changes induced by the application of cADPR with the plasma cell membrane was permeabilized with 250 nM digitonin for 5 min. Figure 8(c) shows the mean time-course of \([\text{Ca}^{2+}]\) changes induced by cADPR. Application of 100 \(\mu\)M cADPR induced persistent increases in \([\text{Ca}^{2+}]\); the mean \([\text{Ca}^{2+}]\) level measured 4 min after application of cADPR was 116 ± 2.3% of the resting (pre-permeabilization) level (mean ± SEM; \(n = 6\)). The amplitude evoked by cADPR was equivalent to or significantly greater than that induced by cADPR: the mean \([\text{Ca}^{2+}]\) level measured 4 min after application of cADPR was 107 ± 2.8% of the resting level (\(n = 10\)). A similar \([\text{Ca}^{2+}]\)-mobilizing pattern was observed in cADPR-applied cells: the mean \([\text{Ca}^{2+}]\) level measured 4 min after application of cADPR was 115 ± 3.1% of the resting level (\(n = 10\)).

These results in NG108-15 neuronal cells indicate that cADPR has potent \([\text{Ca}^{2+}]\)-mobilizing activity, similar to cADPR. Both cADPtr and cADPR have increased efficacy as compared with cADPR, which may be due to their increased metabolic stability.

### Ca\(^{2+}\)-Mobilizing Activity in T Cells

The Ca\(^{2+}\)-mobilizing effect of cADPR (3) was evaluated using saponin-permeabilized Jurkat T cells as reported previously (Guse et al., 1993; Guse et al., 2007; Schwarzmuller et al., 2002). Both cADPtr and cADPR (1) evoked rapid Ca\(^{2+}\) release upon addition to the permeabilized cell suspension indicating similar mechanisms of Ca\(^{2+}\) release (Fig. 8(a)). Though cADPR was somewhat more potent at 100 mM, cADPR and cADPtr had very similar concentration-response curves (Fig. 8(b)) indicating that cADPtr binds to the cADPR receptor protein with similar affinity. Our previous work revealed that replacing the northern ribose of cADPR with the carbocyclic moiety (cADPtr) shifted its \([\text{Ca}^{2+}]\) mobilizing activity to much higher concentrations (Guse et al., 2002). In contrast, the new analog cADPtr was almost as active as cADPR. A difference of cADPtr and analog cADPR are their \(pK_a\) values, amounting to 8.9 and 8.0, respectively. Taking the \(pK_a\) value of cADPR and the indistinguishable biological activity between cIDPR (4) and cADPR (Moreau et al., 2006; Huang et al., 2002; Gu et al., 2004; Xu et al., 2006) in T cells into account, the relatively high biological activity of cADPtr might be in favour of the imino form of cADPR being the active form of the natural second messenger in T cells.

### Conformational Analysis

The three-dimensional structures of biologically active compounds in aqueous solution is very important from the viewpoint of the bioactive conformation. Our study shows that cADPR (1), cADPCr (2), and cADPtr (3) have different activities in sea urchin eggs, neuronal cells and T cells, respectively. To investigate the biological difference based on their conformations, structures of them were constructed by molecular dynamics calculations with a simulated annealing method based on the NOE constraints of the intramolecular proton pairs measured in D\(_2\)O (for details, see Fig. 13). The structures obtained by the calculations based on their observed NOE in the NOESY spectra are shown in Figure 9.

To analyze the structural differences in more detail, these structures were superimposed as shown in Figure 10(a), which shows that the cADPtr structure (red) resembles that of natural cADPR (blue). The cADPCr structure (green), however, is not similar to those of the other two compounds, in which the relative special arrangement of the N1-thioribose ring and the adenine ring is clearly different from those of the other two compounds, as we expected. Thus, the distances between the 4′S between the adenine H2 of cADPR (3.6 Å) is significantly longer than the corresponding distances of cADPR (2.3 Å) and cADPtr (2.5 Å), probably due to the steric repulsion between the H6′β with the adenine H2 in cADPtr.

To confirm the validity of the obtained structures, we used the cADPR structure solved by X-ray crystallographic analysis (2j, Lee et al., 1994). Thus, the X-ray
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Figure 7. Effects of cADPtR on [Ca^{2+}]i increases in permeabilized NG108-15 cells. (a), (b) Cells were permeabilized by the addition of 250 nM digitonin to the bath solution. cADPtR (final concentration, 100 μM) (a) or an equal amount of buffer (b) was added together with digitonin as indicated by the arrows. Representative fields are displayed for each condition. Changes in [Ca^{2+}]i are shown as pseudocolor images, and colors reflecting fluorescence intensities are indicated to the right together with arbitrary units. (c) Time-course of [Ca^{2+}]i changes in Oregon Green-loaded NG 108–15 cells. At about 25 s after the beginning of each trace, cell membranes were permeabilized with buffers containing 250 nM digitonin with 100 μM cADPtR (magenta), cADPcR (blue), cADPR (green), or without nucleotide as control (black). Symbols indicate changes of [Ca^{2+}]i levels for 5 min, represented by the fluorescence intensity at each time (x) divided by resting intensity at time 0 (i.e., F_x/F_0). For calculations, cells with mean fluorescence intensity of 90 to 130 at time 0 were selected. ∗, Values in cells treated with cADPtR or cADPcR were significantly higher than those in control cells at p<0.05. ♦, Values in cells treated with cADPtR were significantly higher than those in cells treated with cADPR at p<0.05. (d) The graph shows concentration-dependent activity of cADPtR (magenta), cADPcR (blue) and cADPR (green) in NG108-15 cells. Symbols indicate changes of [Ca^{2+}]i levels at 280 s after membrane permeabilization, represented by fluorescence intensity at each time divided by the resting state at time 0 (i.e., F_x/F_0). Each symbol is mean ± SEM of 5 to 10 experiments. ∗, values significantly different from a drug-free control value (a gray circle) (P<0.05). ♦, Values in cells treated with cADPtR were significantly higher than those in cells with cADPR at p<0.05.

