# Novel nucleobase-simplified cyclic ADP-ribose analogue: A concise synthesis and $Ca^{2+}$-mobilizing activity in T-lymphocytes†

**Lingjun Li,‡[a,b] Cornelia C. Siebrands,‡[c] Zhenjun Yang,[a] Liangren Zhang,[a] Andreas H. Guse[c] and Lihe Zhang*[a]**

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A purine nucleobase-simplified cyclic ADP ribose (cADPR) analogue **6b** was synthesized, in which a 1,2,3-triazole-4-amide was constructed, instead of a purine moiety, and the northern ribose was replaced by an ether strand. Compound **6b** exhibits calcium release activity in intact T-lymphocytes and indicates that it is a membrane-permeable cADPR mimic. Thus, the cADPR analogue containing 1,2,3-triazole-4-amide provides a novel template for further designing cADPR analogues and elucidating their structure–activity relationships.



## Introduction

Since cyclic ADP-ribose (cADPR) (Fig. 1), a metabolite of $NAD^+$, was discovered by Lee and co-workers in 1987,[1] numerous cell systems have been described to utilize the cADPR/ryanodine receptor (RyR)/$Ca^{2+}$ signaling system to control $Ca^{2+}$-dependent cellular responses, such as fertilization, secretion, contraction, proliferation and many more.[2,3] A large number of key proteins are involved in specifically shaping $Ca^{2+}$ signals. However, it is unclear whether cADPR elicits calcium release by direct binding to RyR, or *via* an additional binding protein. A main approach to explore the molecular mechanism of calcium release is to investigate the relationship of the structure of cADPR and cADPR analogues with their respective biological activities. Much effort has been focused on the syntheses of structural derivatives to elucidate the structure–activity relationship and supply tools for investigating cellular $Ca^{2+}$ signaling.[4–6]

The structural modifications of cADPR include pyrophosphate,[7–9] ribose[10–15] and adenine moieties.[16–21] The drastic reduction in calcium release activity of cADPR($CH_2$) (Fig. 1) indicates that the bridging oxygen in the pyrophosphate moiety is important in cADPR action.[7] A series of $N^1$-glycosyl-subsitituted (cADPcR, cIDPRE, cADPRE) or $N^9$-glycosyl-subsitituted cADPR analogues with carbon riboses[10–13] (cyclic aristeromycin diphosphoribose), or simplified structures, *e.g.* ether stands[14] (cIDPDE), show good agonist activities, indicating that the northern and southern ribose moieties are alterable, to some extent, in cADPR action. Modifications on adenine moieties, such as 8-$NH_2$-cADPR and 8-Br-cADPR, give antagonists of cADPR, which are applied well in different types of cells,[16] and the analogues with the 6-$NH_2$ replaced by an oxygen atom, *e.g.* cIDPR, are fully agonist resistant to enzymatic hydrolysis.[20] It was reported that 7-deaza- and 3-deaza-cADPR are agonists of the cADPR/RyR signaling system, and 3-deaza-cADPR is a 70 times more potent agonist than cADPR itself.[18,21] Therefore, the purine moiety in cADPR plays a very important role for the recognition between cADPR and its receptor. However, as these reported modifications of the nucleobase in cADPR are always based on the purine structure, it would be interesting to synthesize the purine-simplified cADPR analogues and investigate whether the whole purine structure in cADPR is essential for the recognition of cADPR and its receptor. Two cADPR analogues containing 4-amide-1,2,3-triazole have been successfully prepared previously in our group,[22] in which a click reaction was used to construct the 4-amide-1,2,3-triazole nucleobase and simultaneously connect the two building blocks (Scheme 1, Fig. 2). In the current paper, we report a microwave-assisted intramolecular pyrophosphorylation method for the scale-up preparation of **6b**, and a biological study of compound **6b** on the calcium release activity in intact T-lymphocytes, exhibiting that compound **6b** is a membrane-permeable cADPR mimic.

