Trifluoromethylated cyclic-ADP-ribose mimic: synthesis of 8-trifluoromethyl-N^1-[(5″-*O*-phosphorylethoxy)methyl]-5′-*O*-phosphorylinosine-5′,5″-cyclic pyrophosphate (8-CF_3-cIDPRE) and its calcium release activity in T cells†

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A convenient trifluoromethylation method was firstly applied to the synthesis of 8- CF_3-purine nucleosides. On the basis of this method, new protection and deprotection strategies were developed for the successful synthesis of the trifluoromethylated cyclic-ADP-ribose mimic, 8-CF_3-cIDPRE **1**. Using intact, fura-2-loaded Jurkat T cells compound **1** and 2′,3′-*O*-isopropylidene 8-CF_3-cIDPRE **14** were characterized as membrane-permeant cADPR agonists. Contrary to the 8-substituted cADPR analogues that mainly act as antagonists of cADPR in cells, 8-substituted cIDPRE derivatives were shown to be Ca^{2+} mobilizing agonists. Here we report that even compound **1**, the 8-substituted cIDPRE with the strong electron withdrawing CF_3 group, behaves as an agonist in T cells. Interestingly, also the partially protected 2′,3′-*O*-isopropylidene 8-CF_3-cIDPRE activated Ca^{2+} signaling indicating only a minor role for the hydroxyl groups of the southern ribose of cADPR for its biological activity. To our knowledge 8-CF_3-cIDPRE **1** is the first reported fluoro substituted cADPR mimic and 8-CF_3-cIDPRE **1** and compound **14** are promising molecular probes for elucidating the mode of action of cADPR.

Introduction

Cyclic ADP-ribose (cADPR) (Fig. 1) was discovered as a Ca^{2+} mobilizing metabolite of β-nicotinamide adenine dinucleotide (NAD^+) by Lee and coworkers in sea urchin egg homogenates.[1] The structure of cADPR was determined by X-ray crystallography analysis.[2] Besides D-*myo*-inositol 1,4,5-trisphosphate ($InsP_3$) and nicotinic acid adenine dinucleotide phosphate (NAADP), cADPR was found to be one of the principal Ca^{2+}-releasing second messengers involved in cellular Ca^{2+} homeostasis.[3] The important role of cADPR and its Ca^{2+} signaling pathway is illustrated by its phylogenetically widespread occurrence from unicellular protozoa, plants, and animals up to humans.[4] In addition, cADPR-mediated Ca^{2+} signaling has been found in a variety of cellular processes, such as fertilization, insulin secretion in pancreatic islets, hormonal signaling in pancreatic acinar cells, chemotaxis in neutrophils, bone resorption, muscle contraction, long-term synaptic depression in hippocampus, lymphocyte activation and proliferation, abscisic acid signaling in plants and sponges, and in plant circadian clock.[5]

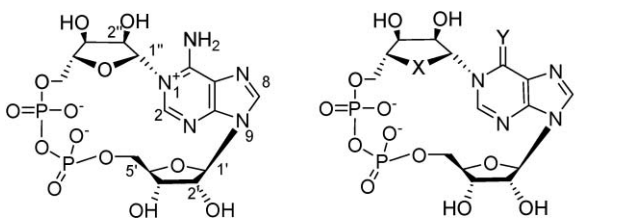

Fig. 1 cADPR and its analogues.

Several lines of evidence, both pharmacological and molecular, argue for activation of ryanodine receptors (RyR) by cADPR. A number of key proteins is involved in specifically shaping RyR-dependent Ca^{2+} signaling.[6] However, it is unclear whether cADPR elicits Ca^{2+} release by direct binding to RyR or *via* an associated protein. A significant approach to explore the molecular mechanism of Ca^{2+} release is to investigate the structure–activity relationship of cADPR and its analogues regarding their calcium mobilizing activities. Agonists or antagonists of cADPR that resulted from such studies are valuable tools to study cellular signal transduction and are also useful as lead structures for drug discovery.[7,8]

Many derivatives of cADPR have been synthesized and studied in terms of biological activity, specifically the analogues with substitutions at the C-8 position of the adenine ring.[7,8] 8-Substituted cADPR, such as 8-Br-cADPR, 8-N_3-cADPR, and 8-NH_2-cADPR are antagonists of cADPR in sea urchin egg homogenates. 8-NH_2-cADPR is the most potent one and has been used to assess cADPR-modulated Ca^{2+} signaling pathways in a variety of biological systems, such as sea urchin eggs, smooth muscle and

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T cells. 8-Br-cADPR was shown to be cell permeant and became an invaluable tool in studying cADPR function in intact cells, although in its antagonist properties not as potent as 8-NH_2-cADPR.[7,9] Shuto *et al.* reported cyclic ADP-carbocyclic-ribose (cADPcR) (Fig. 1) as a stable mimic of cADPR and determined a stronger Ca^{2+} release effect than cADPR in sea urchin egg homogenates, while in Jurkat T-lymphocytes it is less potent as compared to cADPR.[10] 8-Substituted cADPcR analogues are agonists rather than antagonists of cADPR in sea urchin egg homogenates. Potter and Guse reported N^1-cyclized inosine 5′-diphosphoribose (N^1-cIDPR) (Fig. 1) to be as potent as cADPR in mediating Ca^{2+} release in permeabilized human Jurkat T cells. A series of 8-substituted N^1-cIDPR was also synthesized and characterized as cADPR agonists. In particular, 8-I-N^1-cIDPR and 8-N_3-N^1-cIDPR showed even higher activities than the natural cADPR. 8-I-, 8-phenyl- and 8-Br-N^1-cIDPR were reported as membrane permeant agonists of cADPR in intact Jurkat T cells.[11]

A series of modifications on the northern and southern ribose moiety of cIDPR or cADPR was investigated in our group.[12] N^1-Ether cyclic inosine 5′-diphosphoribose (cIDPRE) (Fig. 2) behaved as a stable mimic of cADPR. cIDPRE penetrated the plasma membrane and released Ca^{2+} from an intracellular cADPR-sensitive Ca^{2+} store, and subsequently initiated Ca^{2+} release-activated Ca^{2+} entry in intact Jurkat T cells. Several 8-substituted derivatives of cIDPRE were also synthesized and characterized as membrane-permeant agonists of cADPR in Jurkat T cells.[12b] We found that different functional groups at the 8-position resulted in different biological activities: 8-N_3- and 8-NH_2-derivates showed similar effect in their agonist activity as compared to cIDPRE, while 8-Br- and 8-Cl-cIDPRE had no effect on the free cytosolic Ca^{2+} concentration in Jurkat T cells.[12b]

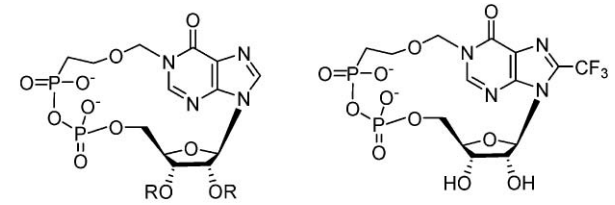


Fig. 2 Structures of cIDPRE and 8-CF_3-cIDPRE (**1**).

To study the structure–activity relationship and further explore the importance of the 8-substitution in cADPR, we report here the synthesis of 8-CF_3-cIDPRE (**1**) (Fig. 2) and its pharmacological characterization with regard to Ca^{2+} signaling. 8-Substituted cADPR analogues, with properties of either an electron withdrawing group or an electron releasing group, such as 8-Br-cADPR, 8-N_3-cADPR, and 8-NH_2-cADPR, are antagonists of cADPR in sea urchin egg homogenate and mammalian cells.[9] In contrast, 8-substitutes cIDPR, 8-I-, 8-N_3-, 8-phenyl- and 8-Br-N^1-cIDPR were reported as membrane permeant agonists of cADPR in intact Jurkat T cells.[11] However, in the cIDPRE series, among the 8-substituted cIDPRE analogues only the 8-N_3- and 8-NH_2-derivatives showed similar effect in their agonist activity as compared to cIDPRE, while 8-Br- and 8-Cl-cIDPRE had no effect on the free cytosolic Ca^{2+} concentration in Jurkat T cells.[12b] Thus, it would be interesting to analyze if the loss of calcium release activity of 8-Br- and 8-Cl-cIDPRE in Jurkat T cells is related to their electronic properties or the potential of hydrogen bonding formation of 8-substitution. In this report, 8-CF_3-cIDPRE was designed. The strong electron withdrawing property and the ability to form the hydrogen bonding of fluorine and its novel role in medicinal chemistry,[13] such as to provide metabolic stability and membrane permeability, 8-CF_3-cIDPRE is an interesting molecule for the investigation of the SAR of cADPR. In addition, although a number of studies on the synthesis and SAR have been reported,[9–12,14–16] fluorinated cADPR derivatives have not yet been investigated.

