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The evolving chemistry of cADPR analogues: from marine discovery to calcium signaling tools

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ABSTRACT

Cyclic adenosine diphosphate ribose (cADPR) is a key second messenger that regulates intracellular Ca^{2+} homeostasis via ryanodine receptor (RyR)-dependent pathways. However, its limited membrane permeability and susceptibility to hydrolysis hinder its application as a molecular probe or therapeutic agent. To address these concerns, extensive efforts have focused on the total synthesis of cADPR analogues, aiming to obtain chemically stable and cell-permeable derivatives with tailored properties. Among the various cellular systems expressing a functional cADPR signaling machinery, Jurkat and neuronal cells are of particular interest, as dysregulation of intracellular calcium levels has been observed in these cellular systems in the contexts of HIV infection and neuroblastoma development, respectively. Accordingly, this review highlights the recent advances in the design and synthesis of cADPR analogues, offering new insights in structure–activity relationship (SAR) studies in Jurkat and neuronal cells, and emphasizing the importance of balancing structural stability, cellular uptake, and receptor affinity to achieve an effective Ca^{2+} mobilization. Overall, the findings discussed herein contribute to a deeper understanding of cADPR chemical biology and provide a synthetic platform for the rational design of cutting-edge Ca^{2+}-modulating agents with potential diagnostic and therapeutic applications.

1. Introduction

Cellular communication depends on the capability to detect and transduce extracellular chemical signals via tightly regulated intracellular pathways [1]. This process is initiated at the plasma membrane, where receptor proteins recognize specific ligands and trigger intracellular signaling cascades. A fundamental feature of these pathways is the production of second messengers—low-molecular-weight compounds that amplify and transmit the signal within the cell [2]. Among them, calcium ions (Ca^{2+}) represent one of the most ubiquitous and functionally versatile second messengers, orchestrating a wide range of cellular processes across all domains of life [3–6]. The finely tuned release of Ca^{2+} from intracellular stores, in response to defined stimuli, governs essential physiological functions such as gene expression, [7] fertilization, [8] cell differentiation, [9,10] and T cell activation [11,12]. Nucleosides, nucleotides, and analogues constitute the fourth principal class of biomolecules and are indispensable for a wide range of biological functions in humans. These include the storage and transmission of genetic information, energy metabolism, [13] intracellular signaling, [14–16] and treatment and prevention of diseases [17–21]. Among them, cyclic nucleotides represent a distinct subclass characterized by two nucleosides joined via a pair of phosphate groups (pyrophosphate) forming a macrocyclic structure. These molecules have emerged as crucial second messengers in both mammalian and bacterial cells, playing pivotal roles in the regulation of numerous physiological processes [22,23]. Several signaling molecules have been identified as

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key mediators of intracellular Ca^{2+} release, [24] among which cyclic adenosine diphosphate ribose (cADPR, **1**, Fig. 1)—a cyclic nucleotide with an 18-membered ring structure [25]—that was firstly isolated from sea urchin egg extracts [26] and detected in various mammalian cell types, including T-lymphocytes, [27] smooth and cardiac muscle cells, [28–30] and cerebellar neurons [31,32]. Structurally, cADPR comprises an adenine base connected via N^9- and N^1-glycosidic bonds to the "southern" and "northern" ribose moieties, respectively. A distinctive feature of cADPR is its cyclic structure, established by a pyrophosphate linkage connecting the 5′-position of the "southern" ribose to the 5″-position of the "northern" ribose.

Following its biosynthesis from nicotinamide adenine dinucleotide (NAD^+), [29] catalyzed by the enzyme CD38 in humans, [33] cADPR facilitates the mobilization of Ca^{2+} from the endoplasmic reticulum (ER) into the cytosol via the ryanodine receptors (RyRs), a large tetrameric calcium release channel located on the ER membrane (Fig. 2). Although the precise mechanism of action remains under investigation, it is widely accepted that cADPR does not bind directly to the RyR but rather exerts its effect through the involvement of auxiliary regulatory proteins [28,34,35]. Beyond its role in facilitating Ca^{2+} release via ryanodine receptors, cADPR has also been implicated in the stimulation of extracellular Ca^{2+} entry, a mechanism in which the transient receptor potential cation channel, subfamily M, member 2 (TRPM2) has garnered increasing attention and is field of increasing debate [36–38]. TRPM2 is a non-selective cation channel permeable to Ca^{2+}, functioning as a sensor of oxidative stress triggered by disturbances in the redox equilibrium and the resulting accumulation of reactive oxygen species (ROS) [39].

Increasing evidence supports a causal association between elevated ROS levels and the development or progression of various pathological conditions, including neurodegenerative diseases, [40,41] and cancer [42]. Neuroblastoma—the pediatric malignancy with the second highest incidence—is characterized by an impaired cellular differentiation, given that spontaneous regression of the disease partially occurs through neuronal differentiation. Therefore, differentiation-inducing therapies are regarded as highly effective therapeutic strategies that in neuroblastoma cell lines correlate with increased intracellular calcium levels [43,44]. Conversely, it has recently been demonstrated that elevated cytosolic Ca^{2+} levels in Jurkat cells, resulting from upregulated CD38 expression in the context of HIV disease, may compromise cell survival and impair their regenerative capacity [45]. Since cADPR regulates calcium homeostasis both in Jurkat and neuronal cell systems, [46] it could serve as a valuable tool for gaining deeper insight into the physiopathological mechanisms underlying calcium-dependent processes. However, the physiological instability of cADPR at the N^1-glycosidic bond, [47,48] along with its limited membrane permeability [49]—likely due to the negative charge of its pyrophosphate group—strongly limits its potential as Ca^{2+} bio-probe. To overcome these limitations, researchers have developed semi-synthetic [50] and synthetic methods

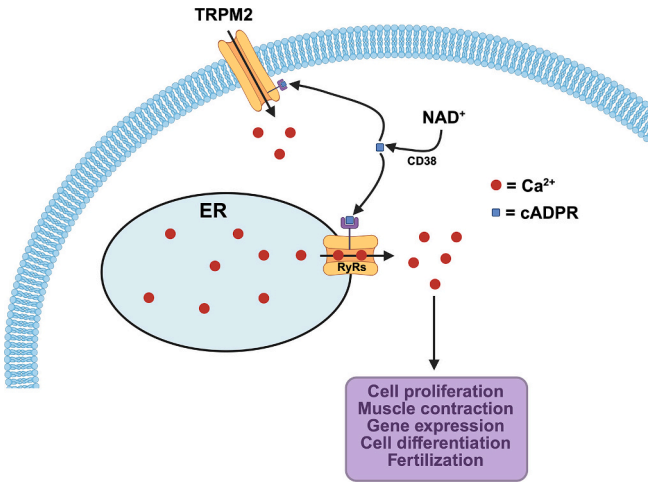


Fig. 2. Schematization of possible intracellular cADPR-mediated Ca^{2+}-release mechanisms.

[25,51] aimed at producing novel analogues that are both non-hydrolysable and capable of permeating cell membranes. Unfortunately, identifying a universal lead compound remains challenging, as Ca^{2+}-releasing activity strongly depends on the specific cellular system under investigation. Accordingly, structure–activity relationship (SAR) studies have been undertaken to determine how structural modifications across the entire molecular scaffold affect agonistic or antagonistic behavior [25,26]. However, the limited knowledge of the cADPR receptor's binding site complicates the rational design of new analogues. The discovery of the Ca^{2+}-releasing properties of cyclic inosine diphosphate ribose (cIDPR, **2**)—a synthetic and stable analogue in which the adenine base of cADPR is replaced by hypoxanthine—[52,53] has provided a powerful lead compound with a tunable structure, enabling the synthesis of a wide range of stable and structurally simplified analogues capable of modulating calcium signaling [25,34]. The present review focuses on recent chemical developments in the synthesis of cIDPR derivatives, particularly those featuring simplified surrogates in the place of "northern" ribose, pyrophosphate bridge, and purine base. The aim is to provide deeper insight into the impact of these structural modifications on biological activity, particularly in Jurkat cells and neuronal cells. This work offers an additional perspective on SAR studies, complementing the seminal contributions of leading researchers working in the field of cADPR analogues [25,34].

2. Total synthesis of cIDPR

cADPR analogues can be obtained through two principal strategies: chemo-enzymatic [50] and total synthesis [25,51]. The chemo-

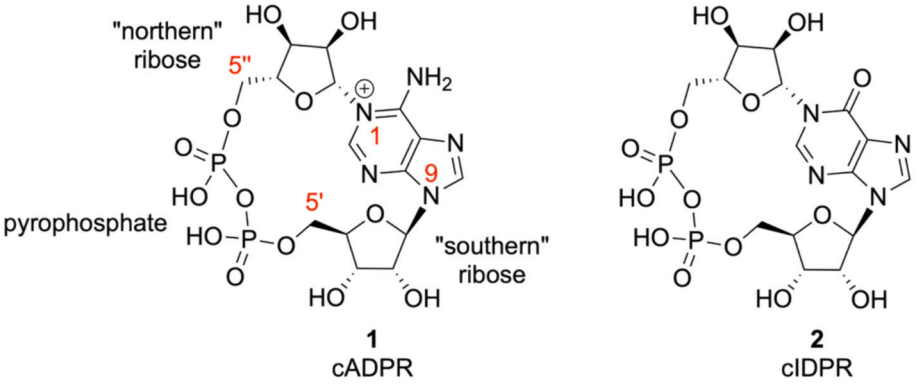


Fig. 1. The structures of cADPR (**1**) and cIDPR (**2**).

enzymatic approach typically involves the enzymatic cyclization of NAD^+ or modified NAD^+ precursors using ADP-ribosyl cyclase, such as Aplysia ADP-ribosyl cyclase, or CD38 [33]. While this latter method is an efficient tool for producing native cADPR and certain close analogues, it is limited by the enzyme's substrate specificity and may not tolerate extensive modifications [54]. Although more labor-intensive, total synthesis provides a powerful route for the generation of structurally diverse and functionally tailored cADPR analogues, enabling complete structural control, and allowing for the incorporation of diverse chemical modifications across the adenine base, ribose moieties, and pyrophosphate bridge. In addition, it provides a full structural flexibility, enabling the introduction of non-natural modifications that are often inaccessible via enzymatic pathways due to substrate specificity limitations [53]. This synthetic control is particularly valuable for exploring structure–activity relationships [55] or for enhancing biological properties such as metabolic stability, membrane permeability and selective receptor interactions. Moreover, total synthesis circumvents issues related to enzyme availability, stability, and scalability, offering a more reproducible and versatile route for analogue development. Notably, total chemical synthesis remains the best viable strategy for the preparation of cIDPR and analogues. In fact, the enzyme-mediated intramolecular cyclization typically yields aberrant and inactive products due to preferential attack by the more nucleophilic N^7-purine atom to the NAD^+ activated "northern" ribose anomeric position (Fig. 3, N^7-cIDPR, **3**) [54].