## Results and discussion

### 1. Microwave-assisted intramolecular pyrophosphorylation for the preparation of cTDPRE 6b

The synthesis of cADPR analogues has been extensively investigated.[23–29] Analogues of cADPR can be prepared from $NAD^+$ derivatives by enzymatic and chemo-enzymatic methods, using ADP-ribose cyclase to catalyze the cyclization reaction.[24,27,28] However, the analogues obtained by these methods are limited because of the substrate specificity of ADP-ribose cyclase.[19,20] Shuto *et al.* developed a chemical method for synthesis of cADPR analogues through intramolecular cyclization for the formation of the pyrophosphate linkage.[11,26,29] The total chemical synthesis allowed extensive modifications of both the ribose and nucleobase moieties.[11–14,25] However, as a disadvantage, this method includes many steps for the protection and deprotection of building blocks in constructing the substituted nucleoside and pyrophosphate

[a]*State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100083, P.R. China. E-mail: zdszlh@bjmu.edu.cn; Fax: 86-10-82802724; Tel: 86-10-82801700; 86-10-82802567*
[b]*College of Chemistry and Environmental Science, Henan Normal University, Xinxiang 453007, P.R. China*
[c]*The Calcium Signaling Group, University Medical Center Hamburg-Eppendorf, Center of Experimental Medicine, Institute of Biochemistry and Molecular Biology I: Cellular Signal Transduction, 20246 Hamburg, Germany*

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‡ These authors contributed equally.

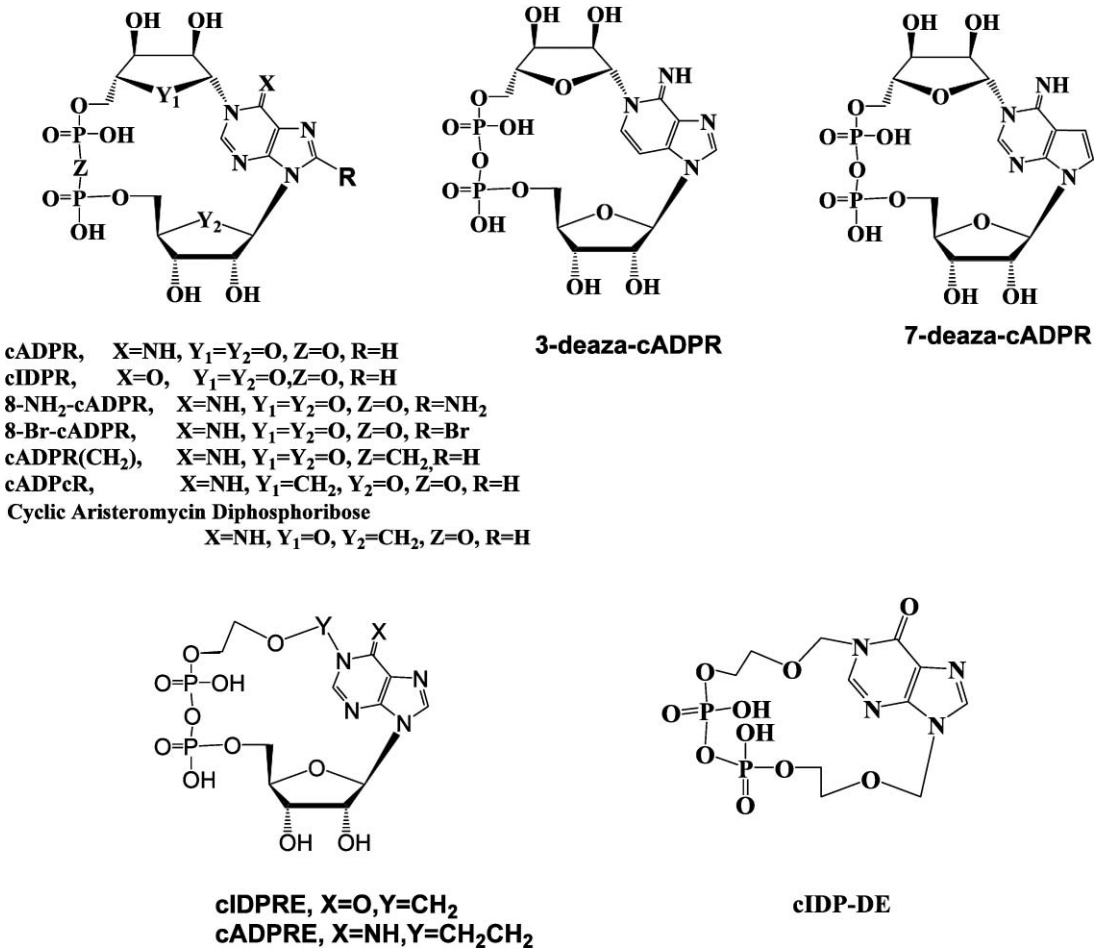
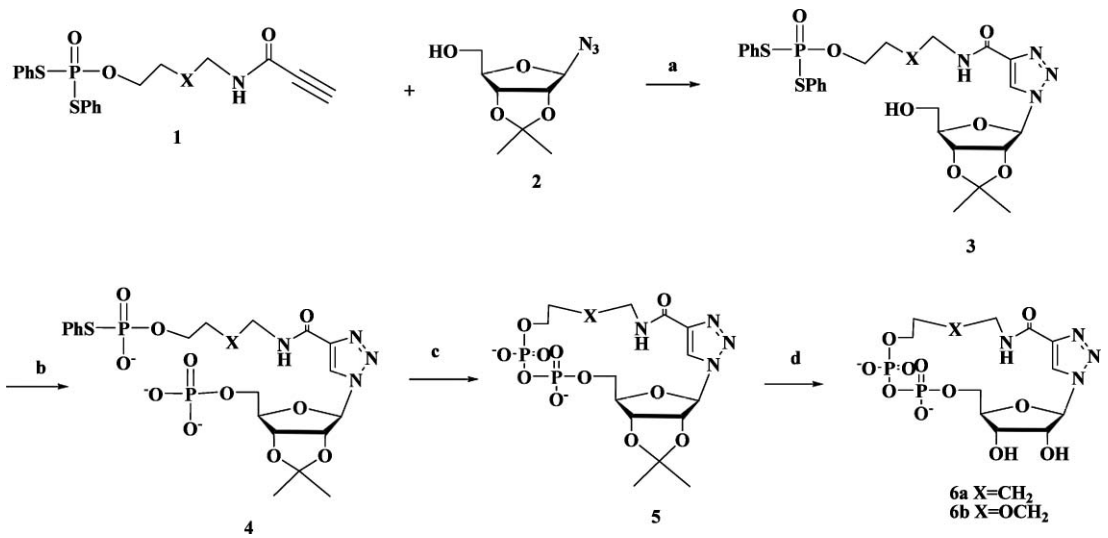