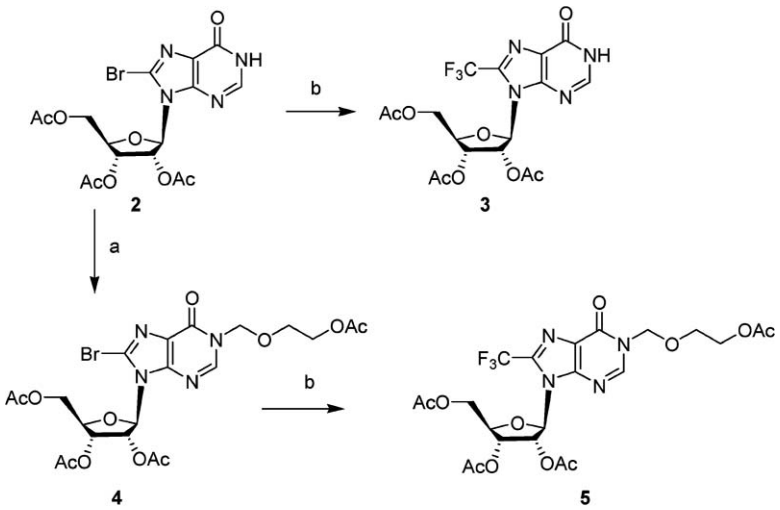
In this paper, the structural position of 8-CF_3-cIDPRE is numbered as follows: a single prime numbering scheme is used for the position of the N^9-ribosyl moiety and a double prime numbering scheme is used for the position of the N^1-substitution (Fig. 1).

Results and discussion

Chemistry

As trifluoromethyl substituted compounds play an important role in medicinal chemistry, the trifluoromethylation reaction has often been studied.[17] However, due to the sensitivity of the glycoside bond under the reaction conditions, only limited work has been reported on the trifluoromethylation of nucleosides and nucleotides. Beal[18] reported $FSO_2CF_2CO_2Me$[19] as a reagent introducing the trifluoromethyl group to the 6-position of purine nucleosides. The first attempt to prepare 8-CF_3 purine nucleosides was described by Kobayashi *et al.*[20] The trifluoromethylation was carried out in the presence of the active reagent CF_3–Cu which was prepared from Cu powder, but the product of trifluoromethylation could only be obtained by using very pure CF_3–Cu and traces of Cu powder interfered with this reaction. Recently, Yamakawa[21] reported that the $CF_3I/FeSO_4/H_2O_2/H_2SO_4$ system could be used to introduce the trifluoromethyl group at the C-8 position of purine base in moderate yields. However, when inosine was used as the substrate, the yield was very low and furthermore, the gaseous reagent CF_3I made the reaction difficult to handle.

We tried to develop a convenient method to introduce CF_3 to the C-8 position of purine. Firstly, 8-bromo-2′,3′,5′-tri-*O*-acetylinosine (**2**) was used as a model compound and the CF_3CO_2Na/CuI[22] system was selected as the reagent. The reaction was performed in NMP at 150 °C or in DMF/HMPA at 135 °C and with the aid of microwave, respectively. However, the desired compound was not detected. When the trifluoromethylation of compound (**2**) was carried out under the condition of $FSO_2CF_2CO_2Me/CuI/HMPA/DMF$,[18] the trifluoromethylation product (**3**) was obtained (data not shown), but the product (**3**) decomposed slightly after storage for several hours in the reaction solution (as monitored by TLC). However, compound (**5**) was synthesized smoothly by trifluoromethylation of compound (**4**) under the same conditions. Thus, it seems that the ether chain at N^1 position makes the trifluoromethyl compound more stable (Scheme 1). To the best of our knowledge, this is the first report using this convenient method to introduce the trifluoromethyl group at the 8-position of purine nucleoside.



[a]Reagents and conditions:a) DBU, $ClCH_2OCH_2CH_2OAc$, CH_2Cl_2.
b) $FSO_2CF_2CO_2Me$, CuI, HMPA

Scheme 1 Model reaction for 8-position trifluoromethylation of purine nucleoside.[a]

With the convenient trifluoromethylation model reaction in hand, a new sequence of suitable protection and deprotection strategies was developed for the synthesis of 8-CF_3-cIDPRE. The special property of the 8-trifluoromethyl group at the purine moiety renders the nucleoside molecule more sensitive to strong acidic or basic reagents. In general, TBDMS, DMT, MMT were used for the protection of hydroxyl groups in the synthesis of cIDPRE and cADPR analogues,[12] but in our case they were sensitive to the condition for trifluoromethylation. (Scheme 2)

2′,3′-*O*-Isopropylidene-8-bromoinosine[23] (**6**) was used as the starting material. At first, *tert*-butyldimethylsilyl chloride (TBDMSCl), DMTCl, and MMTCl were used for the protection of 5′-OH. (Scheme 2) After the N^1 ether chain was introduced successively, substrate (**8**) was trifluoromethylated by the $FSO_2CF_2CO_2Me$/CuI system. However, the desired 8-trifluoromethyl substituted product was not obtained, but instead the 5′-deprotected compound **9**[12a] appeared (Scheme 2). The protecting group TBDPS is 100–250 times more stable than TBDMS in acid condition and could be deprotected selectively by 70% HF·Py.[24] After optimization of the conditions, we found that the desired trifluoromethylated products were obtained only using Ac, Bz or TBDPS as the protecting groups. On the other hand, deprotection in strong basic condition also must be avoided because of the lability of the CF_3 group in alkali.[25] Thus the synthesis of 8-CF_3-cIDPRE was designed as depicted in Scheme 3.

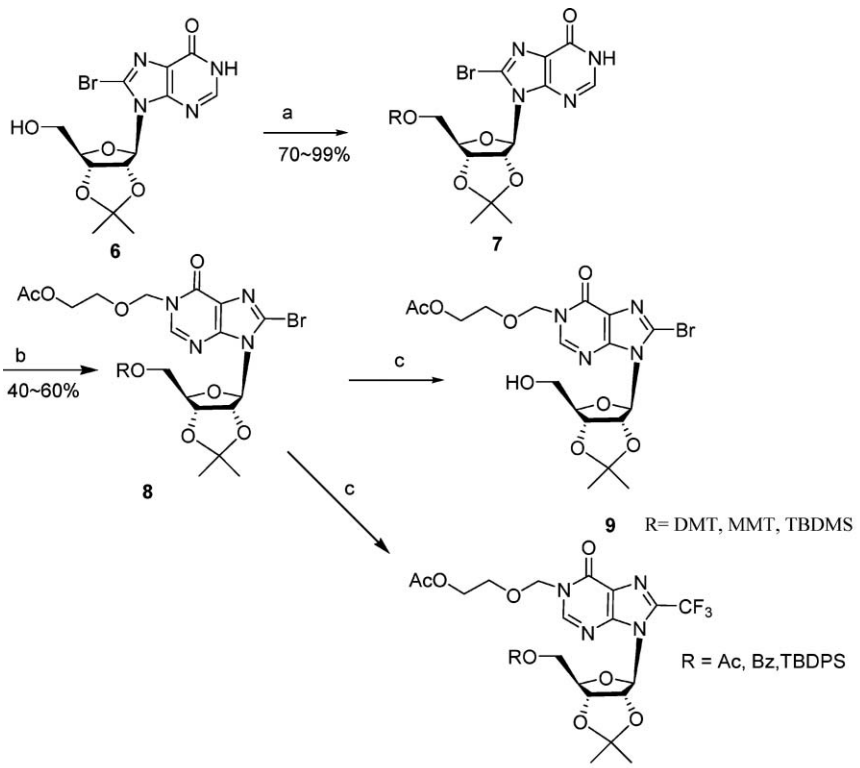
Compound **6** reacted with TBDPSCl to give compound **7**. Then the N^1 substitution was carried out regioselectively with 2-chloromethoxyethyl acetate in the presence of excess DBU to afford **8** in 60% yield. The structure of the N^1-substituted inosine **8** was confirmed by ^{1}H NMR, ^{13}C NMR and HMBC. **8** reacted with $FSO_2CF_2CO_2Me$/CuI in HMPA and the 8-CF_3-substituted compound **10** was achieved successfully in 59% yield. The structure of **10** was confirmed by ^{1}H, ^{13}C, ^{19}F-NMR and HRMS.