The retrosynthetic analysis (Scheme 1) of cIDPR identifies the open-chain intermediates **4a–b** as the key precursors, which can be accessed from nucleoside **5**. This latter is obtainable stereoselectively via a coupling reaction between protected C8—Br inosine **6** and acetate-protected ribose **7** under the silyl-Hilbert–Johnson conditions, [56–58] using trimethylsilyl triflate (TMSOTf) as a Lewis catalyst and 1,8-diazabiciclo[5.4.0]undec-7-ene (DBU) as a base. The intramolecular pyrophosphate bond formation can be accomplished under super diluted conditions either by a 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)-promoted condensation of two phosphomonoesters (**4a**) or by an I_2/Ag^+ promoted condensation of an *S*-phenyl phosphorothioate and a phosphomonoester (**4b**), also known as Hata's reaction [59]. Although this latter method minimizes charge repulsion between the two phosphate groups, it requires two distinct phosphorylation steps at the 5′- and 5″-positions, as well as appropriate protection and deprotection procedures. As a trick to facilitate the macrocyclization reaction, a Br atom can be inserted at the C8 purine position before the coupling step. The halogen atom shifts the equilibrium from the *anti* toward the *syn* conformation, in which the two phosphate groups are brought into closer proximity, facilitating their condensation. Given the difficult introduction of a Br atom at the inosine C8 position, adenosine can be used as the starting material, on which the same reaction can be readily performed. Subsequently, C8—Br adenosine derivative **8** can undergo a diazotization reaction yielding nucleoside **6**. These findings indicate that the key factors that could influence the progression of the macrocyclization reaction are the conformation adopted by the linear precursor and the electrostatic repulsion between the two phosphate groups undergoing condensation.

3. "Northern" ribose modified cIDPR analogues

cIDPR (**2**) exhibited agonistic Ca^{2+}-releasing activity compared to the endogenous congener cADPR in permeabilized Jurkat T cells [52]. These findings paved the way for the synthesis of more hydrophobic and cell-permeant analogues through further structural modifications. The insertion of ether/alkyl chains mimicking the "northern" ribose was among the earliest strategies employed to achieve this objective. Their introduction at the N^1-position of the hypoxanthine ring has proven to be a key modification for obtaining the desired molecular scaffolds, especially considering that hypoxanthine can also undergo alkylation at the purine O^6-position. As shown in Fig. 4, the "northern" ribose in cIDPR (**2**) has been replaced either by an ether linkage (cIDPRE, **9**) [60] or by a pentyl chain (cpIDP, **10**) [61,62]. In both the simplified analogues **9** and **10**, the presence of an N^1—C bond assured the chemical stability under physiological conditions. Further synthetic modifications of the pyrophosphate, purine base moieties and N^1-chains led to the formation of derivatives **14**–**16** from compound **9**, and derivatives **11**–**13** and **17** from compound **10**, respectively.

Zhang et al. reported the first example of a cIDPR mimic featuring a surrogate ether linkage replacing the "northern" ribose moiety (Scheme 2, cIDPRE, **9**) [61]. For its synthesis, they started from a fully ribose-protected inosine derivative (**18**), which was alkylated at the N^1-position with $ClCH_2OCH_2CH_2OAc$ in the presence of DBU as a base, affording nucleoside **19**. Due to the bidentate nature of the hypoxanthine nucleophile, the O^6-regioisomer was also recovered. Subsequent removal of the *tert*-butyldimethylsilyl (TBDMS) group (**19** → **20**) allowed the free hydroxyl group to be phosphorodiamidated, yielding compound **21**. Deacetylation of **21** enabled the introduction on the free hydroxyl group of **22** of a second phosphate group, masked as an *S*,*S*′-diphenylphosphorodithioate (**22** → **23**). The simultaneous conversion of the phosphorodiamidate into a phosphomonoester and the *S*,*S*′-diphenylphosphorodithioate into an *S*-phenylphosphorothioate afforded derivative **24**, which was then subjected to Hata's cyclization protocol, yielding the protected cyclic product **25**. The final deprotection via aqueous hydrolysis gave target cIDPRE (**9**). Notably, the flexibility conferred by the ether linkage allowed for efficient cyclization even in the absence of a Br atom at the C8 position of the purine ring. This result is particularly relevant, as the presence of Br typically affords only a modest increase in cyclization yield. Moreover, its subsequent removal would require an additional hydrogenolysis step, which could be detrimental for the integrity of the pyrophosphate bond in the cyclic

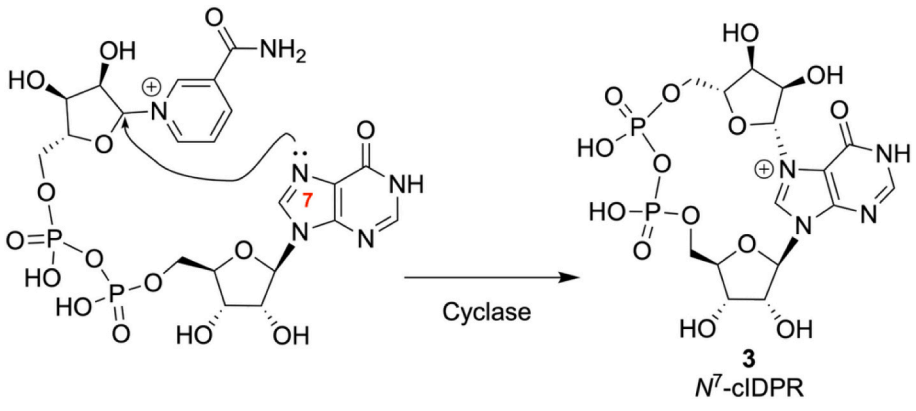
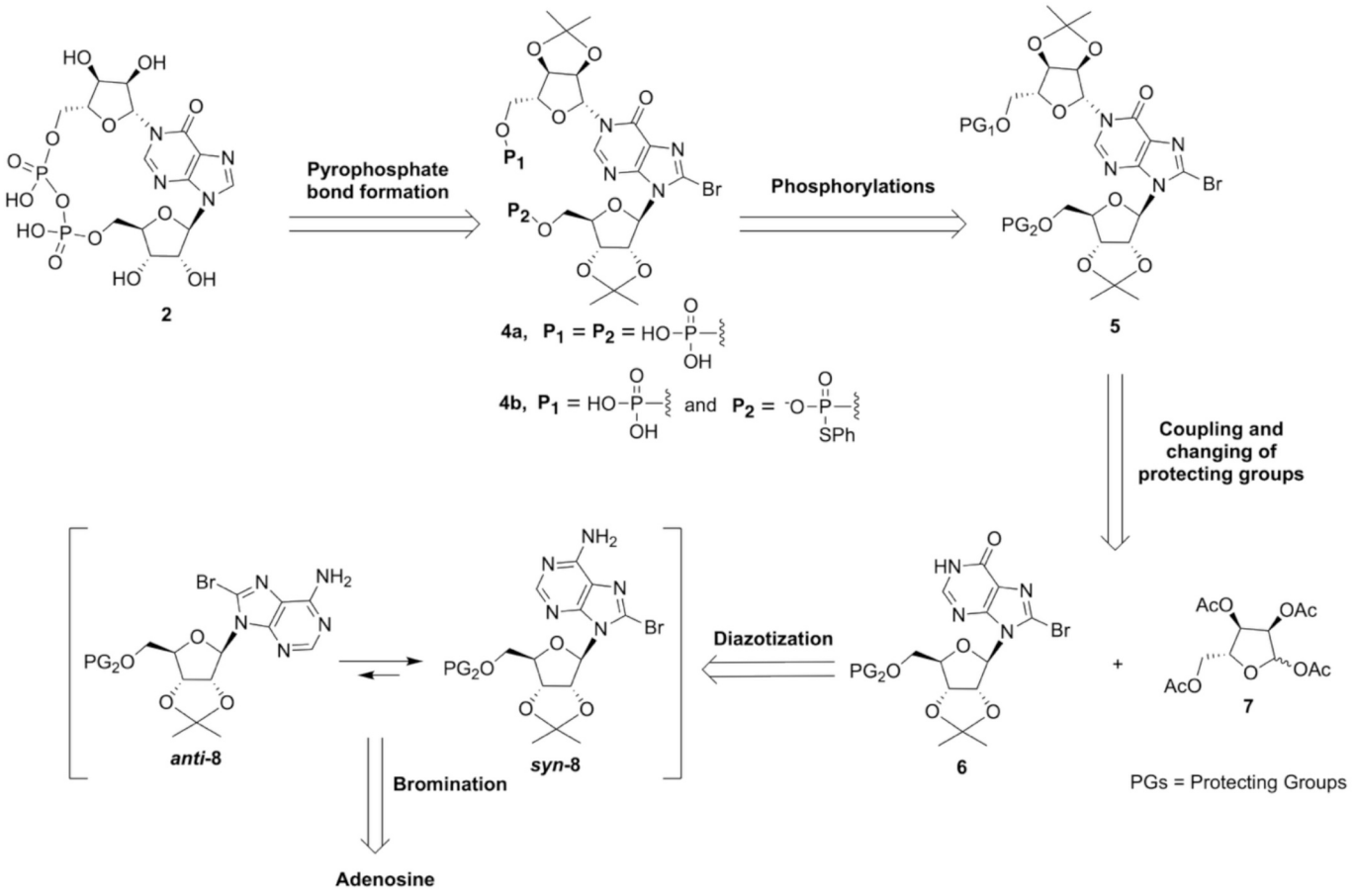


Fig. 3. Simplified representation of the enzyme-mediated synthesis of N^7-cIDPR (**3**).



Scheme 1. Retrosynthetic analysis for the obtainment of cIDPR (**2**).

derivative.

From a spectroscopic point of view, common features which confirm the cyclic structures for all the cIDPR derivatives bearing pyrophosphate bridges are the presence of two doublets ($J \approx 14$ Hz) centered around −10 ppm in the ^{1}H-decoupled ^{31}P NMR spectra, accompanied by a marked deshielding of the 2′-H proton of the ribose ring in the ^{1}H NMR spectra.