**Fig. 1** cADPR and its analogues.




**Scheme 1** Synthesis of cTDPRE derivatives. Reagents and conditions: (a) CuI, DIPEA, $CH_3CN$; (b) 1. $POCl_3$, DIPEA, $CH_3CN$, 0 °C, 12 h 2. 0.1 M TEAB; (c) $I_2$, 3 Å MS, pyridine; (d) 50% HCOOH.

linkage. Furthermore, in most of the cases of total chemical synthesis of cADPR analogues, super-dilute solutions of intermediates are needed for the key intramolecular pyrophosphorylation. Under these super-dilute solution conditions, the reaction is usually completed by using a syringe-pump over 15 h, even for 10 mg of precursor. In addition, the reaction is very sensitive to traces of water.[26] This strict dry environment and the long reaction time in a super-dilute solution render the experimental work-up

**Table 1** Microwave-assisted intramolecular cyclopyrophosphorylation of compound **4b** under different conditions

| Entry[a] | Temperature/K | Molecular sieves/g ml$^{-1}$ of py. | Time/min | Concentration of **4b** | Yield[b] of **5b** (ratio of **7** : **5b** : **8**)[c] |
|---|---|---|---|---|---|
| 1 | r.t. | 0.1 | 1200 | Super-dilute | 82.5 (9 : 83 : 8) |
| 2 | r.t. | 0.1 | 150 | 2.27 mM | 68 (0 : 70 : 30) |
| 3 | 50 °C (MW) | 0.1 | 5 | 2.27 mM | 40 (57 : 40 : 3) |
| 4 | 50 °C (MW) | 0.1 | 10 | 2.27 mM | 45 (51 : 45 : 3) |
| 5 | 50 °C (MW) | 0.1 | 15 | 2.27 mM | 51 (46 : 51 : 3) |
| 6 | 60 °C (MW) | 0.1 | 15 | 2.27 mM | 63 (35 : 63 : 3) |
| 7 | 70 °C (MW) | 0.1 | 15 | 2.27 mM | 83 (15 : 83 : 2) |
| 8 | 80 °C (MW) | 0.1 | 15 | 2.27 mM | 88 (10 : 88 : 2) |
| 9 | 90 °C (MW) | 0.1 | 15 | 2.27 mM | 95 (0 : 98 : 2) |
| 10 | 90 °C (MW) | none | 15 | 2.27 mM | 95 (0 : 98 : 2) |
| 11 | 90 °C (MW) | none | 15 | 4.54 mM | 95 (0 : 98 : 2) |
| 12 | 90 °C (MW) | none | 15 | 6.81 mM | 95 (0 : 98 : 2) |

[a] 100 mg $I_2$ as the catalyst of the reaction was added for 10 mg compound **1**. [b] Isolated yield. [c] The ratio of **7** : **5b** : **8** was based on analysis by HPLC.