70% HF·Py[24] was used to remove the 5′-TBDPS group in compound **10** at 0 °C in high yield (90%). This reaction should be handled carefully since when HF·Py was added at room temperature the de-purine product was detected. The strong electrophilic action of the trifluoromethyl on 8-position of inosine renders the glycosyl bond sensitive to acid conditions. Compound **11** was phosphorylated with cyclohexylammonium S,S-diphenylphosphorodithioate (PSS) in the presence of triisopropylbenzenesulfonyl chloride (TPSCl) and tetrazole in pyridine to give **12** in 86% yield. As the S,S-diphenylphosphorodithioate and the trifluoromethyl group are sensitive to basic conditions, the condition of deacetylation in **12** was optimized. When 0.6 equivalents of AcCl-MeOH[26] was used for the reaction for 24 h at room temperature, **12** was successfully deacetylated in good yield. The free hydroxyl of **12** was phosphorylated by using $POCl_3$/DIEA in CH_3CN at 0 °C for 12 h, and the partial deprotection of S,S-diphenylphosphate was completed successfully in one step by treatment in 1 M TEAB for 6 h.[27] After purification using HPLC, the desired compound **13** was obtained as its triethylammonium form.

The intramolecular cyclization of compound **13** was performed in the presence of excess I_2 and 3 Å molecular sieve in pyridine.[10,12] The cyclic product 8-CF_3-2′,3′-*O*-isopropylidene cIDPRE **14** was purified by HPLC as its triethylammonium salt in 51% yield. The structure of **14** was confirmed by HR-ESI-MS, ^{1}H-NMR, ^{19}F NMR and ^{31}P NMR. Finally, the removal of isopropylidene group of **14** was carried out with 15% HCOOH in water at room temperature for 48 h to obtain the target compound 8-CF_3-cIDPRE **1**. Compound **1** was purified by HPLC as its triethylammonium salt in 64.5% yield, characterized by HR-ESI-MS, ^{1}H NMR, ^{19}F NMR and ^{31}P NMR.

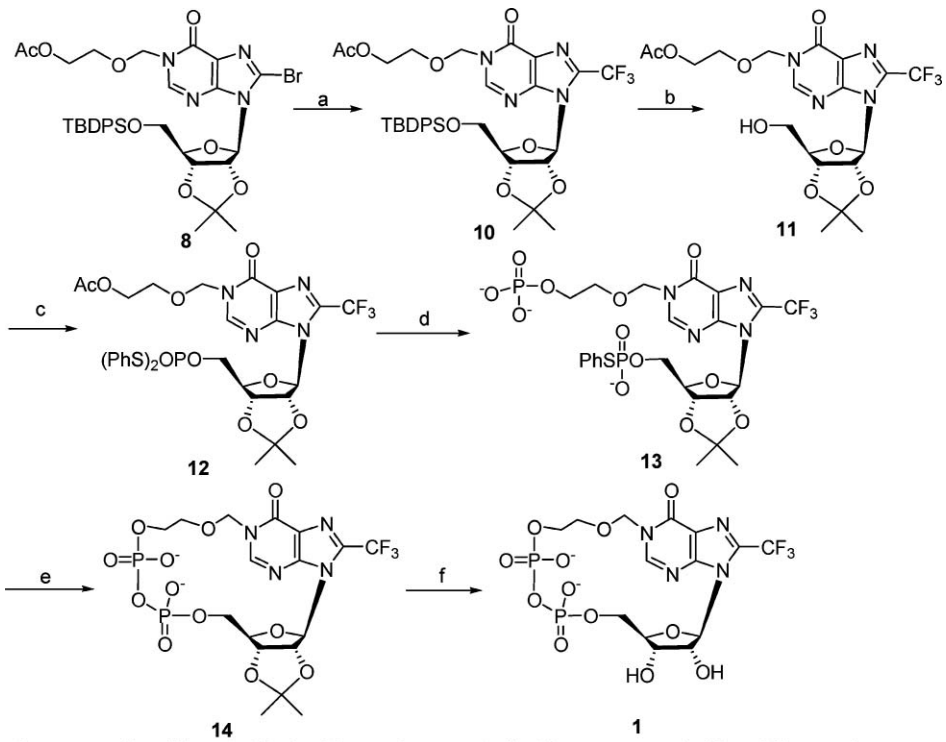
Pharmacology

When added to Fura2-loaded intact Jurkat T cells, compound **1** showed a biphasic type of Ca^{2+} release activity (Fig. 3B). To confer solubility, a small concentration of DMSO had to be used in these experiments. Thus, the corresponding control was carried out using the same carrier solution (Fig. 3A), but with negligible Ca^{2+} signaling activity. Jurkat T cells have been used as a model for T cell signaling since many years; the biphasic nature of the Ca^{2+} response to TCR/CD3 stimulation is well established



[a]Reagents and conditions:a)RCl, Py or imidazole b)DBU, $ClCH_2OCH_2CH_2OAc$, CH_2Cl_2. c) $FSO_2CF_2CO_2Me$, CuI, HMPA

Scheme 2 Selection of the proper protection group of 5′-OH for trifluoromethylation.[a]



[a]Reagents and conditions:a) $FSO_2CF_2CO_2Me$, CuI , HMPA. b) 70% HF·Py, THF. c) PSS, TPSCl, tetrazole, Py, rt; d) 1.AcCl, MeOH. 2.$POCl_3$ DIEA then TEAB e) I_2, 3A MS. f) 15% HCOOH

Scheme 3 Synthesis of 8-CF_3-cIDPRE.[a]