structure of cADPR (white) is superimposed into the three structures as shown in Figure 10(b). This crystal structure resembles the calculated cADPR and cADPtR structures, which suggests our structure determination by the molecular dynamics calculations was done relevantly.

Discussion

cAPDtR (3), which was designed as a stable mimic of cADPR (1), was successfully synthesized. In its synthesis, the key N1-β-thioribosyladenosine structure was effectively constructed by stereoselective condensation between the imidazole nucleoside derivative 8 and the 4-thioribosylamine 7 via equilibrium between the α-anomer (7α) and the β-anomer (7β). Also, the strategy using a S-phenyl phosphorothioate-type substrate in the intramolecular condensation reaction forming the 18-membered pyrophosphate linkage of cADPR-related compounds is demonstrated to be useful in the present case.

Although cADPR is rapidly hydrolyzed by cADPR hydrolase to give ADP-ribose under physiological conditions, cADPIr was shown to be biologically stable. Based on the X-ray crystallographic analysis of the complex of CD38 having cADPR hydrolase activity with nicotinamide guanine dinucleotide (GDP), Lee and Hao presented the reaction mechanism of enzymatic cADPR hydrolysis via an oxocarbenium intermediate, which is stabilized by the hydroxy group of Ser196 of the enzyme catalytic site (Liu et al., 2006). When an oxocarbenium ion, which is often an intermediate in glycosidic bond cleavage, interacts with an electronically negative nucleophilic atom such as oxygen (the hydroxyl oxygen of Ser196 in the case of CD38), it can be effectively stabilized by the adjacent ring oxygen of the sugars due to n→p* hyperconjugation between the non-bonding orbital on the ring oxygen and the vacant p-orbital of anomer, that is known as the anomeric effect (Juariisti and Cuevas, 1992; Thatcher, 1993; Juariisti and Cuevas, 1995; Thibaudeau and Chattopadhyaya, 1999). This stereoelectronic effect would promote enzymatic and also chemical hydrolysis of cADPR at the N1-linkage.
Figure 8. Effect of cADPR (1) and cADPtR (3) on Ca\textsuperscript{2+} signaling in permeabilized Jurkat T cells. After addition of fura-2/free acid (1.5 mM) and charging of the intracellular Ca\textsuperscript{2+}-pools by the addition of ATP (1 mM) and an ATP-regenerating system consisting of creatine phosphate (10 mM) and creatine kinase (20 units/ml), the indicated concentrations of cADPR or cADPtR were added as indicated. (a) Representative tracings. (b) Data presented as mean ± SEM (n = 2 to 8).

On the other hand, the sulfur of thio-sugars, including thioribose, would not so effectively stabilize the corresponding thio-carbenium intermediates, because the hyperconjugation ability of sulfur is remarkably weaker than that of oxygen (Salzner and Salzner, 1993), which can be reason why cADPtR is more stable than cADPR.

On the other hand, the electron-withdrawing effect of sulfur due to its electronically negative property effectively lowered the pK_a of cADPtR, and accordingly, its pK_a is similar to that of cADPR. Under physiological pH, the population of the deprotonated imino form and the protonated amino form in cADPtR would be analogous that in cADPR. Therefore, the pK_a value of cADPr may be favorable to exhibit its biological effects, similarly to natural messenger cADPR.

A difference of cADPr and analog cADPr are their pK_a values, amounting to 8.9 and 8.0, respectively. Taking the pK_a value of cADPR and the indistinguishable biological activity between cADPR (4) and cADPr (Moreau et al., 2006; Huang et al., 2002; Gu et al., 2004; Xu al., 2006) in T cells into account, the relatively high biological activity of cADPr might be in favor of the imino form of cADPR being the active form of the natural second messenger in T cells.

Our conformational analysis revealed that cADPtR has its three-dimensional structure analogous to cADPR due the similar sp\textsuperscript{3} configuration of oxygen and sulfur bearing two non-bonding electron pairs in the N1-ribose or thioribose ring, in which steric repulsion between the three rings (adenine, N9-ribose, and N1-ribose) seems to be minimal. Therefore, the structural and electrostatic features of cADPtR analogous to cADPR make it as biologically active as cADPR in various systems including T-cells, although the target proteins of cADPR in these systems is considered to be different (Kudoh et al., 2005).