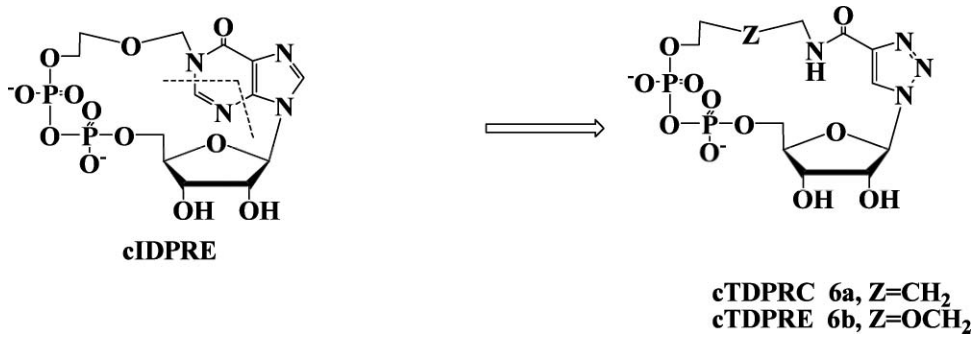



**Fig. 2** Structural design of target compounds cTDPRC **6a** and cTDPRE **6b**.

tedious, and thus, the large-scale preparation of cADPR analogues is limited.

In general, intramolecular reactions are favored in dilute reactions, and intermolecular reactions are favored in concentrated reactions. We first tried the intramolecular pyrophosphorylation of compound **4b** in pyridine at 2.27 mM concentration. Compound **4b** was prepared from **1b** and **2** by the published procedure (Scheme 1).[22] Thus, 5 mg of compound **4b** was reacted in a 2.5 ml dry pyridine solution containing 50 mg $I_2$, at ambient temperature for 3 h; water was added into the reaction mixture for another 1 h to hydrolyze the non-reacted precursor **4b** into compound **7** (Scheme 2). After separation and purification, compound **5b** was obtained as a triethylammonium salt with 69% yield and a dimer **8** (Fig. 3) was separated in 30% yield (entry 2, Table 1). In comparison with the yield of **5b** obtained from the dilute solution (entry 1, Table 1), this is a reasonable yield.