Cytosolic Ca^{2+} concentration is measured by loading the cells with the permeant probe Fura 2-AM, a high-affinity, ratiometric calcium indicator excitable by UV light [63]. Intracellular calcium concentration ($[Ca^{2+}]_i$) is quantified by measuring the fluorescence intensity ratio at distinct excitation or emission wavelengths. Specifically, Fura 2-AM displays an emission maximum at 505 nm, with excitation peaks typically observed between 340 and 380 nm. By employing a Ca^{2+}-free/Ca^{2+}-reintroduction experimental approach, cIDPRE (**9**) was found to induce a transient release of Ca^{2+} from intracellular stores in intact Jurkat cells, that provide a consistent and controllable model to explore calcium signaling. Upon readdiction of extracellular Ca^{2+}—once intracellular Ca^{2+} levels had returned to baseline—a rapid and sustained increase in $[Ca^{2+}]_i$ was observed, indicating activation of Ca^{2+} influx from the extracellular milieu. These findings suggested that cIDPRE (**9**) could permeate the plasma membrane, mobilizing Ca^{2+} from an intracellular store sensitive to cADPR, and subsequently triggering Ca^{2+} release-activated Ca^{2+} entry. A key advantage of using membrane-permeant analogues is that they allow for intracellular delivery without disrupting or mechanically manipulating the cells. This is particularly important for cell types in which mechanical stress alone can independently trigger or suppress Ca^{2+} signaling. In contrast, most previous approaches required invasive techniques to introduce cADPR into cells, such as microinjection or relied on non-intact systems, including permeabilized cells or cell homogenates.

Mayol et al. synthesized the simplified analogue cpIDP (**10**) in which a pentyl chain replaced the “northern” ribose (Fig. 5A) [61,62]. For the alkylation of the purine N^1-atom, they exploited the reaction of ribose-protected N^1–2,4-dinitrophenyl inosine **26** with 5-aminopentan-1-ol. The strong electron-withdrawing 2,4-dinitrophenyl group significantly enhanced the electrophilicity of the purine C2 atom toward amines. The interaction promoted a ring-opening of the pyrimidine ring, followed by the reclosure of the purine ring, ultimately yielding N^1-ω-hydroxyalkylated inosine derivative **27** in high yields, without reporting the recovery of the undesired O^6-alkylated regioisomer. The mechanism of the ring-opening/reclosure reaction has been thoroughly investigated, and it was shown to proceed efficiently in the presence of alkyl, ω-hydroxyalkyl, or ω-aminoalkyl amines (Fig. 5B) [64]. Specifically, the amino group that initially attacked the purine C2 atom was the same involved in the subsequent purine ring reclosure, as demonstrated by experiments performed with labelled ^{14}N amines. Notably, the process exhibited high regioselectivity toward the amino groups, even when hydroxyl groups were present on the same alkyl chain [65].

Starting from intermediate **27**, the bis-phosphorodiamidation reaction yielded compound **28**, which was subsequently converted into the bis-phosphomonoester derivative **29**. The cyclopyrophosphorylation reaction was carried out under highly diluted conditions using EDC as the condensing agent, affording protected cpIDP (**30**). The final deprotection via aqueous hydrolysis gave target cpIDP **10**. The high yield obtained in the macrocyclization step suggested that the flexibility conferred by the pentyl chain played a crucial role during the condensation, likely mitigating the electrostatic repulsion between the two phosphate groups and overcoming the unfavorable *anti* conformation presumably adopted by the linear nucleotide **29** due to the absence of a

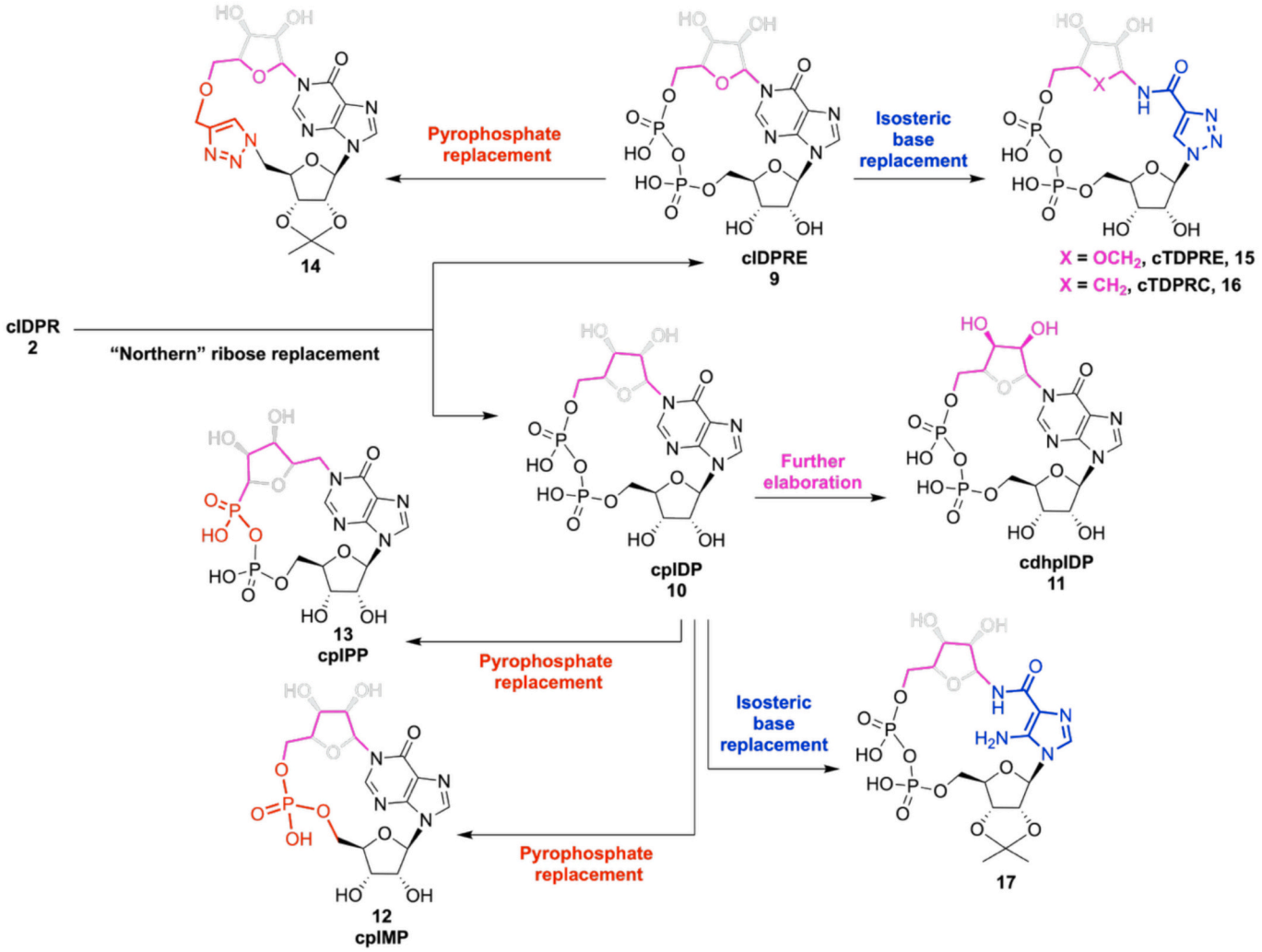
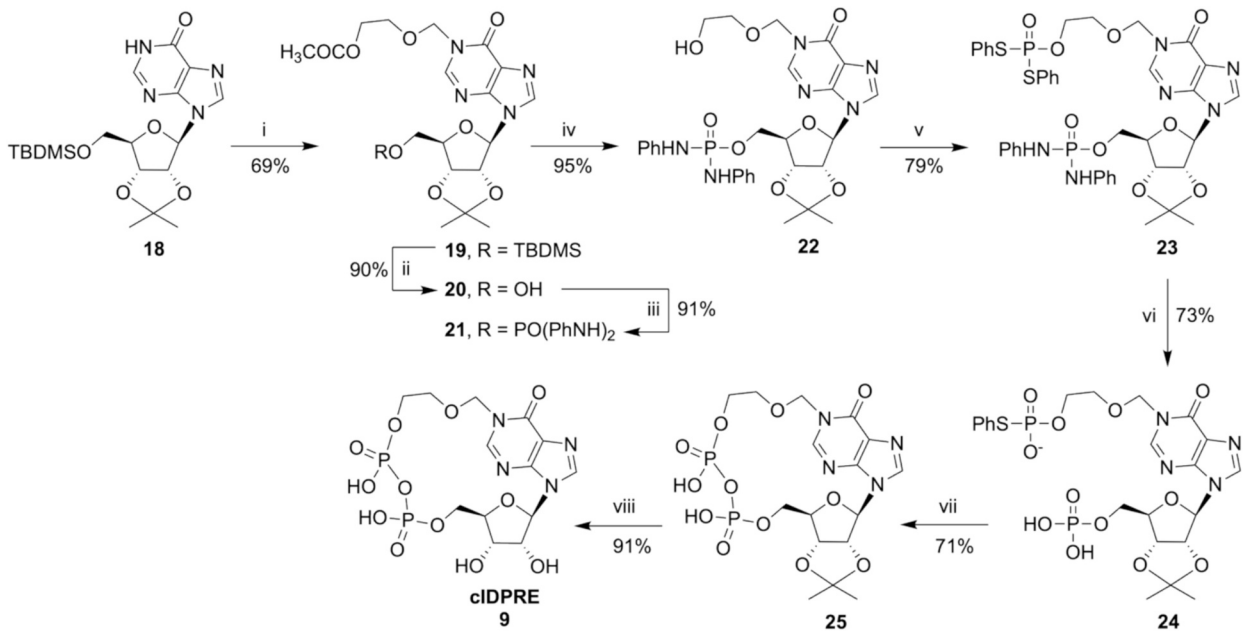


Fig. 4. Generation of cIDPR analogues by replacing the "northern" ribose with an ether linkage (**9**) or a pentyl chain (**10**). Further synthetic elaborations on cIDPRE (**9**) gave **14**, cTDPRE (**15**) and cTDPRC (**16**), whereas on cpIDP (**10**) gave cdhpIDP (**11**), cpIMP (**12**), cpIPP (**13**) and **17**.