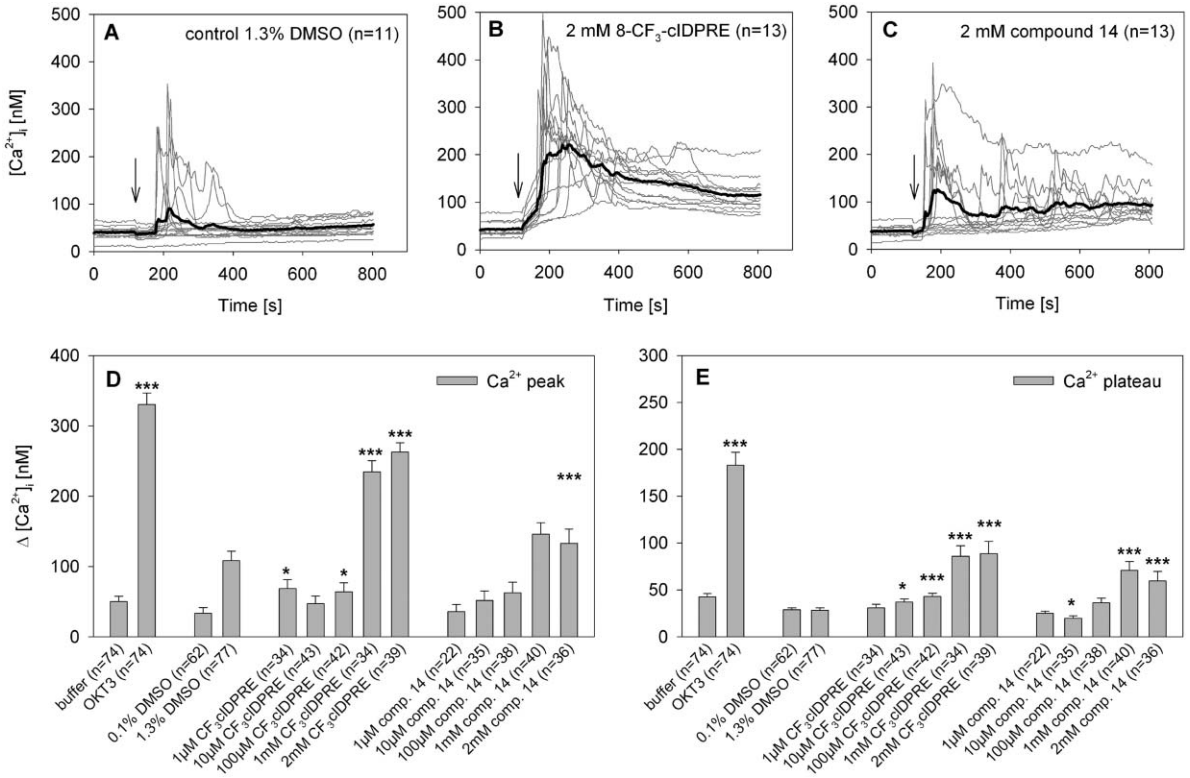


Fig. 3 Ca^{2+} mobilization by compounds **1** (8-CF_3-cIDPRE) and **14** in intact Jurkat T cells. Jurkat T-cells were loaded with Fura-2AM and subjected to Ca^{2+} imaging. **A–C** Characteristic tracings from a representative experiment are shown. The time points of addition of (A) buffer (containing 1.3% DMSO), (B) 2 mM 8-CF_3-cIDPRE (containing 1.3% DMSO) and (C) 2 mM compound **14** (containing 1.6% DMSO) are indicated by arrows. All concentrations are final concentrations. The black tracings indicate the mean from individual cells (grey tracings) from a single experiment; n = cell number analyzed in this experiment. **D**, **E** Combined data representing mean ± S.E.M. (n = 22–77 cells) of single tracings. For control, buffer containing different amounts of DMSO was added instead of the stimulus as described in the experimental section. (D) Ca^{2+} peak is expressed as the difference between the maximal peak amplitude and the basal Ca^{2+} concentration (Δ values). (E) Ca^{2+} plateau is expressed as the difference between the Ca^{2+} plateau (mean at 770–820 s) and the basal Ca^{2+} concentration (Δ values). Significance was tested against corresponding buffer controls. ***, P <0.001; *, P <0.05 (t test).

when cells are analyzed in suspension. However, it is similarly well established that individual cells show Ca^{2+} signaling kinetics differing very much from each other (Fig. 3B,C).[28,29] Interestingly, subcloning of individual Jurkat T cells does not result in cells responding in identical manner, but the same variety of signaling pattern occurs again. The latter indicates that the Ca^{2+} signaling tools used by Jurkat T cells allow the generation of different Ca^{2+} signaling pattern and that minimal differences in signal transduction may result in very different signaling pattern over time.

When compared to the quasi-physiological agonist, the monoclonal *anti*-CD3 antibody OKT3, especially the first phase of Ca^{2+} signaling was considerably smaller with compound **1** (Fig. 3D,E). However, the additional Ca^{2+} releasing second messengers formed upon OKT3 stimulation, D-*myo*-inositol 1,4,5-trisphosphate[30] and nicotinic acid adenine dinucleotide phosphate[31] likely account for the increased Ca^{2+} response upon OKT3 in the first minutes. Importantly, the sustained phase of Ca^{2+} signaling upon both OKT3 or compound **1** appears quite similar indicating ongoing Ca^{2+} release *via* RyR followed by capacitative Ca^{2+} entry (Fig. 3B). Interestingly, the partially protected compound **14** also showed Ca^{2+} release activity in intact Jurkat T cells (Fig. 3C). Saturation of Ca^{2+} signaling was achieved at approx. 1 mM of both compounds **1** and **14** for both the initial Ca^{2+} peak and the sustained Ca^{2+} signaling phase (Fig. 3D,E).

For comparison of the magnitude of Ca^{2+} signaling induced by compound **1**, the parent compound cIDPRE and its partially protected precursor 2′,3′-*O*-isopropylidene cIDPRE[12b] were analyzed in the same series of experiments (Fig. 4). Although the Ca^{2+} signaling amplitudes were somewhat lower as compared to compounds **1** and **14** (Fig. 4E,F *vs.* Fig. 3D,E), similar biphasic signaling pattern were observed (Fig. 4B,D) while the corresponding controls with vehicle showed only negligible signals (Fig. 4A,C).

The 8-substituted cIDPRE analogues reported earlier,[12b] 8-NH_2- and 8-N_3-cIDPRE showed agonist activity in intact human Jurkat T-lymphocytes, but 8-Cl- and 8-Br-cIDPRE were inactive. Thus it seems that the electronegative action of halogen atoms may either directly prevent binding at the binding pocket of the related cADPR receptor or may influence the overall conformation of the whole molecule in a way that it does not resemble cADPR any more. However, the newly synthesized 8-CF_3-cIDPRE **1** retained the agonist effect. This indicates that a strong electron withdrawing substitution at the 8-position does not necessarily interfere negatively with the interaction at the cADPR receptor. One may speculate that this phenomenon may also be attributed