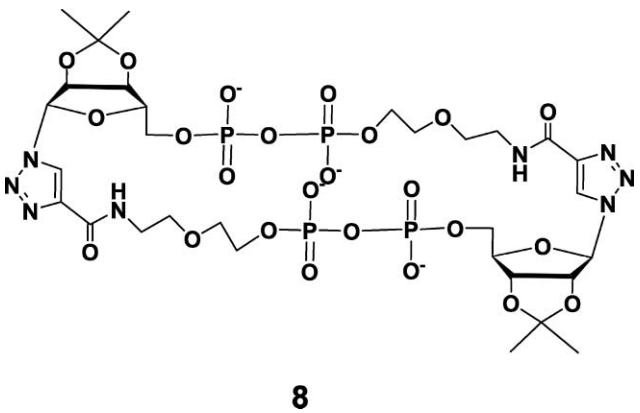
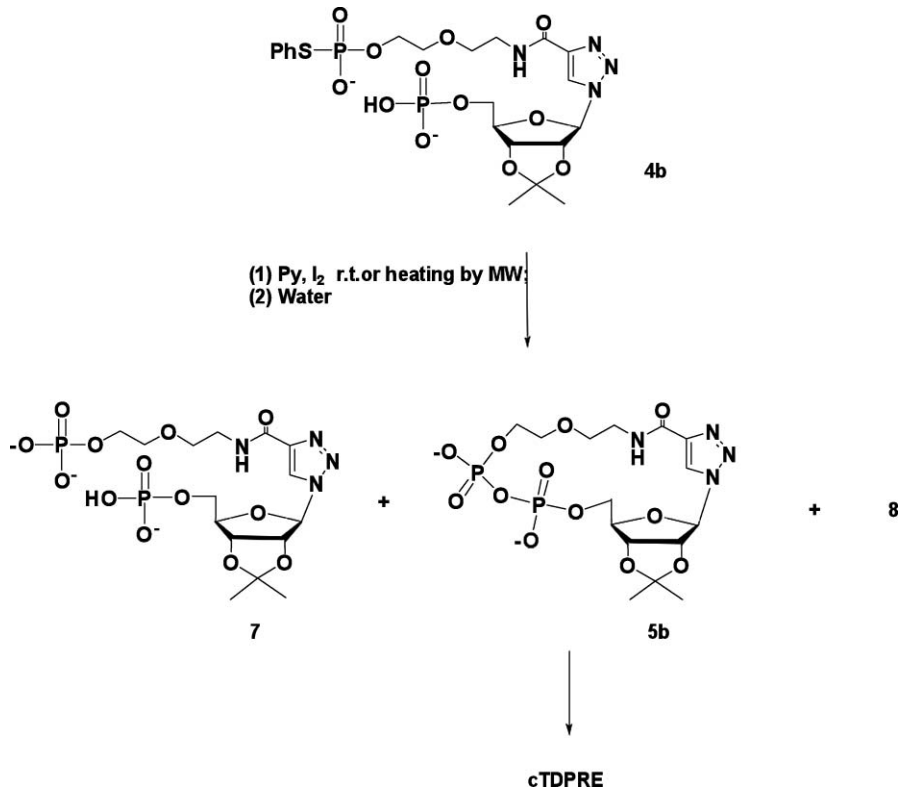



**Fig. 3** Structure of compound **8**.

Microwave-assisted organic chemistry has been recently developed for the fast synthesis of functional compounds and libraries in many areas.[30,31] With the assistance of microwave, the reaction rates and yields were increased greatly in some cases. We applied this technology to the intramolecular pyrophosphorylation of compound **4b** for the preparation of cTDPRE **6b**. Interestingly, the intramolecular pyrophosphorylation of compound **4b** was carried out in a highly concentrated solution, and the amount of dimer **8** was reduced considerably (Table 1). Optimization of these reaction conditions was done by changing temperature and reaction time. Increases of both the temperature and the reaction time under microwave irradiation dramatically increased the yield of compound **5b** (entry 3–9, Table 1). Compound **5b** was obtained in 95% yield when the reaction was carried out at 90 °C for 15 min (entry 9, Table 1). In all of the cases with microwave irradiation, the yield of the intermolecular pyrophosphorylation product **8** was less than 3%. Thus, contamination with by-product **8** was less than in the reaction using the super-dilute solution (entry 1, Table 1). Since water obviously increases the amount of open chain products in the intramolecular pyrophosphorylation reaction in super-dilute solution, the removal of trace amounts of water using the molecular sieves is important.[26] However, in the microwave-assisted intramolecular pyrophosphorylation, molecular sieves are not necessary, as shown in Table 1 (entry 10). Even further increases in the concentration of precursor **4b** up to 4.54 or 6.81 mM resulted in a 95% yield of **5b** (Table 1).




**Scheme 2** Microwave-assisted intramolecular cyclopyrophosphorylation for the preparation of cTDPRE **6b**.

After deprotection of the 2′,3′-*O*-isopropyl group, compound **6b** was obtained from **5b** in quantitative yield. Taken together, an efficient microwave-assisted intramolecular pyrophosphorylation approach was developed and successfully applied to the key step for the preparation of cTDPRE **6b**. The microwave-assisted reaction allows for an efficient cyclopyrophosphorylation that is completed within 15 min at high precursor concentrations, with 95% yield, without requiring super-dilute precursor solutions and molecular sieves. Compound **6b** prepared by this new method was identified by $^{1}$H and $^{31}$P NMR, and HRMS, and compound **6b** is consistent with the compound reported by us previously by HPLC.[22]