halogen atom at the C8 position of the purine ring. In a subsequent paper, the same authors successfully synthesized high yields of cyclic analogues in which the "northern" ribose was replaced by ethyl, butyl, or hexyl chains, using the same EDC-mediated approach [66]. These results demonstrated that flexible linkers forming 15- to 19-membered rings could still facilitate the cyclization reactions, effectively counteracting both the effects of the *anti* conformations and the electrostatic repulsions between the two phosphate groups. To investigate the biological activity of compound **10**, the impact on Ca^{2+} mobilization in NGF-differentiated neuronal cells was evaluated [62]. Notably, the compound induced a rapid and transient elevation in $[Ca^{2+}]_i$ upon administration at 100 nmol/L (Fig. 5C). This response pattern likely reflected an initial release of Ca^{2+} from intracellular stores, followed by a Ca^{2+} influx triggered by membrane depolarization. Moreover, the biological assays confirmed that analogue **10** could permeate the neuronal cell membrane. The promising, yet transient, increase in $[Ca^{2+}]_i$ elicited by analogue **10** in neuronal cells prompted the authors to optimize its structure, making it more closely resemble that of congener **2**. To this end, they designed and synthesized the flexible analogue cdhpIDP (**11**, Fig. 6A), featuring two vicinal hydroxyl groups arranged in a ribose-like configuration on the pentyl chain [32]. For the synthesis of the molecular scaffold of the cyclic structure, the coupling reaction between the protected inosine **31** and the racemic tosylate **32**–obtainable in few steps starting from the propargyl alcohol **34**–was accomplished. Under the optimized reaction conditions, a 7:3 mixture of N^1- and O^6-regioisomers (**35** and **36**, respectively) was obtained, that were separated by column chromatography and identified by the analysis of their HMBC spectra. The presence of two different silyl protecting groups at 5′ and 5″ positions in **35** proved to be crucial for the selective introduction of a phosphotriester (**35** → **37** → **38**) [55] and a *S*,*S*′-diphenyl phosphorodithioate (**38** → **39** → **40**) at the 5″- and 5′-positions, respectively. Upon treatment of compound **40** with an acidic solution, the intermediate **41** was obtained, that was subjected to the Hata's protocol to give the protected cyclic derivative **42.** Despite the very low yields of the cyclization step, it was possible to obtain the cyclic compound **11**, after removal of both acetonide protecting groups. Interestingly, following the final purification step, the two diastereomeric cyclic compounds could be separated; however, only the isomer with the (2″*S*,3″*R*) configuration—tentatively assigned based on 1D and 2D NMR analyses—was obtained in pure form. Inspired by recent evidence that adenosine diphosphate ribose (ADPR) analogues and N^1-*ω*-alkyl or N^1-*ω*-hydroxyalkyl 5′-phosphate inosines promote intracellular Ca^{2+} release, [36,67–69] the authors synthesized also compound **45**, as described in Fig. 6A. Both compounds (2″S,3″R) cdhpIDP (**11**) and **45** were cell permeant and induced a concentration-dependent increase in $[Ca^{2+}]_i$ in the range 1–100 nmol/L in primary cortical neurons (Fig. 6B). Interestingly, open-chain compound **45** displayed superior potency compared to (2″S,3″R) cdhpIDP (**11**) and endogenous cADPR (**1**) at the concentration of 1 nmol/L. The increased biological activity of compound **45** could be attributed to a potentially more accessible interaction



Scheme 2. Reagents and conditions: (i) DBU, $ClCH_2OCH_2CH_2OAc$, CH_2Cl_2, r.t., 0.5 h; (ii) tetrabutylammonium fluoride (TBAF), THF, r.t., 2 h; (iii) $(PhNH)_2POCl$, 1-*H*-tetrazole, pyridine, r.t., 48 h; (iv) CH_3ONa, CH_3OH, r.t., 2 h; (v) *S*,*S*′-diphenylphosphorodithioate cyclohexylammonium salt (PSS), 2,4,6-triisopropyl-benzenesulfonyl chloride (TPSCl), 1-*H*-tetrazole, pyridine, r.t., 12 h; (vi) a) isoamyl nitrite, pyridine/AcOH/Ac_2O, r.t., 8 h, b) H_3PO_2, Et_3N, pyridine, r.t., 11 h; (vii) I_2, 3 Å molecular sieves (MS), pyridine, r.t., 20 h; (viii) 60 % HCOOH, r.t., 8 h.

with its molecular target, or possibly to its ability to engage with distinct intracellular or membrane-associated targets. These latter findings are particularly relevant considering the easier synthetic accessibility of the open-chain derivatives compared to their cyclic counterparts. Although compound (2″S,3″R) cdhpIDP (**11**) was less potent than cADPR (**1**), it still induced a marked increase in intracellular Ca^{2+} levels (Fig. 6C). Notably, the lower plateau phase observed for compound (2″S,3″R) cdhpIDP (**11**) could suggest a reduced efficacy or a less sustained activation of the Ca^{2+}-mobilizing machinery compared to the native cyclic metabolite. This study demonstrated that the insertion of two vicinal hydroxyl groups on the "northern" ribose-mimicking pentyl chain still yielded an effective Ca^{2+}-mobilizing tool capable of efficiently interacting with its putative receptor, despite its molecular flexibility. Therefore, the two hydroxyl groups played a critical role in the biological activity, and the conformational flexibility could favorably influence their spatial arrangement within the receptor binding site. This research paved the way for the synthesis of new linear and cyclic analogues via straightforward derivatization of commercially available propargyl alcohol **34**.

4. Pyrophosphate modified cIDPR analogues

The pyrophosphate bridge in cADPR plays a pivotal role in both its structural integrity and biological function. This moiety not only can contribute to the conformational rigidity required for an efficient receptor binding but also can provide essential electrostatic and hydrogen-bonding interactions necessary for activity at the calcium-mobilizing targets. Modifications of the pyrophosphate—such as substitution with phosphorothioates [70,71] and methylenephosphonates [72]—have been explored to enhance metabolic stability and membrane permeability. These modifications often resulted in diminished biological activity, highlighting the critical importance of the native pyrophosphate unit for maintaining high-affinity interactions with putative intracellular receptors or Ca^{2+}-release machinery. However, simple synthetic modifications are worth to be explored to investigate analogue-gated Ca^{2+}-release in the context of the complex intracellular Ca^{2+}-release machinery. In this context, Oliviero et al. synthesized a doubly structurally reduced cIDPR analogue by replacing the "northern" ribose with a pentyl chain and the pyrophosphate group with a phosphodiester moiety (cpIMP, **12**) [62].

For its synthesis (Fig. 7A), the authors started from nucleoside **18**, which was converted into its N^1-dinitrophenyl derivative (**46**) and subsequently reacted with 5-aminopentan-1-ol, yielding compound **47** [65]. Acetylation of the ω-hydroxy group (**47** → **48**), followed by selective deprotection at the 5′-position, afforded compound **49**. Phosphorylation of the resulting 5′-hydroxy group using the $(^{i}Pr)_2NP(OCE)_2/^{t}BuOOH$ system gave the 5′-*O*-phosphotriester derivative **50**, which was then treated with concentrated aqueous ammonia, to yield intermediate **51**. This latter compound was reacted with EDC to obtain cyclic compound **52**. Finally, treatment of compound **52** with an acidic solution led to the formation of analogue **12**. The activity of compound **12** in modulating $[Ca^{2+}]_i$ was then assessed on PC12 cells previously differentiated with NGF (Fig. 7B). A rapid and transient increase in $[Ca^{2+}]_i$ was observed, likely resulting from an initial release of calcium from intracellular stores, followed by calcium influx triggered by membrane depolarization. These preliminary findings suggested that compound **12**, as its close analogue **10**, exhibited comparable activity, indicating that the pyrophosphate moiety could be not essential for the biological activity. Given its structural simplification and preserved biological activity, compound **12** represented a promising analogue for a further exploration of calcium signaling pathways. The retention of activity, despite the replacement of the pyrophosphate bridge with a phosphodiester, suggested that the key molecular recognition elements for intracellular Ca^{2+} mobilization were preserved. This implied that the negatively charged phosphodiester moiety, while important, may primarily serve to orient the molecule rather than being strictly required for receptor binding or activation [73]. Furthermore, the introduction of the flexible pentyl chain at the N^1-purine position likely enhanced both lipophilicity and membrane permeability, while also enabling favorable accommodation within the binding pocket. The enhanced permeability could also account for the rapid intracellular response observed. To gain deeper insights into the role of the pyrophosphate group in the biological activity of cADPR analogues, the synthesis and biological evaluation of a new cyclic N^1-pentyl inosine phosphono-phosphate analogue, cpIPP

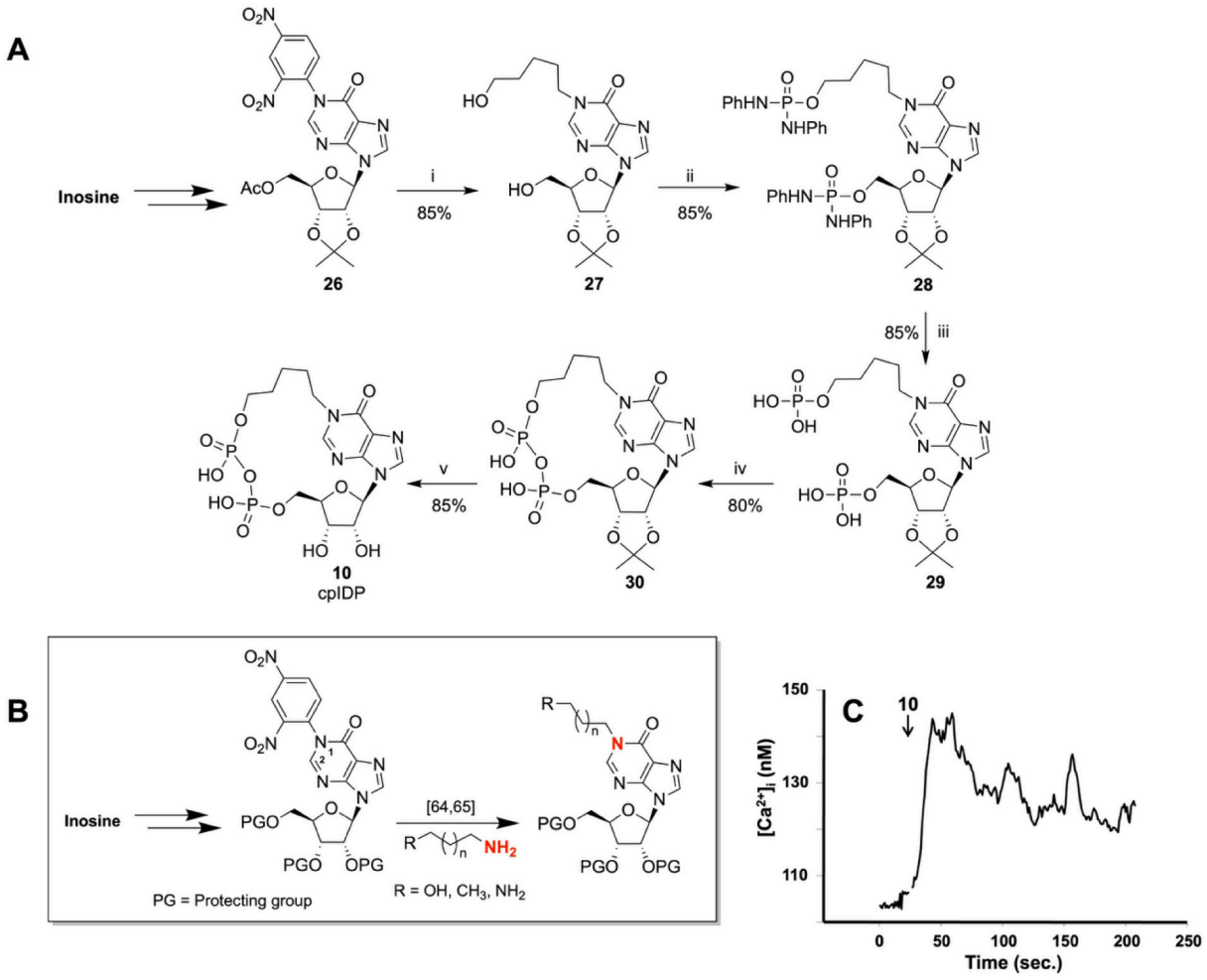


Fig. 5. Panel A: Reagents and conditions: (i) 5-aminopentan-1-ol, DMF, 80 °C, 2.5 h; (ii) $(PhNH)_2POCl$, pyridine, r.t., 12 h; (iii) isoamyl nitrite, pyridine/AcOH/Ac_2O, r.t., 8 h; (iv) EDC, *N*-methylpyrrolidone (NMP), r.t., 60 h; (v) 60 % HCOOH, r.t., 3.5 h, r.t.. Panel B: Inosine N^1-functionalization reaction. Panel C: Effect of compound **10** (100 nmol/L) on $[Ca^{2+}]_i$ in NGF-differentiated PC12 cells.