Conclusion

We have successfully synthesized cADPtR (3) and demonstrated that it is stable and functions as cADPR (1) in various biological systems, i.e., sea urchin egg homogenate, neuronal cells and T cells. Because of the characteristic stability and high potency of cADPtR, it, as a stable equivalent of cADPR, can be useful as an effective tool for identifying target proteins and for investigating cADPR-mediated signaling pathways (Tsuzuki et al., 2013).

EXPERIMENTAL DETAILS

Chemical shifts are reported in ppm downfield from Me_4Si (\textsuperscript{1}H), MeCN (\textsuperscript{13}C) or H_3PO_4 (\textsuperscript{31}P). All of the \textsuperscript{1}H 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
H-5, 4.80 (1 H, br, OH), 5.10 (1 H, dd, J = 5.7, 3.4 Hz, H-3), 5.20 (1 H, ddd, J = 6.3, 5.7, 3.4 Hz, H-2); 13C-NMR (125 MHz, CDCl3) δ 24.61, 27.12, 57.06, 57.06, 58.11, 65.63, 79.76, 82.22, 112.11; HR-MS (EI) calcd for C12H18NaO6S 313.0722 (M+), found 313.0717.

1,5-O-Diacetyl-2,3-O-Isopropylidene-4-Thio-D-Ribose (11)

A solution of 10 (135 mg, 0.44 mmol) in Ac2O (3 mL) was heated at 100 °C for 28 h, and then evaporated. The residue was partitioned between EtOAc and H2O, and the organic layer was washed with aqueous saturated NaHCO3 and brine. The aqueous layers were combined and extracted with CHCl3. The organic layers were combined, dried (Na2SO4), and evaporated. The residue was purified by column chromatography (SiO2, hexane/AcOEt 3/1 then 1/1) to give 11 (93 mg, 64%, J = 11.5, 5.7 Hz, H-5), 4.89–4.91 (2 H, m, H-2, H-3), 6.04 (1 H, s, H-1), for α-anomer, δ 1.36 (3 H, s, isopropylidene-CH3), 1.54 (3 H, s, isopropylidene-CH3), 2.10 (3 H, s, Ac), 2.15 (3 H, s, Ac), 3.90 (1 H, m, H-4), 4.19 (1 H, dd, J = 11.5, 6.3 Hz, H-5), 4.35 (J = 11.5, 5.7 Hz, H-5), 4.89–4.91 (2 H, m, H-2, H-3), 6.04 (1 H, s, H-1), for α-anomer, δ 1.36 (3 H, s, isopropylidene-CH3), 1.54 (3 H, s, isopropylidene-CH3), 2.10 (3 H, s, Ac), 2.15 (3 H, s, Ac), 3.90 (1 H, m, H-4), 4.19 (1 H, dd, J = 11.5, 6.3 Hz, H-5), 4.35 (1 H, dd, J = 11.5, 6.3 Hz, H-5), 4.67 (1 H, dd, J = 6.3, 4.0 Hz, H-3), 4.88 (1 H, m, H-2), 6.09 (1 H, d, J = 5.2 Hz, H-1) 13C-NMR (100 MHz, CDCl3) δ 20.85, 21.21, 24.61, 26.38, 53.85, 65.71, 85.13, 87.17, 88.54, 111.29, 169.16, 170.49; HR-MS (FAB, positive) calcd for C12H18NaO6S 313.0722 [(M+Na)+], found 313.0717.

5-O-Acetyl-2,3-O-Isopropylidene-4-Thio-β-D-Ribofuranosylazide (12)

To a solution of 11 (2.26 g, 7.78 mmol) and TMSN3 (3.09 mL, 23.3 mmol) in CH2Cl2 (20 mL) was added a solution of SnCl4 (223 mL, 1.91 mmol) in CH2Cl2 (10 mL) at 0 °C, and the mixture was stirred at the...
same temperature for 5 min. To the mixture was added aqueous saturated NaHCO₃, and the resulting white precipitate was filtered off with Celite. The filtrate was washed with aqueous saturated NaHCO₃ and brine, dried (Na₂SO₄), and evaporated. The residue was purified by column chromatography (SiO₂, hexane/AcOEt = 20/1) to give 12 (1.84 g, 86%, yellow oil); ¹H-NMR (400 MHz, CDCl₃) δ 1.31 (3 H, s, isopropylidene-CH₃), 1.50 (3 H, s, isopropylidene-CH₃), 2.11 (3 H, s, Ac), 3.63 (1 H, dd, J = 10.0, 5.4 Hz, H-4), 4.12 (1 H, dd, J = 11.3, 10.0 Hz, H-5), 4.27 (1 H, dd, J = 11.3, 5.4 Hz, H-5), 4.64 (1 H, d, J = 5.4 Hz, H-3), 4.86 (1 H, d, J = 5.4 Hz, H-2), 5.18 (1 H, s, H-1); ¹³C-NMR (100 MHz, CDCl₃) δ 20.73, 24.51, 26.26, 54.29, 65.21, 76.13, 85.59, 89.06, 111.23, 140.50; HR-MS (FAB, positive) (EI) calcld for C₁₀H₁₅N₃NaO₄S: 238.0940; IP: 238.0940; 6′-O-(2,3-O-isopropylidene-4-thio-β-D-ribofuranosyl)-5′-O-(tert-butylidimethylsilyl)-2′,3′-O-isopropylideneadenosine (6β)