## 2. Evaluation of $Ca^{2+}$ mobilizing activity of cTDPRE 6b in Jurkat T cells

The biological activity of cTDPRE **6b** was assessed in intact human Jurkat T-lymphocytes as described previously.[12–14] Application of cTDPRE **6b** (1 mM final extracellular concentration) resulted in an increase in the free cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$). Lag periods between approx. 50 and a few hundred seconds were sufficient to reach a threshold concentration of cTDPRE **6b** inside the cell to promote $Ca^{2+}$ release (Fig. 4A and B). Some cells responded with oscillatory $Ca^{2+}$ signaling patterns, while other cells showed a sustained increase in $[Ca^{2+}]_i$ (Fig. 4). Similar to the application of the anti-CD3 monoclonal antibody OKT3, cTDPRE **6b** resulted in a fast increase in $[Ca^{2+}]_i$ (peak), followed by a sustained level of elevated $[Ca^{2+}]_i$ (plateau). This effect was concentration-dependent, resulting in smaller signals with 300 and 100 μM final concentrations of **6b** (Fig. 4C). The linear analogue TDPRE **7** was applied in a concentration of 1 mM, but this compound did not increase $[Ca^{2+}]_i$ significantly (Fig. 4C).

A couple of cADPR analogues modified in the nucleobase have been shown to retain agonist activity. Examples for such biologically active compounds are cIDPR derivatives,[19] 3-deaza-cADPR,[20] and 7-deaza-cADPR.[21] These examples suggest that the nucleobase may be modified at several sites without a major loss of agonist activity. The triazole-based cADPR derivative **6b** presented here represents a much more radical approach to simplify the structure of cADPR analogues, as compared to any other cADPR derivatives. The imidazole moiety of the natural nucleobase adenine is replaced by the similar triazole ring, and the amino-pyrimidine moiety of adenine is replaced by an amide bond partially mimicking C6 and N1 of adenine. Interestingly, we demonstrate here that regardless of this dramatic simplification of the purine nucleobase, the principal ability to mobilize $Ca^{2+}$ is retained in cTDPRE **6b**. Although the peak $Ca^{2+}$ obtained at 1 mM extracellular concentration was below about 200 nM, a sustained $Ca^{2+}$ signal was observed indicating coupling of $Ca^{2+}$ release to capacitative $Ca^{2+}$ entry. Importantly, the relative amplitude (defined as the difference between basal $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ at a certain time point upon addition of the compound) of sustained $Ca^{2+}$ signaling upon cTDPRE addition was almost 100 nM (Fig. 4C), and thus, is clearly comparable to the sustained relative $Ca^{2+}$ signal slightly above 100 nM observed with the benchmark cADPR analogue, cIDPRE.[12]

In conclusion, here we report an efficient microwave-assisted intramolecular cyclopyrophosphorylation approach that was successfully applied to the key step for the preparation of cTDPRE