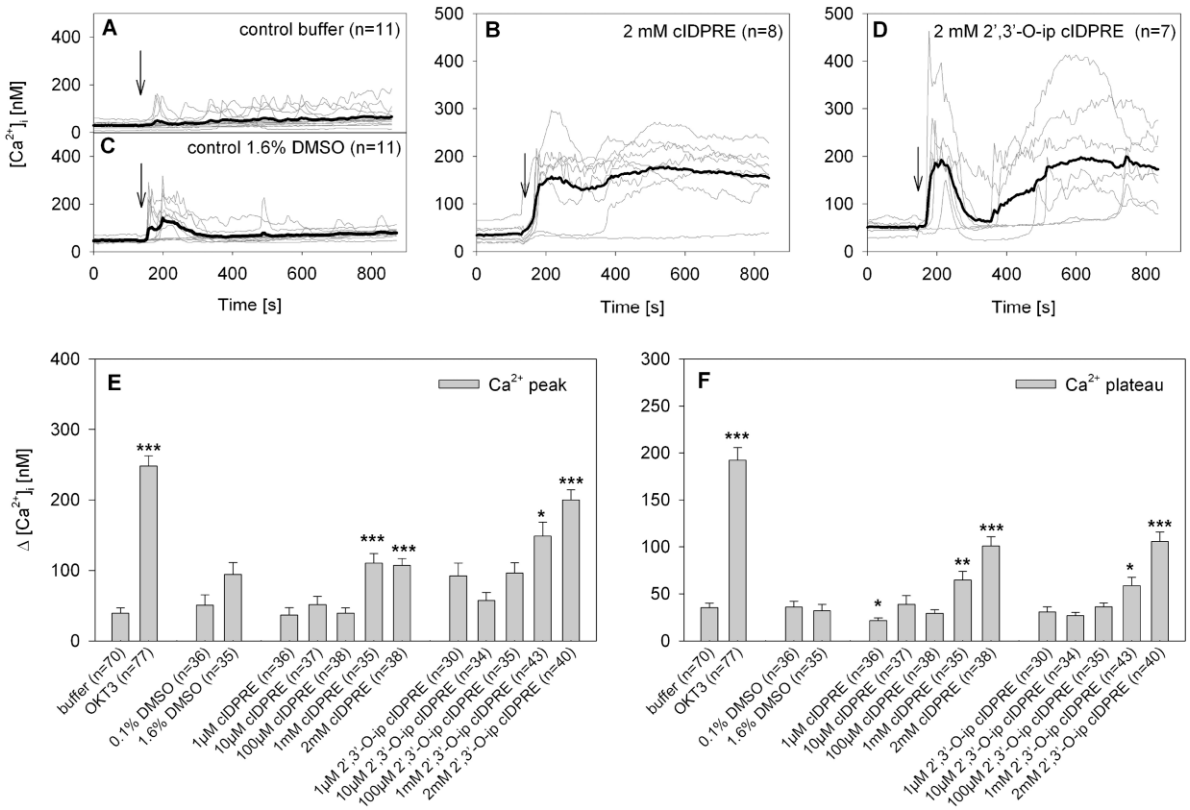


Fig. 4 Ca^{2+} mobilization by cIDPRE and 2′,3′-*O*-isopropylidene cIDPRE [12b] in intact Jurkat T cells. Jurkat T-cells were loaded with Fura-2AM and subjected to Ca^{2+} imaging. **A–D** Characteristic tracings from a representative experiment are shown. The time points of addition of (A) buffer, (B) 2 mM cIDPRE, (C) buffer (containing 1.6% DMSO) and (D) 2 mM 2′,3′-*O*-isopropylidene cIDPRE (2′,3′-*O*-ip-cIDPRE; containing 1.6% DMSO) are indicated by arrows. All concentrations are final concentrations. The black tracings indicate the mean from individual cells (grey tracings) from a single experiment. *n* = cell number analyzed in this experiment. **E,F** Combined data representing mean ± S.E.M. (*n* = 30–77 cells) of single tracings. For control, buffer including different amounts of DMSO was added instead of the stimulus as described in the experimental section. (E) Ca^{2+} peak is expressed as the difference between the maximal peak amplitude and the basal Ca^{2+} concentration (*Δ* values). (F) Ca^{2+} plateau is expressed as the difference between the Ca^{2+} plateau (mean at 770 to 820 s) and the basal Ca^{2+} concentration (*Δ* values). Significance was tested against corresponding buffer control. ***, P <0.001; **, P <0.01; *, P <0.05 (t test).

to potential hydrogen bond formation of trifluoromethyl with the cADPR binding pocket. Theoretical and crystallographic evidences support the idea that fluorine is a poor hydrogen bond acceptor with only a moderate capacity to replace oxygen (or nitrogen) in this role. The C(sp3)–F ⋯ H bond strength is half of that of the O ⋯ H value.[32] However, F ⋯ H hydrogen bonding has been shown to widely exist[33] and relatively short intermolecular O–H ⋯ F–C distances of 2.01 Å were observed with the fluorine of CF_3 group.[34] There are also examples illustrating that the affinity of substrate and receptor was strengthened by a F ⋯ H hydrogen bond.[35]

8-Substituted cADPR analogues, such as 8-Br-cADPR, 8-N_3-cADPR, and 8-NH_2-cADPR, are antagonists of cADPR in sea urchin egg homogenate and mammalian cells.[9] In contrast, 8-substituted cIDPRE analogues are agonists in mammalian cells. Here we report a new member of this agonist family, 8-CF_3 substituted cIDPRE. Structurally, N^1 ribose substituted adenine moiety in cADPR shows a positive charge at N^1 in the presence of protons (Fig. 5).

Obviously, the 8-substituted group in cADPR will influence the electron density of the whole molecule, especially, the N^1 positive charge which may play a key role in the mechanism

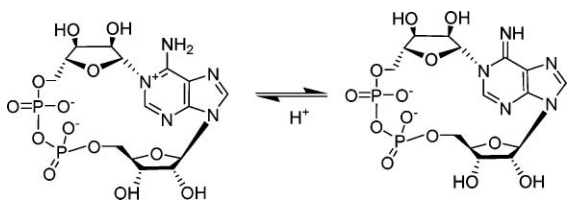


Fig. 5 cADPR and Protonated cADPR.

of antagonist activity and the molecular permeability as well. cIDPRE derivatives do not form the positively charged structure at N^1 and the ether linkage instead of northern ribose in N^1-cIDPRE renders this molecule more stable against enzymatic hydrolysis.[36]

Potter and colleagues systematically analyzed the modification of the 2′- and 3′-hydroxyl groups of the southern ribose of cADPR. They found that 2′-deoxy-cADPR is almost as potent as cADPR in mediating Ca^{2+} release from sea urchin egg homogenates, indicating that 2′-OH has little effect on the agonist activity of cADPR. While the deletion of 2′-OH decreases the antagonist activity of 8-substituted cADPR, even some 8-substituted 2′-deoxy analogues showed agonist activity.[14] 3′-deoxy-cADPR is much less potent than cADPR, indicating that the 3′-hydroxyl group is essential for

Ca^{2+} releasing activity of cADPR in sea urchin eggs. In contrary, 3′-*O*-methyl-cADPR is an antagonist of cADPR-induced Ca^{2+} release in sea urchin eggs. Two other analogues, 3′-phospho-cADPR and 2′,3′-cyclic-cADPR phosphate, were inactive with respect to both agonist and antagonist activities on the cADPR-sensitive Ca^{2+} release mechanism in sea urchin egg homogenates.[15] In our case, 2′,3′-*O*-isopropylidene protection of 8-CF_3-cIDPRE **14** did not much alter the agonist activity of compound **1**; a similar result was obtained for cIDPRE and its protected precursor 2′,3′-*O*-isopropylidene cIDPRE. This indicates on the one hand that such lipophilic masking of two polar hydroxyl groups may render the protected precursors more membrane-permeant, probably leading to a higher intracellular concentration as compared to the non-protected final products. Secondly, it indicates that the southern (and northern) ribose appear to be structural parts of the cADPR molecule that rather are important to hold the adenine ring and the pyrophosphate backbone in a certain distance and orientation than being involved in cADPR receptor recognition on their own. This has been postulated by us previously since the cyclic inosine derivative in which both the northern and the southern ribose were replaced by ether bridges, termed cIDP-DE,[12c] showed full biological activity though at higher concentrations as compared to endogenous cADPR.

Conclusions

A convenient reagent and protection strategy is reported for the synthesis of trifluoromethylated cyclic-ADP-ribose mimic, 8-CF_3-cIDPRE **1**. Compound **1** and 2′,3′-*O*-isopropylidene 8-CF_3-cIDPRE **14** are cell membrane-permeant cADPR agonists. To our best knowledge 8-CF_3-cIDPRE **1** is the first reported fluoro substituted cADPR mimic. Importantly, the data shown here indicate that a strong electron withdrawing substitution at the 8-position does not necessarily interfere negatively with the interaction to the cADPR receptor. This phenomenon may also be attributed to potential hydrogen bond formation of trifluoromethyl with the cADPR binding pocket. On the another hand, 2′,3′-*O*-isopropylidene protection of 8-CF_3-cIDPRE **14** did not much alter the agonist activity of compound **1**; a similar result was obtained for cIDPRE and its protected precursor 2′,3′-*O*-isopropylidene cIDPRE, it may indicate only a minor role for the both 2′ and 3′-hydroxyl groups of the southern ribose of cADPR for its biological activity.

Experimental

Chemistry

HR-ESI-MS (electrospray ionization) were performed with Bruker BIFLEX III. ^{1}H NMR and ^{13}C NMR were recorded with a Bruker AVANCE III 400 or a JEOL AL300 spectrometer. $CDCl_3$, DMSO-*d*6 or D_2O were used as a solvent. Chemical shifts are reported in parts per million downfield from TMS (^{1}H and ^{13}C). ^{31}P NMR spectra were recorded at room temperature by use of JEOL AL300 spectrometer (121.5 MHz); Orthophosphoric acid (85%) was used as an external standard. ^{19}F-NMR spectra were recorded on a Varian VXR-500 spectrometer. Chemical shifts of ^{19}F-NMR are reported in ppm with reference to CF_3-benzene (–63.7 relative to $CFCl_3$) as an external standard except compound **5** (relative to CF_3COOH). Compounds (**13**, **14** and **1**) were purified twice on Alltech preparative C18 reversed phase column (2.2 × 25 cm) with Gilson HPLC by MeCN/TEAB (pH 7.5) buffer systems.