A solution of 7 (381 mg, 1.87 mmol) and 8 (1.79 g, 4.00 mmol) in MeOH (10 mL) was stirred at room temperature for 10 h, and then evaporated. The residue was purified by flash column chromatography (silica gel, hexane/AcOEt = 1/2, AcOEt, then AcOEt/MeOH = 9/1) to give 6β (700 mg, 61%, white amorphous solid), 6α (59 mg, 5%, white amorphous solid), 14 (30 mg, 4%, yellow amorphous solid). 6β: ¹H-NMR (500 MHz, CDCl₃) δ 0.04 (3 H, s, Si-CH₃), 0.05 (3 H, s, Si-CH₃), 0.86 (9H, s, tert-butyl), 1.35 (3 H, s, isopropylidene-CH₃), 1.39 (3 H, s, isopropylidene-CH₃), 1.61 (3 H, s, isopropylidene-CH₃), 1.63 (3 H, s, isopropylidene-CH₃), 3.77 (1 H, dd, J = 11.4, 4.0, 4.0 Hz, H-5), 3.83–3.88 (2 H, m, H-5′, H-5″), 3.90 (1 H, m, H-4″), 4.01 (1 H, dd, J = 11.4, 3.7 Hz, H-5″), 4.39 (1 H, m, H-4″), 4.88 (1 H, dd, J = 6.3, 3.4 Hz, H-3′), 4.99 (1 H, dd, J = 5.2, 1.7 Hz, H-3″), 5.06 (1 H, dd, J = 6.3, 2.3 Hz, H-2″), 5.51 (1 H, dd, J = 5.2, 3.4 Hz, H-2″), 6.00 (1 H, d, J = 3.4 Hz, H-1″), 6.02 (1 H, d, J = 2.3 Hz, H-1″), 7.85 (1 H, s, H-8), 8.81 (1 H, s, H-2″); ¹³C-NMR (125 MHz, CDCl₃) δ −5.58, −5.47, 18.25 25.25, 25.80, 27.13, 27.92, 55.53, 63.37, 64.75, 74.54, 81.19, 85.23, 85.75, 86.22, 86.95, 91.15, 111.66, 114.09, 123.75, 137.20, 140.30, 146.81, 153.31; UV (MeOH) λ₅₅₆₅ = 260 nm; LR-MS (FAB, positive) m/z 610 [(M+H)⁺]: Anal. Calcd for C₇₂H₁₄₅N₅O₇SSi: C, 53.18; H, 7.11, N, 11.48. Found C, 52.88; H, 6.95; N, 11.35. NOE irradiated H-2′ observed H-2″ (3.8%), irradiated H-1″ observed H-4″ (2.3%), irradiated H-2″ observed H-2 (4.7%), irradiated H-4″ observed H-1″ (3.2%). 6α: ¹H-NMR (500 MHz, CDCl₃) δ 0.05 (3 H, s, Si-CH₃), 0.05 (3 H, s, Si-CH₃), 0.87 (9H, s, tert-butyl), 1.35 (3 H, s, isopropylidene-CH₃), 1.39 (3 H, s, isopropylidene-CH₃), 1.61 (3 H, s, isopropylidene-CH₃), 1.63 (3 H, s, isopropylidene-CH₃), 3.56 (1 H, d, J = 4.1, 1.1 Hz, H-4″), 3.76 (1 H, dd, J = 11.3, 3.6 Hz, H-5″), 3.84–3.88 (2 H, m, H-5′, H-5″), 3.93 (1 H, dd, J = 10.9, 4.1 Hz, H-5″), 4.39 (1 H, dd, J = 6.3, 3.6 Hz, H-4″), 4.90 (1 H, dd, J = 6.3, 2.7 Hz, H-3″),

2,3-O-Isopropylidene-4-Thio-D-Ribofuranosylamine (7)

A mixture of 12 (1.25 g, 4.57 mmol) and Pd/C (10%, 630 mg) in MeOH (45 mL) was stirred under atmospheric pressure of H₂ at room temperature for 1 h, and then the catalysts were filtered off with Celite. The filtrate was evaporated, and the residue was purified by column chromatography (NH-silica gel, hexane/AcOEt = 6/1, 2/1 then 1/3) to give 7 (1.09 g, quant., α/β = 1/2, brown oil); ¹H-NMR (300 MHz, CDCl₃) δ 1.30 (2 H, s), 1.36 (1 H, s), 1.52, (2 H, s), 1.57 (1 H, s), 1.83 (2 H, brs), 3.58 (2/3 H, dd, J = 7.7, 5.4 Hz), 3.73 (1/3 H, ddd, J = 6.3, 5.9 Hz), 4.32 (2/3 H, dd, J = 11.7, 7.7 Hz), 4.43 (2/3 H, dd, J = 11.7, 5.4 Hz), 4.49 (1/3 H, dd, J = 11.3, 5.9 Hz), 4.60–4.66 (2/3 H, m), 4.71–4.74 (1 H, m), 4.76 (2/3 H, d, J = 5.2 Hz, b–H–1), 4.92 (1/3 H, dd, J = 4.5 Hz), 5.04 (2/3 H, d, J = 4.5 Hz); ¹³C-NMR (100 MHz, CDCl₃) δ −6.54, −5.51, 18.23, 24.36, 25.27, 25.80, 26.50, 27.22, 57.20, 63.68, 64.31, 64.63, 81.06, 85.99, 86.45, 87.57, 91.20, 91.65, 95.13, 110.33, 113.74, 117.26, 132.20, 148.52, 151.04; HR-MS (EI) calcld for C₈H₁₅NO₅S: 205.0773 (M⁺), found 205.0773.