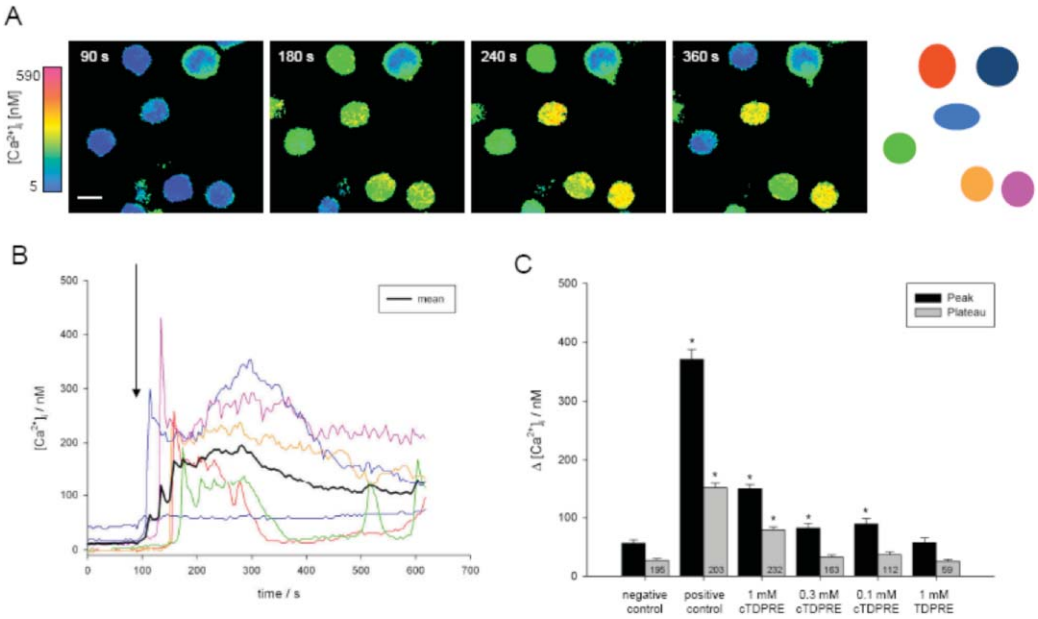



**Fig. 4** Quantitative analysis of the $Ca^{2+}$ mobilizing effects of cTDPRE **6b** and TDPRE **7**. A representative field of six cells was imaged for $[Ca^{2+}]_i$ at different time points. cTDPRE (1 mM final concentration) was added as indicated by the arrow (90 s). Changes in $[Ca^{2+}]_i$ are shown as pseudocolor images (A; scale bar 10 μm) and by quantification of the mean $[Ca^{2+}]_i$ in each single cell. (B) Color encoding of localization of individual cells in the microscopy field is as indicated in the inset in (A). (C) Changes in mean $[Ca^{2+}]_i$ after the application of compound or buffer control were averaged for at least three independent experiments (number of cells are indicated in the bars) for each condition. The peak was analyzed as the maximum within 200 s after application, the plateau was analyzed 500 s after application. Data are presented as mean ± SD. * Indicates a significant difference compared to the respective negative control ($p < 0.01$). The response to the application of the anti-CD3 monoclonal antibody OKT3 served as a positive control while addition of buffer was used as a negative control.

**6b**. With this microwave-assisted reaction, an efficient cyclopyrophosphorylation can be completed in 15 min at high precursor concentrations, resulting in 95% yield. To our best knowledge, this is the first report about a high-yield intramolecular cyclization in highly concentrated precursor solutions for the preparation of cADPR analogues. Together with its $Ca^{2+}$ release activity, cell membrane-permeant property, and the concise and efficient synthesis, cTDPRE **6b** is a promising valuable tool for the investigation of cADPR-mediated $Ca^{2+}$ signals. The 1,2,3-triazole-modified cADPR mimics may open a completely new direction for the medicinal chemistry and pharmacology of cADPR.

## Experimental

### (i) Preparation of cTDPRE 6b by MW-assisted intramolecular pryophosphorylation reaction

Mass spectra were obtained on either VG-ZAB-HS or Bruker APEX. HR-ESI-MS (electrospray ionization) were performed with a Bruker BIFLEX™ III. $^1$H NMR was recorded with a JEOL AL300 spectrometer using $D_2O$ as solvent. Chemical shifts were reported in parts per million downfield from TMS. $^{31}$P NMR spectra were recorded at room temperature by use of a Bruker Avance 200 spectrometer (81 MHz); orthophosphoric acid (85%) was used as an external standard. Compound **6b** was purified twice on an Alltech preparative $C_{18}$ reversed phase column (2.2 × 25 cm) using a Gilson HPLC by buffer system: MeCN–TEAB (pH 7.5) and MeCN–TEAA (pH 7.0) before the structural identification and biological study.

Compound **4b** was prepared based on the method that we reported previously.[22] The mixture of 10 mg of compound **4b** (12.4 μmol) and 100 mg of iodine in pyridine (5 ml) was stirred at 90 °C, assisted by microwave irradiation, for 15 min. Then the pyridine 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 a C18 reversed-phase column (2.2 × 5 cm). The column was eluted using a linear gradient of 0–50% $CH_3CN$ in TEAB buffer (0.05 M, pH 7.5) within 50 min to give **5b** at 34.1 min (8.2 mg, 95%) as a triethylammonium salt. $^1$H NMR (300 MHz, $D_2O$), $\delta$ 8.48 (s, 1H, H-5), 5.96-5.98 (d, 1H, $J = 8$ Hz, H-1′), 4.81-4.89 (m, 2H, H-2′, 3′), 4.57-4.60 (m, 1H, H-4′), 4.21-4.26 (d, $J =$ 13 Hz, 1H, H-5′a), 3.83-3.88 (d, $J = 13$ Hz, 1H, H-5′b), 3.39-3.60 (m, 8H, H-1″,2″,4″,5″), 1.30, 1.45 (each s, each 3H, -C($CH_3$)$_2$). $^{13}$C NMR (100 MHz, $D_2O$), $\delta$ 127.9, 124.3, 123.6, 113.3, 94.9, 85.4, 84.1, 80.8, 68.9, 67.0, 64.8, 63.8, 45.6, 38.5, 24.9, 23.2. $^{31}$P NMR ($D_2O$, 81 MHz), $\delta$ –9.11 (br, s), –11.4 (br, s). HRMS (ESI) calculated for (M + $H^+$) $C_{15}H_{25}N_4O_{12}P_2$ 515.0939, found 515.0925, (M + $Na^+$) $C_{15}H_{24}N_4O_{12}P_2$ 537.0758, found 537.0746.