***N*^{1}-[(5″-Acetoxyethoxy)methyl]-2′,3′,5′-tri-*O*-acetyl-8-trifluoromethyl-inosine 5.** To a solution of 8-bromo derivative **4** (62 mg, 0.105 mmol) and CuI (24 mg, 0.126 mmol) in DMF (2 mL), HMPA (90 μL, 0.52 mmol) and $FSO_2CF_2CO_2Me$ (134 μL, 1.054 mmol) were added successively. The mixture was stirred for 27 h at 75 °C under nitrogen. The reaction mixture was then cooled to room temperature, 6 mL of saturated aq. NH_4Cl was added and the mixture was extracted with 10 mL of EtOAc–hexane (7 : 3). The organic layer was washed successively with sat. aq. $NaHCO_3$, water and brine, dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Flash chromatography (PE: Acetone = 5 : 1~3 : 1) afforded the title compound **5** (21 mg, 34%) as a light yellow foam. ^{1}H NMR (300 MHz, DMSO) δ 1.94, 1.99, 2.07, 2.12 (each s, each 3H, 4 × Ac), 3.79 (t, 2H, J = 4.5 Hz OCH_2), 4.10 (t, 2H, J = 4.5 Hz, CH_2OAc), 4.22 (m, 1H, H-5′), 4.41–4.45 (m, 2H, H-5′,H-4′), 5.49 (s, 2H, 2 × H-1″), 5.68 (m, 1H, H-3′), 6.09 (m, 2H, H-1′, H-2′), 8.73 (s, 1H, H-2); ^{19}F NMR (470 MHz, Acetone) δ 24.8; (relative to CF_3COOH). HRMS (ESI-TOF$^+$): calcd for $C_{22}H_{25}F_3N_4O_{11}$ [(M + H)$^+$], 579.1545; found, 579.1550.

5′-*O*-TBDPS-2′,3′-*O*-isopropylidene-8-bromoinosine 7. To a solution of **6** (0.65 g, 1.68 mmol) in DMF (10 mL) imidazole (1.14 g, 16.8 mmol) and TBDPSCl (2.31 mL, 8.4 mmol) were added under N_2, and the mixture was stirred at room temperature for 12 h. Ice water (10 mL) was added to stop the reaction and the mixture was evaporated under reduce pressure, the residue was partitioned between H_2O and CH_2Cl_2. The aqueous phase was extracted again with CH_2Cl_2, the organic layers were combined and washed with brine, dried (Na_2SO_4), and evaporated. The residue was purified by silica gel column chromatography (PE:Acetone = 3 : 1) to give compound **7** (1.047 g, 99%) as a yellow foam.^{1}H NMR (400 MHz, $CDCl_3$) δ 1.02 (s, 9H, $(CH_3)_3C$-), 1.40, 1.63 (each s, each 3H, $(CH_3)_2C$), 3.72–3.84 (m, 2H, H-5′), 4.39 (m, 1H, H-4′), 5.09 (dd, 1H, $J_{H3',H4'}$ = 3.6, $J_{H2',H3'}$ = 6.4 Hz, H-3′), 5.56 (dd, 1H, $J_{H1',H2'}$ = 2.0, $J_{H2',H3'}$ = 6.4 Hz, H-2′), 6.15 (d, 1H, $J_{H1',H2'}$ = 2.0 Hz, H-1′), 7.24–7.40 (m, 6H, Ar H), 7.54 (d, J_{AB} = 6.4 Hz, 2H, A of aryl A_2B_2), 7.60 (d, J_{AB} = 6.4 Hz, 2H, B of aryl A_2B_2) 7.87 (s, 1H, H-2) 13.07 (s, 1H, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ,157.9, 149.4, 145.0, 135.5, 133.3, 133.0, 129.8, 129.7, 127.6, 127.5, 126.9, 125.4, 114.4, 91.5, 88.0, 83.2, 81.7, 63.8, 27.2, 26.8, 25.4, 19.2; HRMS (ESI-TOF$^+$): calcd for $C_{29}H_{33}BrN_4O_5Si$ [(M + H)$^+$], 625.1476; found, 625.1480.

***N*^{1}-[(5″-Acetoxyethoxy)methyl]-5′-*O*-TBDPS-2′,3′-*O*-isopropylidene-8-bromoinosine 8.** To the solution of **7** (510 mg, 0.82 mmol) and DBU (1.23 mL, 8.2 mmol) in CH_2Cl_2 (10 mL) $ClCH_2OCH_2CH_2OAc$ (0.56 mL, 4.1 mmol) was added at 0 °C. After being stirred for 1 h, the solvent was evaporated *in vacuo* and the residue was purified by silica gel column chromatography (PE:Acetone = 4 : 1) to give compound **8** (365 mg, 60%) as a white foam. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.02 (s, 9H, $(CH_3)_3C$-), 1.39, 1.62 (each s, each 3H, $(CH_3)_2C$), 2.03 (s, 3H, AcO), 3.77–3.88 (m, 4H, OCH_2, 2 × H-5′), 4.15–4.17 (m, 2H, CH_2OAc), 4.32–4.36 (m, 1H, H-4′), 5.06 (dd, 1H, $J_{H3',H4'}$ = 4.4, $J_{H2',H3'}$ = 6.4 Hz, H-3′), 5.31 (d, 1H, $J_{H1''a,H1''b}$ = 10.0 Hz, H-1″a), 5.48 (dd, 1H, $J_{H1',H2'}$ = 2.4, $J_{H2',H3'}$ = 6.4 Hz, H-2′), 5.51 (d, 1H, $J_{H1''a,H1''b}$ = 10.0 Hz, H-1″b), 6.15 (d, 1H, $J_{H1',H2'}$ = 2.4 Hz, H-1′), 7.23–7.63 (m, 10H, Ar H),

7.63 (s, 1H, H-2); ^{13}C NMR (100 MHz, $CDCl_3$) δ170.7, 155.2, 147.9, 146.9, 135.5, 133.5, 132.9, 129.7,127.6, 126.3, 125.2, 114.6, 91.2, 87.5, 83.2, 81.4, 74.9, 68.2, 63.8, 62.9, 27.3, 26.7, 25.5, 20.8, 19.2; HRMS (ESI-TOF$^+$): calcd for $C_{34}H_{41}BrN_4O_8Si$ [(M + H)$^+$], 741.1950; found, 741.1953.

N^1-[(5″-Acetoxyethoxy)methyl]-5′-*O*-TBDPS-2′,3′-*O*-isopropylidene-8-trifluoromethyl inosine 10. To a solution of 8-bromo derivative **8** (300 mg, 0.405 mmol) and CuI (93 mg, 0.486 mmol) in DMF (15 mL) HMPA (0.36 mL, 2.025 mmol) and $FSO_2CF_2CO_2Me$ (258μL, 2.025 mmol) were added successively. The reaction mixture was stirred for 12 h at 70 °C under nitrogen, then cooled to room temperature. 10 mL of saturated aq. NH_4Cl were added and the mixture was extracted with 40 mL of EtOAc–hexane (7 : 3). The organic layer was washed successively with sat. aq. $NaHCO_3$, water and brine, dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Flash chromatography (PE:Acetone = 5 : 1) afforded the title compound **10** (174 mg, 59%) as a light yellow foam.^{1}H NMR (400 MHz, $CDCl_3$) δ 1.02 (s, 9H, $(CH_3)_3$C-), 1.38, 1.62 (each s, each 3H, $(CH_3)_2$C), 2.03 (s, 3H, AcO), 3.75 (t, 2H, J = 4.6 Hz OCH_2), 3.82–3.93 (m, 2H, 2 × H-5′), 4.17 (t, 2H, J = 4.6 Hz, CH_2OAc), 4.35–4.38 (m, 1H, H-4′), 5.05 (dd, 1H, $J_{H3',H4'}$ = 4.4, $J_{H2',H3'}$ = 6.4 Hz, H-3′), 5.31 (d, 1H, $J_{H1''a,H1''b}$ = 10.4 Hz, H-1″a), 5.42 (dd, 1H, $J_{H1',H2'}$ = 2.4, $J_{H2',H3'}$ = 6.4 Hz, H-2′), 5.52 (d, 1H, $J_{H1''a,H1''b}$ = 10.4 Hz, H-1″b), 6.13 (d, 1H, $J_{H1',H2'}$ = 2.0 Hz, H-1′), 7.21–7.42 (m, 10H, Ar H), 7.44 (s, 1H, H-2); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.7, 156.1, 148.5, 148.1, 138.3 (d, J_{CF} = 41 Hz) 135.6, 133.5, 132.9, 129.7,127.5, 123.6, 118.1 (d, $^1J_{CF}$ = 270 Hz) 114.8, 90.5, 87.7, 83.7, 81.3, 74.9, 68.3, 63.8, 62.9, 27.3, 26.7, 25.5, 20.8, 19.2; ^{19}F NMR (470 MHz, $CDCl_3$) δ –62.2; HRMS (ESI-TOF$^+$): calcd for $C_{35}H_{41}F_3N_4O_8Si$ [(M + H)$^+$], 731.2719; found, 731.2720.