(**13**), in which the northern phosphate of compound **10** was replaced by C-phosphonate group, have been reported [74].

The retrosynthetic analysis of cpIPP (**13**) afforded as the key intermediate linear precursor **53**, obtainable from the reaction between usual protected inosine **18** and iodide **54** (Fig. 8A). These latter compounds were coupled in the presence of K_2CO_3 as a base, affording compound **55** as the sole regioisomer (Scheme 3). The authors also attempted to carry out the coupling reaction under Mitsunobu conditions, [75] using alcohol **56** as the electrophile, readily obtainable starting from 5-bromopentan-1-ol (**57**). However, the O^6-regioisomer was recovered as the major product, likely favored by a hard–hard interaction between the alkoxyphosphonium intermediate and the purine O^6-atom [76]. After deprotection at the 5′-position (**55** → **60**) and phosphorylation, key intermediate **61** was obtained. The phosphonate and phosphate groups were then deprotected (**61** → **62**) and condensed using EDC, thus obtaining the ribose protected cyclic compound **63**. The presence of two doublets (19.3 ppm for the phosphonate and − 11.2 ppm for the phosphate) in the ^{1}H-decoupled ^{31}P NMR spectrum confirmed the cyclic structure of **63**. This latter compound was finally deprotected at the 2′- and 3′-ribose positions by an acidic hydrolysis, giving cpIPP (**13**).

Ca^{2+} mobilization assays were performed on NGF-differentiated PC12 cells to compare the potency of cpIPP (**13**) with that of the pyrophosphate analogue cbIDP (**64**), [66] which shared the same ring size as cpIPP (**13**) due to the presence of a butyl chain replacing the "northern" ribose. Unfortunately, both the cyclic compounds **13** and **64** were found to be inactive. Considering that minor conformational differences among analogues **10**, **13** and **64** were unlikely to account for the lack of biological activity of compounds **13** and **64** compared to the active analogue cpIDP (**10**) (Fig. 8B), a plausible explanation could lie in the reduced membrane permeability of the inactive compounds. This hypothesis was supported by calculated AlogP values, which indicated progressively lower lipophilicity: cpIDP = −2.0 > cbIDP = −2.4 > cpIPP = −2.5. Lower AlogP values suggested poorer passive diffusion across the cell membrane, potentially limiting intracellular accumulation and, consequently, biological efficacy. These observations, along with previous findings, suggested that the presence of a single phosphate group combined with an N^1-linked pentyl chain represented the minimal structural requirement for generating a biologically active cIDPR analogue in the NGF-differentiated PC12 cell model. A balance between hydrophilicity and adequate lipophilicity along with a favorable accommodation within the binding pocket appeared to be critical for the biological activity.

The design of drug analogues remains central to medicinal chemistry, aiming to overcome resistance, improve selectivity, and refine pharmacokinetic profiles. In nucleic acid chemistry, a successful strategy to address these challenges involves replacing the phosphodiester moiety with functionalities that resist to the degradation by endo- and exonucleases, while offering a favorable hydrophilicity/hydrophobicity balance [77]. One such approach consists of neutralizing the backbone charge by substituting phosphodiesters with 1,2,3-triazoles, which are

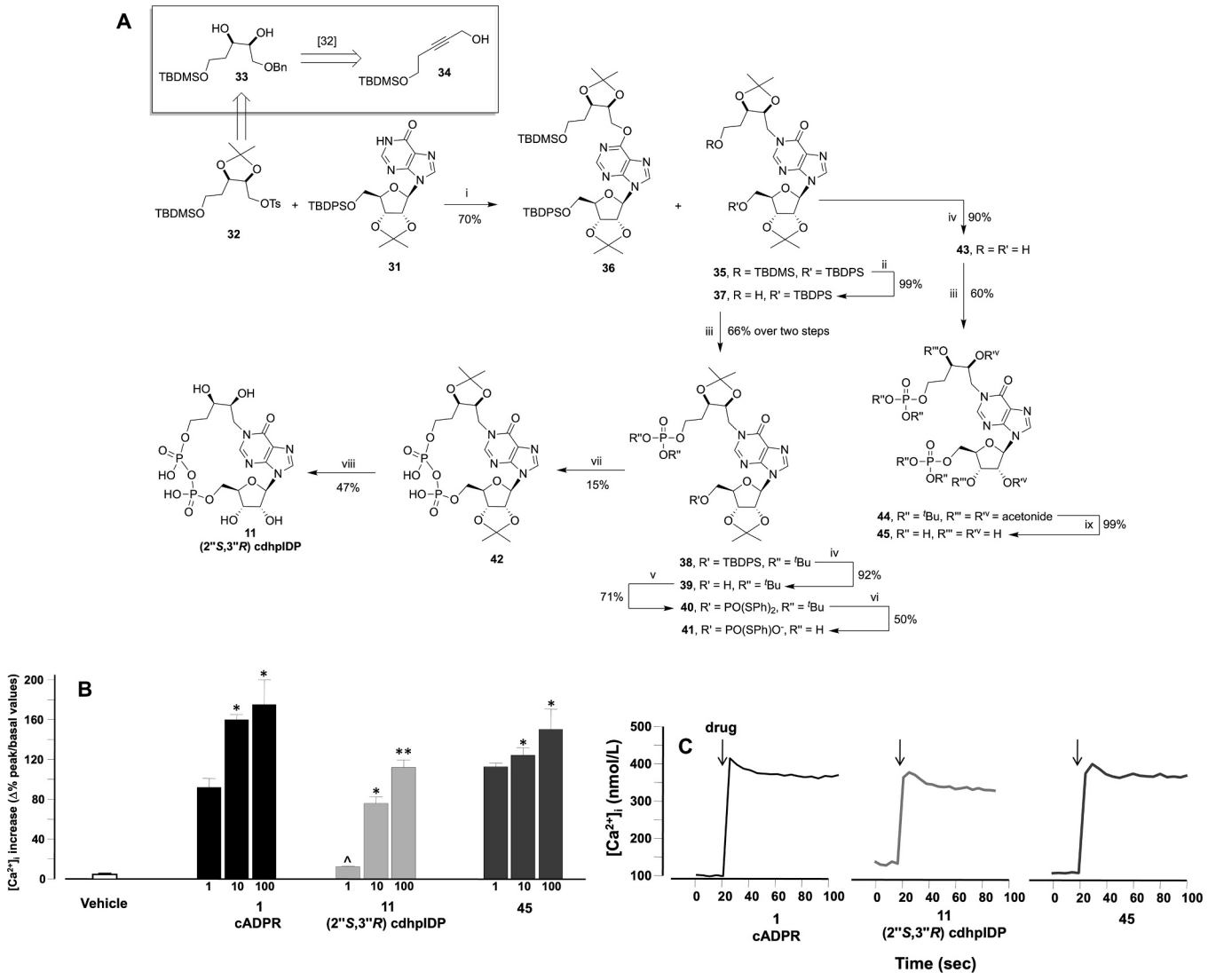


Fig. 6. Panel A: Reagents and conditions: (i) DBU, DMF, 80 °C, 12 h; (ii) pyridinium *p*-toluensulfonate (PyOTs), EtOH, 40 °C, 1 h; (iii) a) $(({}^{t}BuO)_2PN({}^{i}Pr)_2)$, 1-*H*-tetrazole, THF, r.t., 6 h, b) ${}^{t}BuOOH$, THF, r.t., 1 h; (iv) tetrabutylammonium fluoride (TBAF), THF, r.t., 2 h; (v) PSS, TPSCl, pyridine, 40 °C, 16 h; (vi) 50 % TFA in CH_2Cl_2, 0 °C → r.t., 2 h; (vii) I_2, molecular sieves (MS) 3 Å, pyridine, r.t., 16 h; (viii) 60 % aqueous HCO_2H, r.t., 4 h; (ix) 50 % TFA in H_2O, 0 °C → r.t., 4 h. Panel B: Quantification of $[Ca^{2+}]_i$ increase calculated as the percentage change of plateau/basal value after the addition of each compound. Each bar represents the mean (±S.E.M.) of the values obtained in three independent experimental sessions. For each experiment, 20 to 30 individual neurons were monitored. Krebs-Ringer saline solution was used as vehicle. Calculated EC50 for **1** was 0.9 ± 0.005 nmol/L; for (2″S,3″R) **11** 6.3 ± 0.05 nmol/L; for **45** 0.3 ± 0.005 nmol/L. *, $p < 0.05$ versus 1 nmol/L; **, $p < 0.05$ versus previous concentration; ^, $p < 0.05$ versus 1 nmol/L of **1**. Panel C: Representative single-cell traces of the effect of cADPR (**1**) (100 nmol/L), (2″S,3″R) cdhpIDP **11** (100 nmol/L) and **45** (100 nmol/L) on $[Ca^{2+}]_i$. Figures in Panels B and C are republished with permission of Elsevier, from [32]; permission conveyed through Copyright Clearance Center, Inc. License Number: 6034101271245.

among the most versatile bio-isosteres [78]. Their distinctive properties—including high chemical stability, hydrogen-bonding capacity, and geometric similarity to various functional groups—make them ideal surrogates in drug design. The incorporation of triazole linkages is readily achieved via copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC) [79]. These triazole-based internucleotide linkages retain the oligonucleotide functionality while improving stability and imparting structural rigidity. The first example of a such bio-isosteric replacement in a cIDPR analogue appeared in the work of Potter et al., which synthesized "click" cIDPR (**65**, Fig. 9A) [80]. Although intracellular Ca^{2+}-release was only marginal in sea urchin egg homogenates, compound **65** was able to inhibit CD38-mediated hydrolysis of cADPR (**1**), likely by blocking the access of the endogenous molecule to the active site. To investigate the molecular basis of this activity, compound **2** (cIDPR) was removed from the co-crystal structure of CD38 (PDB ID: 2PGJ), and analogue **65** was subsequently docked into the binding site (Fig. 9B). Most docking poses showed a strong overlap between the "northern" ribose of analogue **65** and that of cIDPR (**2**), with the 1,4-disubstituted triazole positioned between the two phosphate-binding regions. These results suggest that the triazole moiety can partially mimic the pyrophosphate group, engaging in key interactions with amino acid residues within the CD38 active site.