A solution of **5b** (5 mg, 7.1 μmol) in 50% HCOOH (1.5 ml) was stirred for 2 h and then evaporated under reduced pressure. The purification of the residue was performed with the same procedure as for compound **5b** by HPLC on a C18 reversed-phase column, eluted with a linear gradient of 0–65% $CH_3CN$ in TEAB buffer (0.05 M, pH 7.5) to give the target molecule **6b** in quantitative yield. $^1$H NMR (300 MHz, $D_2O$), $\delta$ 8.67 (s, 1H, H-5), 6.03-6.05 (d, $J = 9$ Hz, H-1′), 4.54-4.56 (m, 2H, H-2″, 3″), 4.14-4.18 (m, 1H, H-4″), 3.93-4.02 (m, 2H, H-5′a, 5′b), 3.80-3.66 (m, 8H, H-1″,2″,

4″,5″). $^{31}$P NMR ($D_2O$, 81 MHz), $\delta$ –8.91 (br, s), –11.39 (br, s). HRMS (ESI) (M – $H^+$) calculated for $C_{12}H_{20}N_4O_{12}P_2$ 473.0480, found: 473.0477.

**HPLC analysis of the microwave-assisted reactions.** The reaction mixture 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 a C18 reversed-phase column (2.2 × 5 cm). The column was eluted using a linear gradient of 0–50% $CH_3CN$ in TEAB buffer (0.05 M, pH 7.5) within 50 min to give **7** at 24.2 min, **5b** at 34.1 min, and **8** at 38.0 min; the molecular weights of **7**, **5b** and **8** were given by ESI-TOF$^-$ with 531.0, 513.0, and 1027.1, respectively.

### (ii) Evaluation of cADPR analogues by ratiometric $Ca^{2+}$ imaging

Jurkat T lymphoma cells were loaded with Fura-2/AM as described (ref. 12) and kept in the dark at room temperature until use. Thin glass coverslips (0.1 mm) were coated with bovine serum albumin (5 mg ml$^{-1}$) and poly-L-lysine (0.08 mg ml$^{-1}$). Silicon grease was used to seal small chambers consisting of a rubber O-ring on the glass coverslips. 60 µl of 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 and 20 mM HEPES, pH 7.4, and a 40 µl cell suspension ($2 \times 10^6$ cells ml$^{-1}$) suspended in the same buffer were added into the small chamber. The coverslip with cells slightly attached to the bovine serum albumin/poly-L-lysine coating was mounted on the stage of a fluorescence microscope (Leica DM IRE2). Ratiometric $Ca^{2+}$ imaging was performed as described (ref. 12). We used an Improvision imaging system (Tübingen, Germany) built around the Leica microscope at 40-fold magnification. Illumination at 340 and 380 nm was carried out using a monochromator system (Polychromator IV, TILL Photonics, Gräfelfing, Germany). Images were taken with a gray-scale CCD camera (type C4742-95-12ER; Hamamatsu, Enfield, United Kingdom). Data processing was performed using Openlab software (Improvision, Tübingen, Germany).

### Abbreviations

| | |
|---|---|
| cADPR | cyclic adenosine 5′-diphosphoribose |
| cIDPR | cyclic inosine 5′-diphosphoribose |
| cADPcR | cyclic adenosine 5′-diphospho- carboribose |
| cIDPRE | cyclic inosine diphosphoribose-ether |
| cIDP-DE | cyclic inosine diphospho-diether |
| DCC | dicyclohexylcarbodiimide |
| DIPEA | diisopropylethylamine |
| $^{31}$P NMR | phosphorus-31 nuclear magnetic resonance |
| HR-ESI-MS | high resolution electrospray ionization mass spectrometry |
| HPLC | high performance liquid chromatography |
| TOF | time of flight |
| THF | tetrahydrofuran |
| Py | pyridine |
| TEAA | triethylammonium acetate |
| TEAB | triethylammonium bicarbonate |
| TMS | tetramethylsilane |

## Acknowledgements

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