Figure 13. Important correlations in NOESY spectra of (a) cADPR (1), (b) cADPcR (2), (c) cADPtR (3), used for the calculations.
4.95 (1 H, d, J = 5.9 Hz, H-3′), 5.01 (1 H, dd, J = 5.9, 4.5 Hz, H-2′), 5.09 (1 H, dd, J = 6.3, 2.7 Hz, H-2′), 6.05 (1 H, d, J = 2.7 Hz, H-1′), 7.02 (1 H, d, J = 4.5 Hz, H-1′), 7.83 (1 H, s, H-8), 8.42 (1 H, s, H-2); 13C-NMR (125 MHz, CDCl3) δ −5.54, −5.44, 18.29, 24.22, 25.33, 25.97, 27.16, 54.36, 61.15, 63.34, 65.17, 80.59, 81.19, 85.30, 85.87, 86.99, 91.16, 111.38, 114.09, 122.77, 136.83, 140.88, 147.76, 150.00; UV (MeOH) λ_max = 259 nm; HR-MS (FAB, positive) calcd for C_{25}H_{39}N_{2}O_{5}SSi 610.2731 (M+) found 610.2727. NOE irradiated H-2′ observed H-4′ (10%), irradiated H-1′/observed H-2′ (10.7%), irradiated H-2′/observed H-1′ (8.4%), irradiated H-4′/observed H-2′ (1.7%).

13°H-NMR (500 MHz, CDCl3) δ 0.00 (3 H, s, Si-CH3), 0.01 (3 H, s, Si-CH3), 0.83 (9H, s, tert-butyl), 1.32 (3 H, s, isopropylidene-CH3), 1.40 (3 H, s, isopropylidene-CH-3), 1.59 (3 H, s, isopropylidene-CH3), 1.62 (3 H, s, isopropylidene-CH3). 3.69 (1 H, m, H-4′), 3.73 (1 H, dd, J = 11.3, 4.5 Hz, H-5′), 3.83–3.87 (2 H, m, H-5′, H-6′), 3.90 (1 H, br, OH), 4.07 (1 H, dd, J = 10.4, 2.7 Hz, H-5′), 4.39 (1 H, m, H-4′), 4.87 (1 H, d, J = 5.0 Hz, H-2′), 4.94–4.96 (2 H, m, H-3′, H-3′), 5.28 (1 H, dd, J = 6.3, 2.3 Hz, H-2′), 6.13 (1 H, d, J = 2.3 Hz, H-1′), 6.16 (1 H, br, H-1′), 7.49 (1 H, d, J = 9.1 Hz, NH), 7.97 (1 H, s, H-2′), 8.45 (1 H, s, H-8); 13C-NMR (125 MHz, CDCl3) δ −5.64, −5.51, 18.23, 24.36, 25.27, 25.80, 26.50, 27.22, 57.20, 63.68, 64.31, 66.63, 81.06, 85.99, 85.46, 87.57, 91.20, 91.65, 95.13, 110.33, 113.74, 117.76, 132.60, 148.52, 151.04; UV (MeOH) λ_max = 273 nm; HR-MS (FAB, positive) calcd for C_{25}H_{39}N_{2}O_{5}SSi 610.2731 (M+) found 610.2741.

N1,(2,3-O-isopropylidene-4-thio-5-O-dimethoxytrityl-β-D-ribofuranosyl)-5′-O-(tert-butylidemethylsilyl)-2′,3′-O-Isopropylidenedenosine (14)