Considering that replacing the "northern" ribose moiety in cIPDR (**2**) with simplified surrogates yielded cell-permeant compounds capable of mobilizing Ca^{2+} ions from intracellular stores in Jurkat cells, Zhang et al. designed and synthesized analogue **14**, which incorporated the same ether linkage used in the preparation of cIDPRE (**9**) in the place of the "northern" ribose (Fig. 10A) [81]. Through a convergent approach, building blocks **66** and **67**—the latter obtained in few steps starting from ethane-1,2-diol (**68**)—were coupled to afford key intermediate **72**, which featured an N_3 group at the 5′-ribose position and an alkyne moiety as a pendant functionality at the terminus of the ether chain.

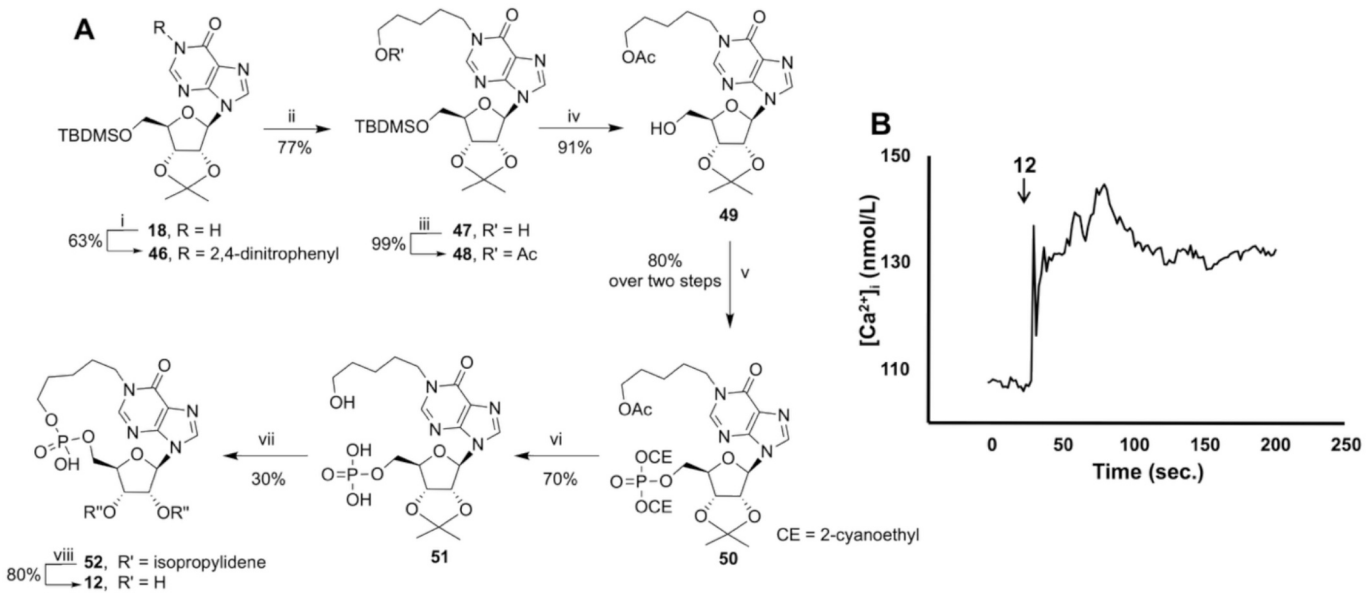


Fig. 7. Panel A: Reagents and conditions: (i) 2,4-dinitrochlorobenzene, K_2CO_3, DMF, 4 h, 80 °C; (ii) 5-aminopentan-1-ol, DMF, 16 h, 50 °C; (iii) Ac_2O, pyridine, 2 h, rt., (iv) NH_4F, MeOH, 16 h, reflux; (v) a) $(^iPr)_2NP(OCE)_2$, 1-*H*-tetrazole, THF, 2 h, rt., b) ^{t}BuOOH, 2 h, r.t.; (vi) conc. NH_4OH (aq.), MeOH, 50 °C, 16 h; (vii) EDC, DMF, r.t.; (viii) TFA, H_2O, 16 h, r.t.. Panel B: Representative single-cell trace of the effect of cpIMP (**12**) (100 nmol/L) on $[Ca^{2+}]_i$ in NGF-differentiated PC12 cells.

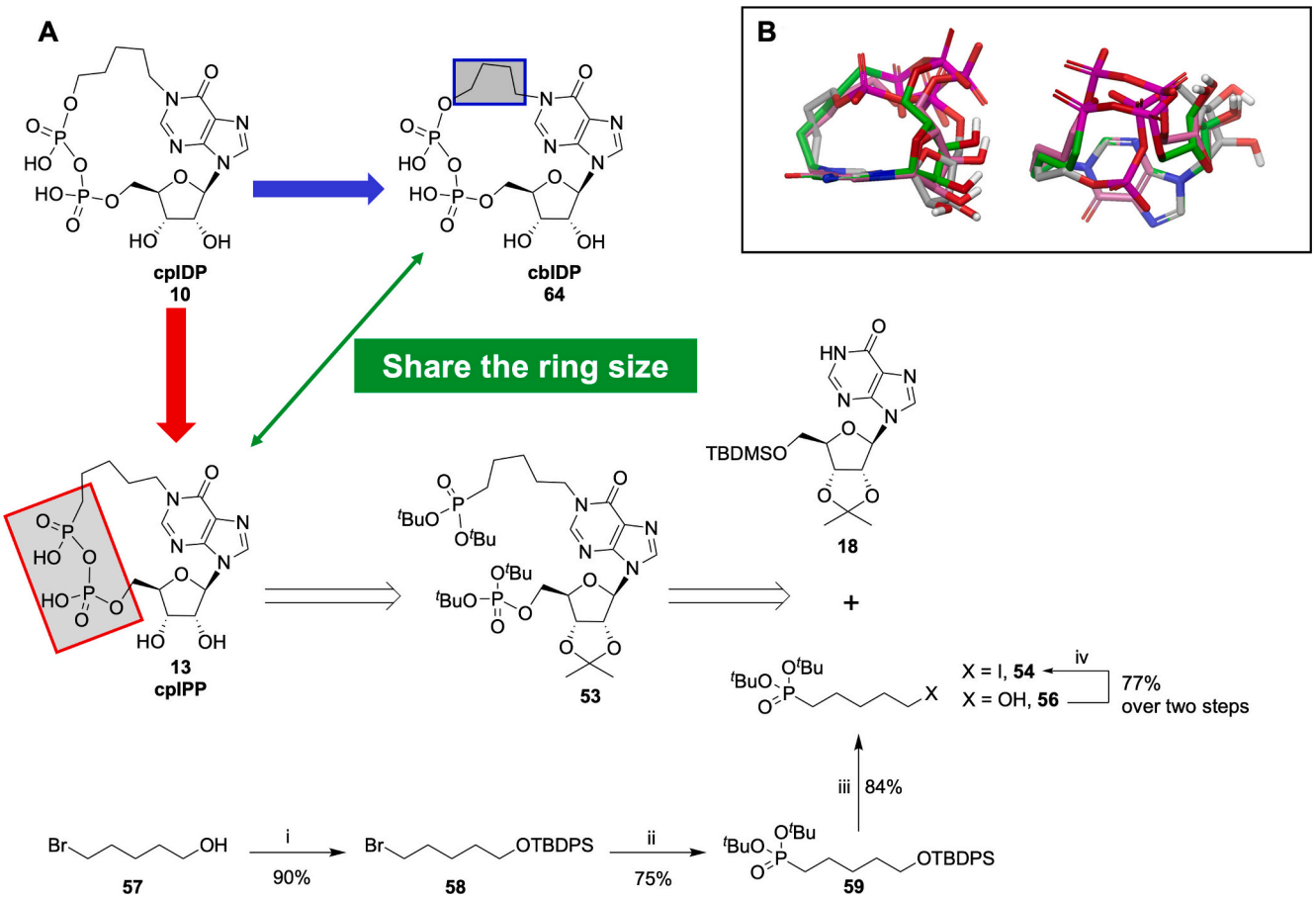
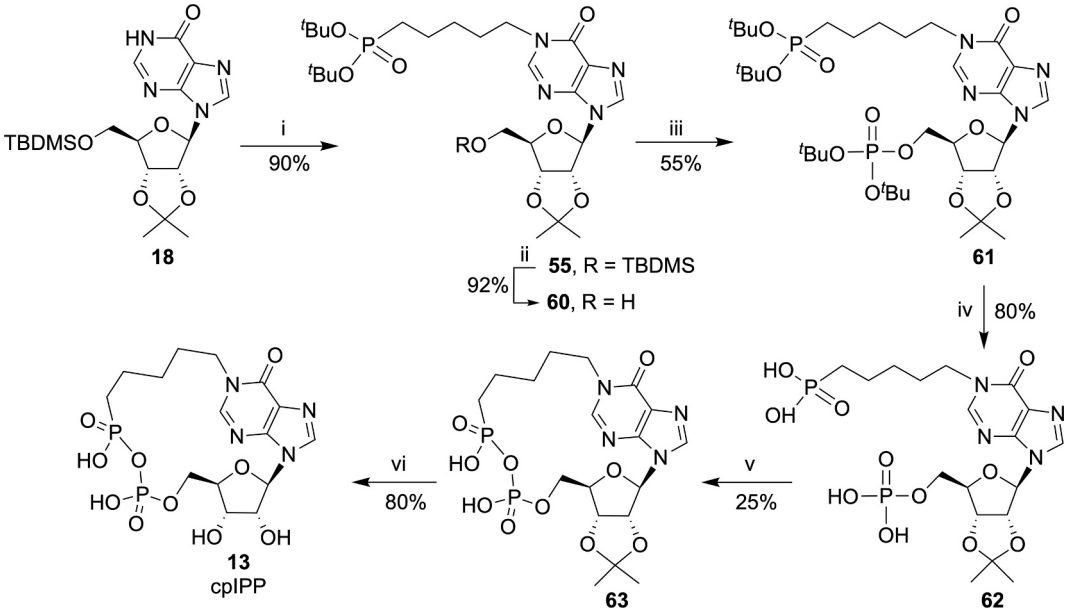


Fig. 8. Panel A: Reagents and conditions: (i) *tert*-butyldiphenilsilyl chloride (TBDPSCl), DMF, r.t., 2 h; (ii) NaH, $HPO(O^tBu)_2$, DMF, 0 °C, 16 h; (iii) tetrabutylammonium fluoride (TBAF), THF, r.t., 2 h; (iv) a) mesyl chloride (MsCl), TEA, CH_2Cl_2, 0 °C, 1 h; b) KI, DMF, 70 °C, 5 h. Panel B: Side (left) and top (right) view of the lowest energy conformers of cbIDP (**64**, grey), cpIDP (**10**, pink) and cpIPP (**13**, green) superimposed on the hypoxanthine ring. Non-polar hydrogens were omitted for sake of clarity. Oxygens were reported in red, nitrogens in blue, phosphates in magenta, hydrogens in white. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



Scheme 3. Reagents and conditions: (i) **54**, K_2CO_3, DMF, r.t., 16 h; (ii) TBAF, THF, r.t., 1 h; (iii) a) $(^tBuO)_2PN(^iPr)_2$, 1-*H*-tetrazole, THF, r.t., 4 h, b) ^tBuOOH, THF, r.t., 1 h; (iv) 5 % TFA in CH_2Cl_2, 0 °C, 5 h; (v) EDC, DMF, r.t., 72 h.; (vi) 60 % HCO_2H in H_2O, r.t., 16 h.