N^1-[(5″-Acetoxyethoxy)methyl]-2′,3′-*O*-isopropylidene-8-trifluoromethylinosine 11. A solution of **10** (165 mg, 0.226 mmol) in THF (30 mL) was added to 70% HF·Py 1 ml at 0 °C. The mixture was stirred at 0 °C for 1 h and at room temperature over night. The reaction mixture was quenched with saturated aq. $NaHCO_3$ at 0 °C and diluted with ethyl acetate, then separated and the water layer was washed with ethyl acetate again. The organic layer was combined, washed with brine, dried, filtered and concentrated under reduced pressure. Flash chromatography (PE:EA = 1 : 2) afforded the title compound **11** (100 mg, 90%) as a white foam. ^{1}H NMR (400 MHz, DMSO) δ 1.31, 1.53 (each s, each 3H, $(CH_3)_2$C-), 1.95 (s, 3H, AcO), 3.50–3.58 (m, 2H, 2 × H-5′), 3.76 (t, 2H, J = 4.6 Hz OCH_2), 4.09 (t, 2H, J = 4.6 Hz CH_2OAc), 4.16 (dd, 1H, $J_{H3',H4'}$ = 3.6, $J_{H4',H5'}$ = 6.0 Hz, H-4′), 4.94 (t, 1H, $J_{OH,H5'}$ = 5.6, OH), 4.98 (dd, 1H, $J_{H3',H4'}$ = 3.6 Hz, $J_{H2',H3'}$ = 6.4 Hz, H-3′), 5.47 (q, 2H, J = 10.4 Hz, H-1″), 5.54 (dd, 1H, $J_{H1',H2'}$ = 2.4, $J_{H3',H2'}$ = 6.4 Hz, H-2′), 6.01 (d, 1H, $J_{H1',H2'}$ = 2.4 Hz, H-1′), 8.66 (s, 1H, H-2); ^{13}C NMR (100 MHz, DMSO) δ 170.2, 155.6, 151.1, 148.5, 136.1 (q, J_{CF} = 40 Hz), 123.0, 118.1 (q, $^1J_{CF}$ = 270 Hz), 113.7, 90.3, 87.7, 82.9, 81.2, 75.0, 67.2, 62.8, 61.3, 27.0, 25.2, 20.5; ^{19}F NMR (470 MHz, DMSO) δ –56.6; HRMS (ESI-TOF$^+$): calcd for $C_{19}H_{24}F_3N_4O_8$ [(M + H)$^+$], 493.1541; found, 493.1542.

N^1-[(5″-Acetoxyethoxy)methyl]-5′-*O*-[bis(phenylthio)phosphoryl]-2′,3′-*O*-isopropylidene-8-trifluoromethylinosine 12. To a solution of **11** (100 mg, 0.203 mmol) in pyridine (10 mL) TPSCl (184 mg, 0.609 mmol), PSS (278 mg, 0.732 mmol), and tetrazole (51 mg, 0.732 mmol) were added, and the mixture was stirred at room temperature for 12 h. The mixture was evaporated, and the residue was partitioned between H_2O and CH_2Cl_2. The organic layer was washed with brine, dried (Na_2SO_4), and evaporated. The residue was purified by silica gel column chromatography (PE:Acetone = 3 : 1) to give compound **12** (132 mg, 86%). ^{1}H NMR (400 MHz, DMSO) δ 1.34, 1.56 (each s, each 3H, $(CH_3)_2$C), 1.94 (s, 3H, AcO), 3.72 (m, 2H, OCH_2), 4.05 (m, 2H, CH_2OAc), 4.40–4.50 (m, 3H, H-4′, 2 × H-5′), 5.09 (dd, 1H, $J_{H3',H4'}$ = 2.8 Hz, $J_{H2',H3'}$ = 6.4 Hz, H-3′), 5.47 (q, 2H, J = 10.4 Hz, 2 × H-1″), 5.62 (dd, 1H, $J_{H1',H2'}$ = 2.0, $J_{H3',H2'}$ = 6.4 Hz, H-2′), 6.10 (d, 1H, $J_{H1',H2'}$ = 2.0 Hz, H-1′), 7.34–7.46 (m, 10H, Ar H), 8.66 (s, 1H, H-2); ^{13}C NMR (100 MHz, DMSO) δ 170.2, 155.6, 151.2, 148.4, 143.1, 136.1 (q, J_{CF} = 39 Hz), 135.0, 134.9, 134.8, 129.8, 129.6, 125.5, 125.4, 125.3, 125.2, 123.0, 118.2 (q, $^1J_{CF}$ = 269 Hz), 114.0, 90.3, 85.6, 83.3, 80.5, 75.0, 67.2, 66.7, 66.6, 62.8, 26.9, 25.2, 20.6; ^{19}F NMR (470 MHz, DMSO) δ-56.6; ^{31}P NMR (D_2O, 121.5 MHz, decoupled with ^{1}H) δ 51.27 ppm (s). HRMS (ESI-TOF$^+$): calcd for $C_{31}H_{32}F_3N_4O_9PS_2$ [(M + H)$^+$], 757.1373; found, 757.1375.

N^1-[(5″-Phosphonoxyethoxy)methyl]-5′-*O*-[(phenylthio)phosphoryl]-2′,3′-*O*-isopropylidene-8-trifluoromethylinosine 13. To a solution of **12** (79 mg, 0.104 mmol) in MeOH (4 mL) acetyl chloride (4.4 μL, 0.062 mmol) was added at 0 °C. The mixture was stirred at the same temperature for 30 min and raised to room temperature for 24 h, then quenched by addition of a sat. aq $NaHCO_3$ solution. The mixture was extracted with CH_2Cl_2, dried (Na_2SO_4), filtered and concentrated under reduced pressure. Flash chromatography (PE:EA = 1 : 2) afforded the deacetylate product (44 mg) as a white foam. The deacetylate product (44 mg, 0.061 mmol) was dissolved in CH_3CN (8 mL). DIEA (63.7 μL, 0.369 mmol) and $POCl_3$ (34.5 μL, 0.302 mmol) was added to the solution successively at 0 °C, and the mixture was stirred at the same temperature for 14 h, then 5 mL of TEAB (1 M, pH 7.5) was added at 0 °C and stirred for 6 h at r.t. After evaporation in vacuo, the residue was partitioned between H_2O and ethyl acetate, and the aqueous layer was washed with EA (5 mL × 3) and evaporated in vacuo. The residue was dissolved in 2 mL of TEAB buffer (0.05 M, pH 7.5), then applied to a C18 reversed-phase column (2.2 cm × 25 cm) developed by a linear gradient of 0–50% CH_3CN in TEAB buffer (0.05 M, pH 7.5) within 30 min to give **13** (26 mg, 36% for two steps) as a triethylammonium salt. ^{1}H NMR (400 MHz, D_2O) δ1.31, 1.52 (each s, each 3H, $(CH_3)_2$C), 3.70 (m, 2H, OCH_2), 3.84 (m, 2H, CH_2OP), 4.02–4.57 (m, 3H, H-4′, 2 × H-5′), 5.16 (dd, 1H, $J_{H3',H4'}$ = 2.8 Hz, $J_{H2',H3'}$ = 6.4 Hz, H-3′), 5.42 (q, 2H, J = 10.8 Hz, 2 × H-1″), 5.67 (dd, 1H, $J_{H1',H2'}$ = 1.6, $J_{H3',H2'}$ = 6.4 Hz, H-2′), 6.10 (s, 1H, H-1′), 6.92–7.03 (m, 5H, Ar H), 8.46 (s, 1H, H-2). ^{19}F NMR (470 MHz, D_2O) δ-60.0; ^{31}P NMR (D_2O, 121.5 MHz, decoupled with ^{1}H) δ2.80 ppm (s), 17.74 ppm (s). HRMS (ESI-TOF-) calcd for $C_{23}H_{27}N_4O_{12}P_2S_1F_3$ [(M – H)$^-$], 701.0701; found, 701.0705.