A solution of 6β (889 mg, 1.46 mmol) and DMTcCl (989 mg, 2.92 mol) in pyridine (5 mL) was stirred at room temperature for 12 h. After addition of MeOH, the resulting mixture was partitioned between EtOAc and 1 M aqueous HCl, and the organic layer was washed with H2O and brine, dried (Na2SO4), and evaporated. The residue was purified by column chromatography (silica gel, hexane/AcOEt = 6/1, 3/1, 2/3, and 2/3) to give 14 (1.08 g, 81%, white amorphous solid); 1H-NMR (500 MHz, CDCl3) δ 0.05 (3 H, s, Si-CH3), 0.06 (3 H, s, Si-CH3), 0.88 (9H, s, tert-butyl), 1.30 (3 H, s, isopropylidene-CH3), 1.38 (3 H, s, isopropylidene-CH3), 1.59 (3 H, s, isopropylidene-CH3), 1.62 (3 H, s, isopropylidene-CH3), 1.77 (3 H, s, isopropylidene-CH3), 1.80–1.86 (2 H, m, H-5′–5′), 2.65 (2 H, m, H-4′–4′), 4.38 (1 H, ddd, J = 5.7, 4.0, 3.9 Hz, H-4′), 4.72 (1 H, dd, J = 5.7, 4.0 Hz, H-3′), 4.86 (1 H, dd, J = 6.3, 5.7 Hz, H-3′), 4.89 (1 H, dd, J = 5.7, 2.3 Hz, H-2′), 4.97 (1 H, dd, J = 6.3, 2.9 Hz, H-2′) 6.02 (1 H, d, J = 2.9 Hz, H-1′), 6.81–7.47 (13 H, m, Ar), 7.82 (1 H, s, H-8), 8.12 (1 H, s, H-2); 13C-NMR (125 MHz, CDCl3) δ −5.58, −5.47, 18.22, 25.21, 25.28, 25.79, 27.13, 27.35, 54.95, 55.05, 63.30, 64.65, 66.78, 81.06, 84.82, 85.25, 86.53, 86.79, 89.22, 90.86, 112.38, 113.03, 113.05, 114.01, 123.23, 126.66, 127.73, 127.95, 129.92, 129.96, 135.46, 135.60, 136.51, 140.35, 144.47, 145.55, 153.98, 158.37; LR-MS (FAB, positive) m/z 882 [M+H]+; UV (MeOH) λ_max = 259 nm; Anal. Calcd for C_{25}H_{39}N_{2}O_{5}SSi: C, 63.20; H, 6.74, N, 7.68. Found C, 63.19; H, 6.91; N, 7.39.

N1,(2,3-O-isopropylidene-4-thio-5-O-dimethoxytrityl-β-D-ribofuranosyl)-5′-O-[bis(phenylthio)phosphoryl]-2′,3′-O-Isopropylidenedenosine (15)

To a solution of 15 (845 mg, 1.06 mmol) in pyridine (5 mL) was added a solution of PSS (809 mg, 2.12 mmol) and TPSCI (693 mg, 1.91 mmol) in pyridine (5 mL) at −15 °C, and the mixture was stirred at the same temperature for 2.5 h. After addition of H2O, the resulting mixture was partitioned between EtOAc and 1 M aqueous HCl, and the organic layer was washed with H2O and brine,
A solution of 16 (23 mg, 22 mmol) in aqueous 60% AcOH (2 mL) was stirred at room temperature for 4 h, and then evaporated. The residue was purified by column chromatography (silica gel, hexane/AcOEt = 1/1, 1/3, then AcOEt) to give 17 (15 mg, colorless amorphous solid): $^1$H-NMR (500 MHz, CDCl$_3$) δ 1.34 (3 H, s, isopropylidene-CH$_3$), 1.38 (3 H, s, isopropylidene-CH$_3$), 1.42 (3 H, s, isopropylidene-CH$_3$), 1.50 (9 H, t, J = 7.4 Hz, isopropylidene-CH$_3$), 2.9 Hz, isopropylidene-CH$_3$), 3.76–3.83 (3 H, m, H-4a, H-5′ × 2), 4.30 (1 H, dd, J = 11.4, 9.7, 5.2 Hz, H-5′), 4.43 (1 H, d, J = 6.3, 3.2, 2.9 Hz, H-4′), 4.47 (dd, J = 12.6, 9.7, 6.3 Hz, H-3′), 4.83 (1 H, d, J = 5.2 Hz, H-5′), 5.04 (1 H, dd, J = 6.3, 2.9 Hz, H-3′), 5.09 (1 H, d, J = 5.2, 1.1 Hz, H-2′), 5.18 (1 H, d, J = 6.3, 1.7 Hz, H-2′), 5.50 (1 H, d, J = 1.7 Hz, H-1′), 6.07 (1 H, d, J = 1.1 Hz, H-1′), 7.27–7.54 (10H, m, Ar), 7.63 (1 H, s, H-8), 8.55 (1 H, s, H-2); $^{13}$C-NMR (125 MHz, CDCl$_3$) δ 24.88, 25.08, 26.87, 27.28, 57.09, 64.26, 66.26, 66.32, 73.96, 81.93, 84.52, 85.65, 85.73, 89.68, 91.39, 110.99, 114.10, 123.84, 125.28, 125.34, 125.46, 125.52, 129.27, 129.29, 129.38, 129.40, 129.58, 129.60, 129.73, 129.75, 135.04, 135.09, 135.37, 135.40, 138.01, 140.22, 147.09, 154.09; $^{31}$P-NMR (202 MHz, CDCl$_3$) δ 52.23 (s); UV (MeOH) $\lambda_{max}$ = 251, 258 nm; HR-MS (FAB, positive) calcd for C$_{77}$H$_{138}$N$_5$O$_{12}$P$_2$S$_7$46.1126 [(M-H)$_-$], found 746.1106.