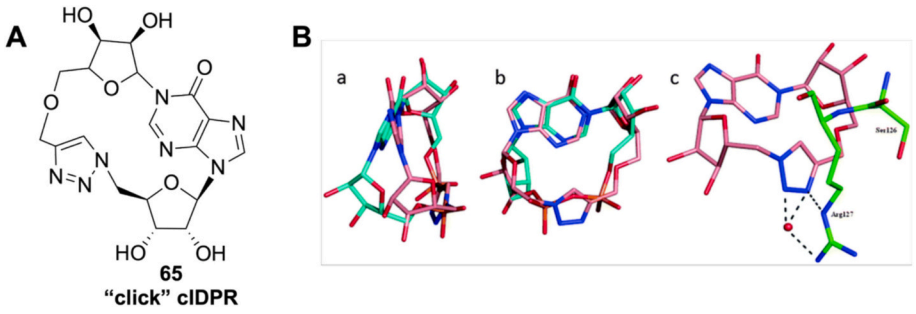


Fig. 9. Panel A: The structure of "click" cIDPR (**65**). Panel B: Docked ligand poses (a and b) of **65** into the 2PGJ crystal structure from which cIDPR (**2**) was removed, and simulated protein-ligand (**65**) complex (c). The protein is shown in green, cIDPR (**2**) in cyan and "click" cIDPR (**65**) in pink. Figure in Panel B is reproduced from [80] with permission from the Royal Society of Chemistry. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Under CuI-catalyzed Huisgen 1,3-dipolar cycloaddition conditions, the target compound **14** was then obtained. The formation of the 1,4-disubstituted 1,2,3-triazole ring system was evidenced by the presence of a singlet at 7.19 ppm in the ^1H NMR spectrum.

Attempts to remove the protecting groups at the 2′- and 3′-ribose positions under conventional acidic conditions were unsuccessful, leading exclusively to decomposition products. Given that the 2′- and 3′-ribose positions are not critical for Ca^{2+}-mobilizing activity in Jurkat cells, [25] and considering a gain in lipophilicity in derivative **14** due to the presence of the acetonide group, the capacity of the newly synthesized mimic to trigger Ca^{2+} release in Jurkat cells was then examined. Compound **14** was able to penetrate cells and elicited a more sustained increase in cytosolic Ca^{2+} levels than cIDPRE (**9**) in the presence of an external Ca^{2+} source (Fig. 10B), inducing an immediate Ca^{2+} peak and a subsequent plateau phase. The involvement of RyRs was further investigated by treating Jurkat cells with ryanodine, a known RyR antagonist. The significant inhibition of the Ca^{2+} elevation in the presence of ryanodine supported the conclusion that compound **14** promoted Ca^{2+} release through activation of RyRs. Since the pyrophosphate moiety appeared not to be critical for receptor interaction, this study paves the way for the development of new generation analogues incorporating a triazole ring in place of the pyrophosphate. Moreover, a smart biological reassessment of previously synthesized low-activity analogues could be conducted following the introduction of a such modification in their structures, to determine whether the increased lipophilicity/rigidity of the molecular scaffold conferred by the triazole moiety could play favorable roles for their biological activity.

5. Base modified cIDPR analogues

Since the replacement of the "northern" ribose in cIDPR analogues with simplified surrogates has led to the development of cell-permeant molecules capable of mobilizing Ca^{2+} ions from intracellular stores, the final section of this review will focus on a new class of doubly modified cIDPR mimics featuring ether or alkyl chains linked to purine base isosteres. Isosteres can preserve essential molecular recognition features while altering metabolic stability, electronic distribution, hydrogen bonding capacity, and membrane permeability [82]. The replacement of natural purine bases with isosteres has become a well-established strategy in the design of modified nucleosides and analogues with tailored physicochemical and biological properties [17]. A prominent example is the nucleoside ribavirin, a broad-spectrum antiviral agent whose 1,2,4-triazole-3-carboxamide moiety acts as an isostere of the purine base, allowing it to mimic both adenosine and guanosine in viral RNA synthesis [83]. The 4-amido-1,2,3-triazole motif, [78] synthesizable through the well-established CuI-catalyzed Huisgen

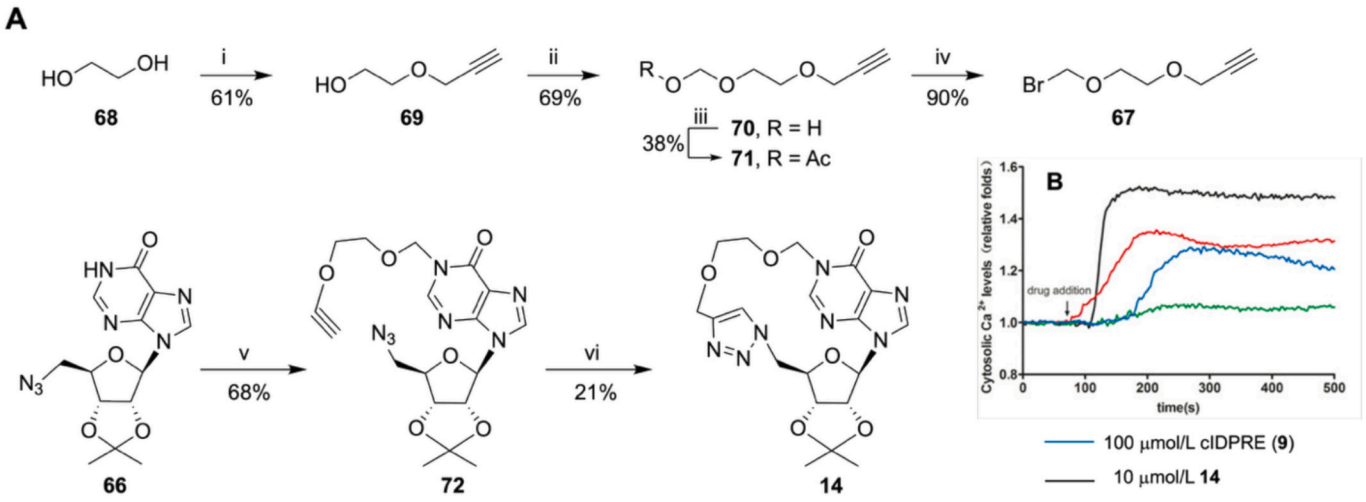
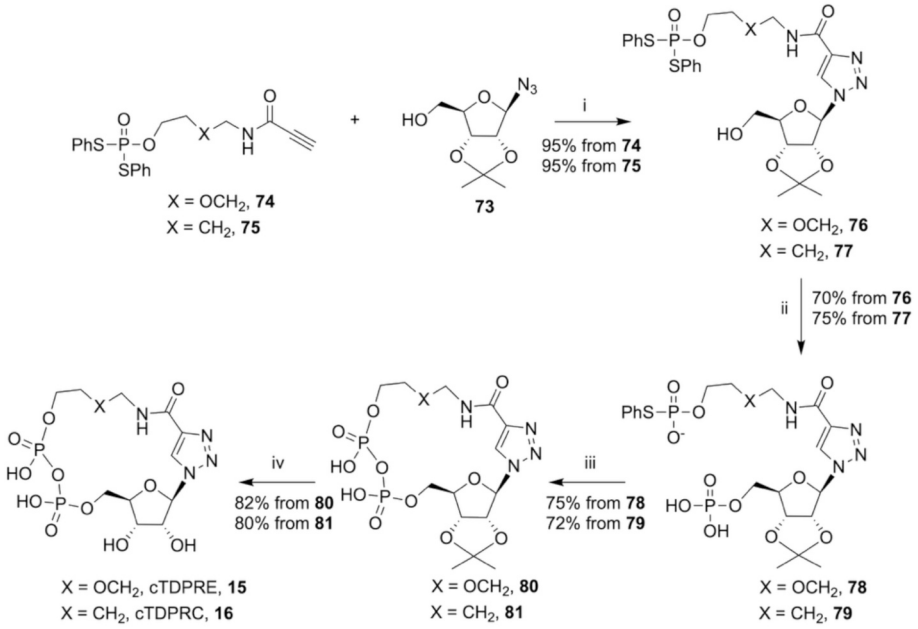


Fig. 10. Panel A: Reagents and conditions: (i) Propargyl bromide, KOH, H_2O, r.t., 18 h; (ii) $(MeO)_2CH_2$, P_2O_5, CH_2Cl_2, r.t., 24 h; (iii) Ac_2O, $BF_3{\cdot}OEt_2$, 0 °C, 2 h; (iv) trimethylsilyl bromide (TMSBr), CH_2Cl_2, reflux, 18 h; (v) **67**, DBU, CH_2Cl_2, −10 °C, 4 h; (vi) CuI, DIPEA, THF, reflux, 16 h. Panel B: Effects of **14** (10 μmol/L) and cIDPRE (**9**) (100 μmol/L) on cytosolic Ca^{2+} increase in Jurkat cells in the presence of an external Ca^{2+} source. Data are means ± S.D. (n = 3, 20–40 cells in each independent experiment). Figure in Panel B is republished with permission of John Wiley and Sons, from [81]; permission conveyed through Copyright Clearance Center, Inc. License Number: 6034110132327.

1,3-dipolar cycloaddition reaction, [79] has attracted growing interest in medicinal chemistry as a versatile bio-isostere of both amide and purine functionalities. Notably, it can function as an isosteric replacement of the purine base in guanosine due to its close structural and electronic resemblance to guanine. Accordingly, Zhang et al. synthesized cIDPR analogues **15** and **16** (Scheme 4), in which the 4-amido-1,2,3-triazole isostere was linked to an ether or an alkyl chain, respectively [84].