N^1-[(5″-*O*-Phosphorylethoxy)methyl]-2′,3′-*O*-isopropylidene-5′-*O*-phosphoryl-8-trifluoromethylinosine 5′,5″-cyclicpyrophosphate 14. A solution of **13** (26 mg, 37 μmol) in pyridine (4 mL) was added slowly over 20 h, using a syringe pump, to a mixture of I_2 (254 mg, 1 mmol) and MS 3 Å (1.5 g), in pyridine (40 mL) at room temperature in the dark. The MS 3 Å was filtered off with Celite and washed with H_2O. The combined filtrate was evaporated, and the residue was partitioned between $CHCl_3$ and H_2O. The aqueous layer was evaporated, and the residue was dissolved in

0.05 M TEAB buffer (1.0 mL), which was applied to C18 reversed phase column (2.2 × 25 cm). The column was developed using a linear gradient of 0–80% CH_3CN in TEAB buffer (0.05 M, pH 7.5) within 30 min to give **14** as a triethylammonium salt. (11.2 mg, 51%). ^{1}H NMR (400 MHz, D_2O) δ 1.37, 1.54 (each s, each 3H, 2 × CH_3), 3.73–3.98 (m, 6H, CH_2O, 2 × H-5′, CH_2OP), 4.50 (m, 1H, H-4′), 5.19 (d, 1H, $J_{H1''a,H1''b}$ = 10.8 Hz, H-1″a) 5.41 (dd, 1H, $J_{H2',H3'}$ = 2.4 Hz, $J_{H3',H4'}$ = 6.0 Hz, H-3′), 5.90–5.94 (m, 2H, H-1″b, H-2′), 6.29 (s, 1H, H-1′), 8.56, (s, 1H, H-2); ^{19}F NMR (470 MHz, D_2O) δ-60.1; ^{31}P NMR (D_2O 121.5 MHz, decoupled with ^{1}H) δ-9.77 (d, $J_{P,P}$ = 14.64 Hz), −10.71 (d, $J_{P,P}$ = 14.64 Hz). HRMS (ESI-TOF-) Calcd for $C_{17}H_{21}N_4O_{12}P_2F_3$ [(M −H)$^-$], 591.0511; found, 591.0510.

***N*^{1}-[(5″-*O*-Phosphorylethoxy)methyl]-5′-*O*-phosphoryl-8-trifluoromethylinosine 5′,5″-Cyclic pyrophosphate 1.** The solution of **14** (9 mg, 15.2 μmol) in 15% HCOOH (6 mL) was stirred for 48 h, and then 9 mL TEAB (1M, pH 7.5) was added. The solution was evaporated under reduced pressure. The residue was dissolved in 0.05 M TEAB buffer (2.0 mL), which was applied to C18 reversed phase column (2.2 × 25 cm). The column was developed using a linear gradient of 0–30% CH_3CN in TEAB buffer (0.05 M, pH 7.5) within 30 min to give the designed compound **1** as a triethylammonium salt. (5.4 mg. 64.5%). ^{1}H NMR (400 MHz, D_2O) δ 3.70–3.74 (m, 2H, CH_2O-), 3.86–3.90 (m, 2H, OCH_2OP), 3.96–3.99 (m, 1H, H-5′a), 4.23 (m, 1H, H-5′b), 4.29–4.31 (m, 1H, H-4′), 4.69–4.71 (m, 1H, H-3′), 5.17 (d, 1H, $J_{H1''a,H1''b}$ = 11.2 Hz, H-1″a), 5.59 (t, 1H, $J_{H1',H2'}$ = 5.2 Hz, $J_{H3',H2'}$ = 5.2 Hz, H-2′), 5.99 (d, 1H, $J_{H1''a,H1''b}$ = 11.2 Hz, H-1″b), 6.00 (d, 1H, $J_{H1',H2'}$ = 5.2 Hz, H-1′), 8.52 (s, 1H, H-2); ^{31}P NMR (D_2O, 121.5 MHz, decoupled with ^{1}H) δ-9.65 (d, $J_{P,P}$ = 14.76 Hz), −10.23 (d, $J_{P,P}$ = 14.76 Hz); ^{19}F NMR (470 MHz, D_2O) δ-60.1; HRMS (ESI-TOF$^-$) Calcd for $C_{14}H_{17}N_4O_{12}P_2F_3$ [(M − H)$^-$] 551.0198; found, 551.0216.

Pharmacology

Cell culture. Jurkat T-lymphocytes (subclone JMP) were cultured as described previously[37] at 37 °C in the presence of 5% CO_2 in RPMI 1640 medium containing Glutamax I and HEPES (25 mM) and supplemented with 7.5% (v/v) newborn calf serum (NCS), 100 units/ml penicillin and 100 μg ml^{-1} streptomycin.

Ratiometric Ca^{2+} imaging. Jurkat T cells were loaded with Fura-2/AM as described[37] and kept in the dark at room temperature until use. For Ca^{2+} imaging of Jurkat T cells thin glass coverslips (0.1 mm) were coated first with BSA (5 mg ml^{-1}) and subsequently with poly-L-lysine (0.1 mg ml^{-1}). Silicon grease was used to seal small chamber slides consisting of a rubber O-ring on the glass coverslip. Then, 30 μl buffer A containing 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM NaH_2PO_4, 5.5 mM glucose, 20 mM HEPES, pH 7.4 and 30 μl Fura-2 loaded T cell suspension were added into the small chamber. The loaded coverslip was mounted on the stage of a fluorescence microscope (Leica DMIRBE). For control buffer or buffer containing a small amount of DMSO were used. In detail, for control (i) pure buffer was added instead of OKT3 or cIDPRE, (ii) buffer containing 0.1% DMSO was used instead of 1 μM, 10 μM or 100 μM 8-CF3-cIDPRE, compound **14** or 2′,3′-*O*-isopropylidene cIDPRE, (iii) buffer containing 1.3% DMSO was used instead of 1 mM or 2 mM 8-CF3-cIDPRE and compound **14**, or (iv) buffer containing 1.6% DMSO was applied instead of 1 mM or 2 mM 2′,3′-*O*-isopropylidene cIDPRE. Ratiometric Ca^{2+} imaging was performed as described recently[36] We used an improvision imaging system (Tübingen, Germany) built around the Leica microscope usually at 40-fold magnification. Illumination at 340 and 380 nm was carried out using a monochromator system (Polychrom II, TILL Photonics, Gräfelfing, Germany). Images were taken with a gray-scale CCD-camera (type C 4742–95; Hamamatsu, Enfield, United Kingdom) operated in 8-bit mode. The spatial resolution was 512 × 640 pixels. The acquisition rate was adjusted to ~14 ratios per minute. Raw data images were stored on a hard disk and ratio images (340/380 nm) were constructed pixel by pixel. Finally, ratio values were converted into Ca^{2+} concentrations by external calibration.[28] Data processing was performed using Openlab software, version 3.5.2 (Improvision, Tübingen, Germany).

Abbreviations.

Ac	acetyl
Bz	benzoyl
cADPR	cyclic adenosine 5′-diphosphoribose
cADPcR	cyclic adenosine 5′-diphosphocarboribose
cIDPR	cyclic inosine 5′-diphosphoribose
cIDPRE	*N*1-[(5″-*O*-phosphorylethoxy)methyl]-5′-*O*-phosphoryl inosine 5″,5″-cyclicpyrophosphate
NAD^+	nicotinamide adenosine dinucleotide
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DMTrCl	dimethoxytrityl chloride ^{1}H NMR, proton nuclear magnetic resonance
^{13}C NMR	carbon-13 nuclear magnetic resonance
^{19}F NMR	fluorine-19 nuclear magnetic resonance. ^{31}P NMR, phosphorus nuclear magnetic resonance
HMBC	heteronuclear multiple bond correlation
HMPA	Hexamethyl phosphoric triamide
HPLC	high performance liquid chromatography
HR-ESI- MS	high-resolution electro spray ionization mass spectrometry
3 Å MS	3 Å molecular sieve
MMTrCl	monomethoxytrityl chloride
PE	petroleum ether
PSS	cyclohexylammonium *S*,*S*-diphenylphosphorodithioate
Py	pyridine
TBDMSCl	*tert*-butyldimethylsilyl chloride
TBDPSCl	*tert*-butyldiphenylsilyl chloride
TEAB	triethylammonium bicarbonate
THF	tetrahydrofuran
T.L.C.	Thin Layer Chromatography
TPSCl	triisopropylbenzenesulfonyl chloride
TMS	tetramethyl silicane;.

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