Cyclic ADP-4-Thio-Ribose 2′,3′- Bisacetamide (19)

To a mixture of AgNO$_3$ (36 mg, 0.21 mmol), Et$_3$N (29 ml, 0.21 mmol), and MS 3A (powder, 1.0 g) in pyridine (8 mL), a solution of 5 (9 mg, 50 OD$_{250}$ unit, 4 mmol) in pyridine (8 mL) was added slowly over 15 h, using a syringe-pump, at room temperature under shaking. To the mixture was added TEAA buffer (2.0 M, pH 7.0, 2 mL), and the resulting mixture was filtered with Celite and evaporated. The residue was partitioned between EtOAc and H$_2$O, and the aqueous layer was evaporated, and the residue was purified by column chromatography (ODS, 1.2 × 16 cm, 0–35% CH$_2$CN/0.1 M TEAA buffer (0.1 M, pH 7.0, 400 mL), linear gradient). The excess TEAA included in the residue was removed by column chromatography (ODS, 1.2 × 16 cm, CH$_2$CN/H$_2$O = 1/1). The product was lyophilized to give 19 (37 mg, 512 OD$_{250}$ unit, 46%) as a triethylammonium acetate (TEAA) buffer (0.5 M, pH 7.0, 3 mL) then H$_2$PO$_4$ (101 μL, 2.0 mmol) and Et$_3$N (140 μL, 1.0 mmol), and the mixture was stirred at room temperature for 13 h, and then evaporated. The residue was partitioned between EtOAc and H$_2$O, and the aqueous layer was evaporated. The residue was purified by column chromatography (ODS, 1.2 × 16 cm, 0–37% CH$_2$CN/0.1 M TEAA buffer (0.1 M, pH 7.0, 400 mL), linear gradient). The excess TEAA included in the residue was removed by column chromatography (ODS, 1.2 × 16 cm, CH$_2$CN/H$_2$O = 1/1). The product was lyophilized to give 5 (37 mg, 512 OD$_{250}$ unit, 46%) as a triethylammonium salt: $^1$H-NMR (500 MHz, D$_2$O) δ 1.26 (9H, t, J = 7.4 Hz, (CH$_2$CH$_2$)$_3$N), 1.40 (3 H, s, isopropylidene-CH$_3$), 1.43 (3H, s, isopropylidene-CH$_3$), 1.69 (3H, s, isopropylidene-CH$_3$), 3.18 (6H, q, J = 7.4 Hz, (CH$_2$CH$_2$)$_3$N), 4.10–4.13 (4H, m, H-4′, H-5′ × 2, H-5′), 4.70 (1 H, m, H-4′), 4.70 (1 H, m, H-4′), 4.72 (1 H, m, H-5′), 4.74 (1 H, m, H-5′), 4.71 (1 H, m, H-4′), 4.94 (1 H, d, J = 5.2 Hz, H-3′) 5.12–5.15 (2 H, m, H-2′, H-3′), 5.42 (1 H, dd, J = 6.3, 2.9 Hz, H-3′), 5.39 (1 H, d, J = 5.2 Hz, H-2′), 5.95 (1 H, s, H-1″), 6.37 (1 H, s, H-1′), 7.19–7.33 (5H, m, Ar), 8.41 (1 H, s, 8-H), 9.24 (1 H, s, 2-H); $^{13}$C-NMR (125 MHz, D$_2$O) δ 8.30, 24.32, 24.35, 25.95, 26.41, 46.71, 54.48, 54.55, 65.90, 65.95, 66.59, 66.62, 75.84, 81.39, 84.11, 86.19, 86.27, 86.52, 89.30, 90.88, 113.51, 114.72, 119.08, 127.91, 129.08, 129.47, 129.51, 132.75, 132.79, 143.02, 145.48, 146.84, 150.53; $^{31}$P-NMR (202 MHz, D$_2$O) δ 17.54 (s), 0.80 (s); UV (D$_2$O) $\lambda_{max}$ = 258 nm; HR-MS (FAB, negative) calcd for C$_{77}$H$_{138}$N$_5$O$_{12}$P$_2$S$_7$46.1126 [(M-H)$_-$], found 746.1106.
Cyclic ADP-4-Thioribose (3)