For their synthesis, following the coupling reaction between protected azido ribose **73** and alkynes **74** and **75**—both of which already embodying the *S*,*S*-diphenyl phosphoroditioate groups bonded at the end of the chains—intermediates **76** and **77** were obtained. The retention of the sugar β-configuration as well as the formation of the triazole ring were supported by 1D and 2D NMR analyses. On both the derivatives **76** and **77** the introduction of a phosphomonoester moiety at the 5′-ribose position, followed by partial deprotection of the *S*,*S*′-diphenyl phosphoroditioate groups afforded intermediates **78** and **79**, that were subjected to the modified Hata's protocol, yielding cyclic analogues **80** and **81**, respectively. Interestingly, when the pyrophosphate bond formation was carried out on compound **78** under microwave irradiation in a highly concentrated solution, the yield of the cyclization reaction significantly increased (from 82 % to 95 %), while the reaction time was markedly reduced (minutes instead of hours). Final deprotection of ribose 2′- and 3′-hydroxyl groups under conventional acidic conditions furnished target cyclic compounds **15** and **16**. cTDPRE (**15**) proved to be cell-permeant and elicited a sustained increase in cytosolic Ca^{2+} levels in intact Jurkat cells. This finding is particularly significant, as it demonstrated that the 4-amido-1,2,3-triazole moiety—despite its marked structural divergence from the natural purine ring—was still able to preserve key molecular interactions with the receptor, thereby enabling Ca^{2+} mobilization.

5-aminoimidazole-4-carboxamide ribonucleotide (ZMP, Fig. 11) is



Scheme 4. Panel A: Reagents and conditions: (i) CuI, DIPEA, CH_3CN; (ii) $POCl_3$, DIPEA, CH_3CN, 0 °C, 12 h; (iii) I_2, 3 Å MS, pyridine; (iv) 50 % HCOOH in H_2O.

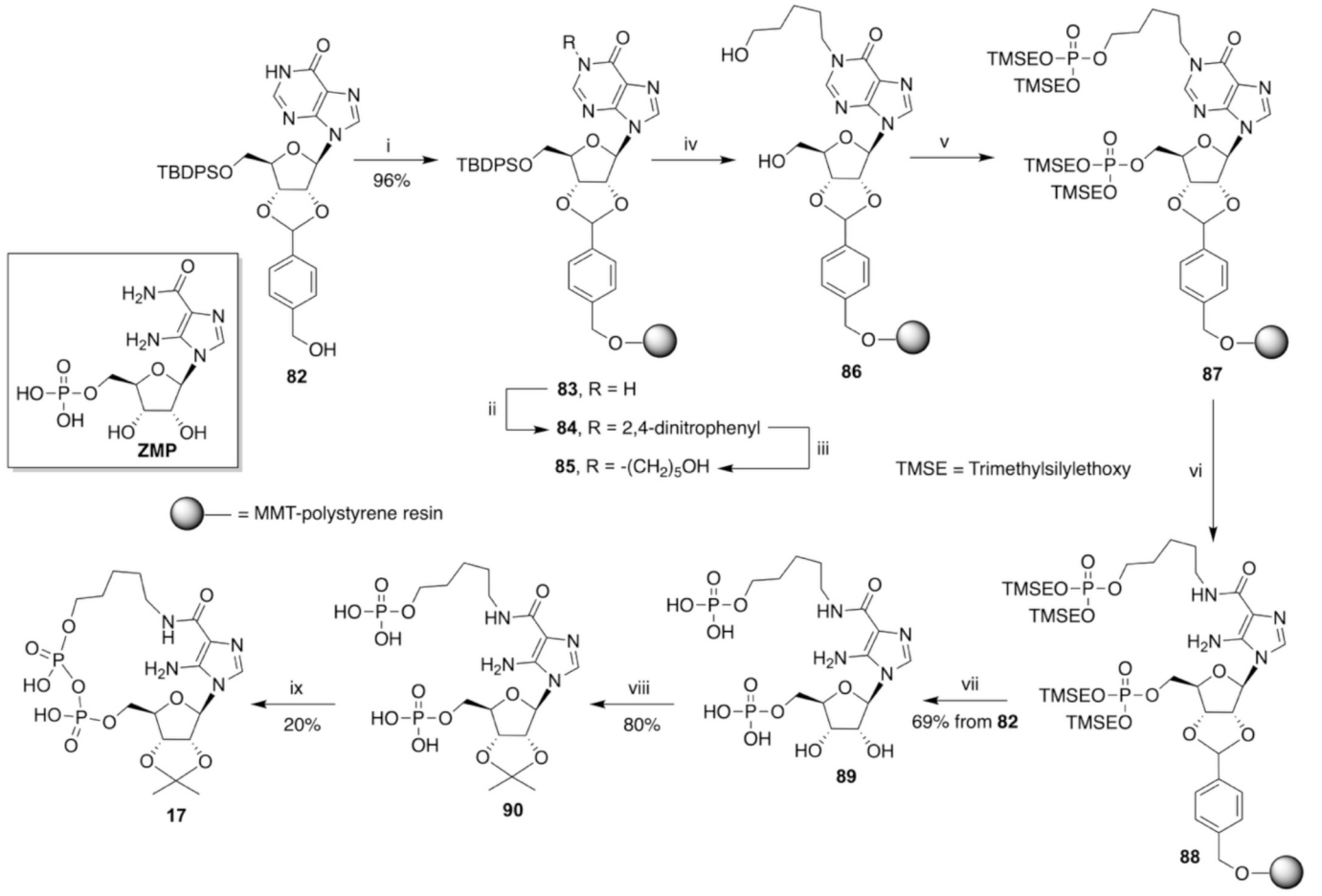


Fig. 11. Reagents and conditions: (i) MMT-Cl resin, pyridine, DMAP, 24 h, r.t.; (ii) 2,4-dinitrochlorobenzene, K_2CO_3, DMF, 2 h, 80 °C; (iii) 5-aminopentan-1-ol, DMF, 80 °C, 8 h; (iv) NH_4F, MeOH, reflux, 12 h; (v) a) $(^iPr)_2NP(OTMSE)_2$, 1-*H*-tetrazole, THF/DCM, r.t., 15 h; b) ^{t}BuOOH, THF, r.t., 2.5 h; (vi) NaOH, EtOH, reflux, 5 h; (vii) TFA 2 % in DCM, r.t., 20 min followed by H_2O washings; (viii) *p*-toluelsulfonic acid (*p*-TsOH), 2,2′-dimethoxypropane (DMP), DMF, r.t., 2 h; (ix) 1,1′-carbonyldimidazole (CDI), DMF, r.t., 48 h.

an adenosine 5′-monophosphate (AMP) mimic that activates AMP-activated protein kinase (AMPK), thereby modulating cellular energy metabolism and demonstrating potential in metabolic disorders and ischemic conditions [85,86]. Given these important biological functions, Oliviero et al. employed a combined solution-phase/solid-phase strategy to synthesize the doubly modified analogue **17**, featuring a pentyl chain attached at the N^4-position of the 5-aminoimidazole-4-carboxamide base moiety [87]. For its synthesis, nucleoside **82** was bound through the benzylic OH group to a monomethoxytrityl (MMT) polystyrene resin, giving the solid support **83**.

After introduction of the 2,4-dinitrophenyl group at the N^1-purine position, support **84** was treated with 5-aminopentan-1-ol, affording resin **85** [64,65]. The removal of the TBDPS protecting group (**85** → **86**) allowed to carry out the bis-phosphorylation reaction on the two free OH groups, and the solid support **87** was recovered. Thereafter, pyrimidine ring degradation was performed by exposing resin **87** to ethanolic NaOH, [65] affording support **88**. This step particularly benefitted from the solid-phase approach, as the work-up procedure only required simple resin washings. Conversely, performing the same reaction in solution would have posed significant issues during the work-up, due to the high base concentration required for pyrimidine ring opening. Since the final intramolecular pyrophosphate bond formation proved challenging on solid phase, [66] this reaction was instead carried out in solution. To this aim, the treatment of support **88** with an acidic solution allowed the one-pot removal of all trimethylsilyl ethoxy (TMSE) groups on phosphates, cleavage of the benzylidene, and detachment of the crude nucleotidic material from the resin, from which pure compound **89** could be obtained after HPLC purification. The presence of the imidazole ring in compound **89** was confirmed by analysis of its ^{1}H NMR spectrum, in which the singlet at 7.45 ppm accounted for the 2-H proton. The final cyclization reaction was performed on the isopropylidene derivative **90** upon treatment with 1,1′-carbonyldimidazole (CDI) as condensing agent, thus obtaining the target compound **17**. Biological assays for compound **17** have not been yet reported.

6. Conclusions

The discovery that cADPR acts as a second messenger involved in Ca^{2+} homeostasis across various cellular systems has prompted extensive efforts to elucidate the molecular mechanisms underlying its activity. However, its instability in physiological conditions has strongly limited the knowledge of processes in which the molecule is involved. Accordingly, the design and synthesis of cADPR analogues has emerged as an active area of research at the interface of bioorganic chemistry and intracellular signaling. A simple isosteric replacement of adenine base with hypoxanthine yielded cIDPR, a stable yet non-cellular permeant analogue, which retained the biological properties of the endogenous molecule. Total synthesis has enabled fine-tuned modifications around the cIDPR scaffold, yielding analogues with enhanced stability and membrane permeability. Substitution of the “northern” ribose with ether or alkyl chains has proven effective in improving cellular uptake while maintaining Ca^{2+}-mobilizing activity. Similarly, the replacement

of the native pyrophosphate bridge with a phosphodiester group has shown that a full structural conservation is not always necessary for retaining the biological activity. Notably, the introduction of base isosteres—such as 1,2,3-triazoles and substituted imidazoles—has further expanded the chemical diversity of active mimics, suggesting a degree of structural tolerance at the receptor site. Altogether, these synthetic efforts have yielded valuable molecular tools for probing calcium signaling pathways through systematic modifications of the endogenous molecule cADPR. Furthermore, the recent discovery of Ca^{2+}-mobilizing properties of N^1-ω-alkyl and N^1-ω-hydroxyalkyl 5′-phosphate inosines offers promising, synthetically more accessible bio-probes that could complement their cyclic counterparts, offering a potential better understanding of diseases in which Ca^{2+} dyshomeostasis is involved. In conclusion, the challenging chemical modifications introduced herein may give rise to next-generation probes or therapeutic candidates, that could be explored for a better comprehension of the molecular targets of this class of compounds.

CRediT authorship contribution statement

Andrea Patrizia Falanga: Writing – original draft. **Ahmed Mahal:** Writing – original draft. **Maria Marzano:** Data curation. **Stefano D'Errico:** Conceptualization, Supervision. **Agnese Secondo:** Data curation. **Ilaria Piccialli:** Data curation. **Nicola Borbone:** Conceptualization, Writing – review & editing, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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