A solution of 19 (280 OD_{260} unit, 25 mmol) in aqueous 60% HCO_2H (1 mL) was stirred at room temperature for 40 h and then evaporated. After co-evaporation with H_2O, the residue was purified by column chromatography (ODS, 1.2 x 16 cm, TEAA buffer (5 mM, pH 7.0)). The excess TEAA included in the residue was removed by column chromatography (ODS, 1.2 x 16 cm, CH_3CN/H_2O = 1/1). The product was lyophilized to give 19 (6 mg, 36 OD_{260} unit, 72%) as a triethylammonium salt: ^{1}H-NMR (500 MHz, D_2O) δ 1.24 (9H, t, J = 7.4 Hz (CH_3CH_2)3N), 1.41 (3H, s, isopropylidene-CH_3), 1.43 (3H, s, isopropylidene-CH_2), 1.63 (3H, s, isopropylidene-CH_3), 1.65 (3H, s, isopropylidene-CH_3), 3.17 (6H, q, J = 7.4 Hz, (CH_2CH_2)3N), 3.91 (1H, m, H-5′), 4.13 (1H, m, H-4′), 4.20 (1H, m, H-5′), 4.22 (1H, m, H-5′), 4.32 (1H, m, H-5′), 4.58 (1H, m, H-4′), 5.99 (1H, s, 2-H), 5.89 (1H, dd, J = 4.8 Hz, H-3′) 5.13 (1H, d, J = 4.8 Hz, H-2′), 5.42 (1H, dd, J = 6.3, 2.9 Hz, H-3′), 5.22 (1H, d, J = 6.3, 1.7 Hz, H-2′), 5.99 (1H, s, H-1′), 6.38 (1H, d, J = 1.7 Hz, H-1′), 8.38 (1H, s, H-8′), 9.59 (1H, s, 2-H); ^{13}C-NMR (125 MHz, D_2O) δ 8.29, 24.39, 24.42, 26.07, 26.26, 46.73, 54.50, 54.59, 64.80, 68.03, 68.43, 68.00, 77.65, 81.36, 83.25, 86.65, 87.63, 87.34, 90.82, 91.45, 113.11, 114.59, 119.58, 144.91, 145.55, 147.20, 150.67; ^{31}P-NMR (202 MHz, D_2O) δ = 9.15 (d, J = 12.2 Hz), δ = 10.20 (d, J = 12.2 Hz); UV (D_2O) λ_{max} = 258 nm; HR-MS (FAB, negative) calcd for C_{21}H_{28}N_{5}O_{12}P_{2}S 636.0936, found 636.0947.

Rat brain microsomes were prepared by a procedure according to the previous method (Murayama, T. and Ogawa Y. (1996). J. Biol. Chem. 271, 5079–5084.). cADPR or cADPR (1.6 OD_{260} unit) was preincubated in 20 mM MOPS buffer (pH 7.1, 160 μL) at 37 °C for 5 min. This was added to the solution of the microsome fraction of rat brain extract (14.9 mg/mL, 140 μL), and the mixture was incubated at 37 °C. The reaction mixture was sampled (25 μL) at every 30 min afterwards and diluted with water (175 μL), which was frozen in liquid nitrogen to stop the reaction. After the samples were a centrifuged at 12000 rpm at 4 °C for 15 min, the supernatants were filtered using centrifugal filter at 12000 rpm at 4 °C for 15 min, and the resulting filtrates (70 μL) were analyzed by ion exchange HPLC (TSK-GEL DEAE-2SW, 4.6 x 250 mm; 5–35% 1 M HCO_2NH_2/20% MeCN, 20 μL; 260 nm). The results are shown in Figure 2.

**Stability in Rat Brain Microsomes**

These bioassays were carried out as reported previously (Kudoh et al., 2005).

**Biological Evaluations with Neuronal Cultured Cells**

NG108-15 neuroblastoma x glioma hybrid cells were cultured as reported previously (Higashida et al., 1990). Oregon Geen-loaded NG108-15 cells were incubated for 2 min in the following calcium-free medium (140 mM K-glutamate, 20 mM PIPES, 5 mM EGTA, 2 mM Mg-ATP, 10 mM glucose, 1 mM magnesium chloride, 0.01% bovine serum albumin, pH 6.8) at 37 °C, and subsequently permeabilized with 250 nM digitonin in the calcium-free medium. cADPR (1–100 μM), cADPcr (1–100 μM), or cADPcr (1–100 μM) were applied together with the digitonin-containing permeabilization buffer to allow free passage of these nucleotides into the cytoplasm. Concentrations of [Ca^{2+}], were determined microspectrofluorometrically using fura-2 in differentiated NG108-15 cells cultured on polyllysine-coated glass coverslips. The cells were loaded with fura-2 using 5 μM Oregon Green 488 1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetate acetyoxymethyl ester (BAPTA-1 AM). Fluorescence was measured at 37 °C with excitation wavelengths of 485 nm and emission wavelengths of 538 nm using an Argus 50 Ca^{2+} microspectrofluorometric system (Hamamatsu Photonics, Hamamatsu, Japan) and images were collected every 10 s for up to 5 min.
The changes in fluorescence intensity of each cell were expanded into an X-t plane, and data were performed in fluorescence intensity at each time (x) divided by resting intensity at time 0, i.e., $F(t)/F_0$ (Amina et al., 2010). Data are expressed as mean ± s.e.m. Statistical analysis was performed using a Student’s t test. The criterion for significance in all cases was $p < 0.05$.

### Computational Calculations

For structure determination, molecular calculations were carried out by AMBER11 (Case et al., 2010). cADPR (1), cADPRcR (2) and cADPRtR (3) were modeled by the General AMBER Force Field (Wang et al., 2004). AM1-BCC charge (Jakalian, A., Jack, D. B., and Bayly, C. I. (2002). J. Comput. Chem. 23, 1623–1641) was used for cADPR and its analogs assigned by Antechamber module (Wang et al., 2006) of AMBER11. In the calculations, interatomic distances were determined by the integrated volumes of the NOEY cross-peaks. As cross peaks of cADPR and cADPRcR, previously published data were used (Kudoh, et al., 2005). For generating conformations, we carried out 400 ps simulated annealing molecular dynamics simulations: temperature was decreased from 1000 to 300 K. These simulations were repeated 100 times with different initial velocity for each compound. In calculated conformations, we chose and analyzed the lowest energy conformation.

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