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Rapid Synthetic Route toward Structurally Modified Derivatives of Cyclic Adenosine 5′-Diphosphate Ribose

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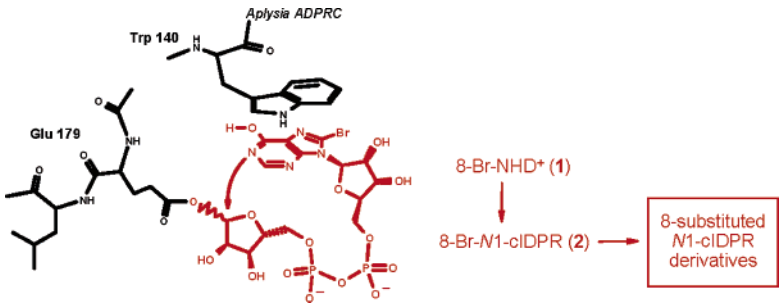

A concise synthesis of five new analogues of the second messenger cADPR (cyclic adenosine 5′-diphosphate ribose) is presented. The synthetic plan centered around the key derivative 8-Br-*N*1-cIDPR (cyclic 8-Br-inosine 5′-diphosphate ribose, **2**), which was prepared in only three steps from IMP (inosine 5′-monophosphate) via an unusual enzymatic cyclization reaction. The enhanced stability of **2** allowed for the direct modification of this cyclic dinucleotide at the 8 position, providing the unsubstituted parent *N*1-cIDPR (**4**) as well as the 8-phenyl (**5**), 8-azido (**6**), and 8-amino (**7**) *N*1-cIDPR analogues. In Jurkat T-lymphocytes, *N*1-cIDPR **4** induced Ca^{2+} release with an almost identical profile as the natural agonist cADPR, illustrating the value of this approach.

Introduction

Intracellular Ca^{2+} is a highly regulated signal, which governs or modulates a multitude of cellular processes from cell proliferation to apoptosis.[1] In both invertebrate and mammalian cell types, the release of Ca^{2+} from internal stores is mediated through three second messengers: Ins(1,4,5)P_3 (D-*myo*-inositol-1,4,5-trisphosphate), NAADP (nicotinic acid 5′-adenine dinucleotide 2′-phosphate), and cADPR (cyclic adenosine 5′-diphosphate ribose, cf. Figure 1 for structure). The interplay between these three pathways is complex, and an understanding, in particular, of the roles of NAADP and cADPR in this network is only just emerging.[2] To investigate cADPR mediated Ca^{2+} signaling, synthetic cADPR analogues that can act as potent agonists or antagonists, ideally membrane-permeant and resistant to both enzymatic and chemical hydrolysis, are required.[3,4] In addition, a close structural similarity between such analogues and the cADPR parent is desired, so that the importance of individual structural features for biological activity can be assessed.

To date, the generation of such analogues has been a difficult process. The main choice for synthetic chemists has been between the total synthesis approach of Shuto et al.,[5−7] and a chemo-enzymatic synthesis developed

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(1) Reviews on all aspects of Ca^{2+} signaling have recently appeared in Focus on Calcium. *Nature Rev. Mol. Cell Biol.* **2003**, *Vol. 4*, No. 7 and Regulators of Calcium Signaling and Cellular Function. *Curr. Mol. Med.* **2004**, *Vol. 4,* No. 4.

(2) Lee, H. C. *Curr. Mol. Med.* **2004**, *4*, 227−237.
(3) Potter, B. V. L.; Walseth, T. F. *Curr. Mol. Med.* **2004**, *4*, 303−311.
(4) Shuto, S.; Matsuda, A. *Curr. Med. Chem.* **2004**, *11*, 827−845.
(5) Shuto, S.; Shirato, M.; Sumita, Y.; Ueno, Y.; Matsuda, A. *J. Org. Chem.* **1998**, *63*, 1986−1994.
(6) Fukuoka, M.; Shuto, S.; Minakawa, N.; Ueno, Y.; Matsuda, A. *J. Org. Chem.* **2000**, *65*, 5238−5248.
(7) Shuto, S.; Fukuoka, M.; Manikowsky, A.; Ueno, Y.; Nakano, T.; Kuroda, R.; Kuroda, H.; Matsuda, A. *J. Am. Chem. Soc.* **2001**, *123*, 8750−8759.

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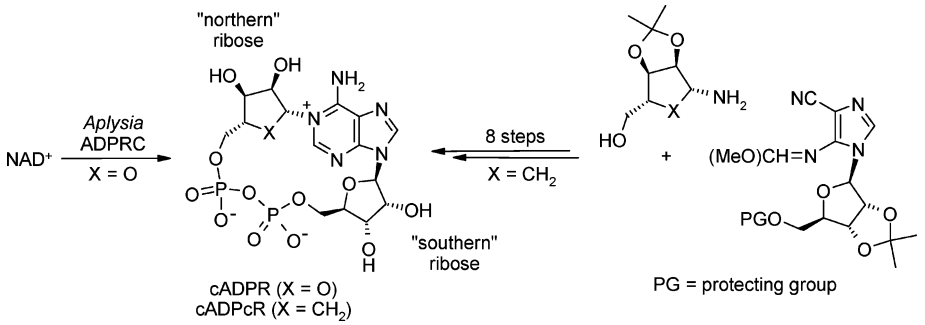


FIGURE 1. Outline of the chemo-enzymatic (left) and total synthesis (right) approach toward the preparation of cADPR[8,9] and its carbocyclic analogue cADPcR.[7] Note that the starting materials for the total synthesis of cADPcR have to be prepared from commercially available compounds in another two and five synthetic steps, respectively.[13]

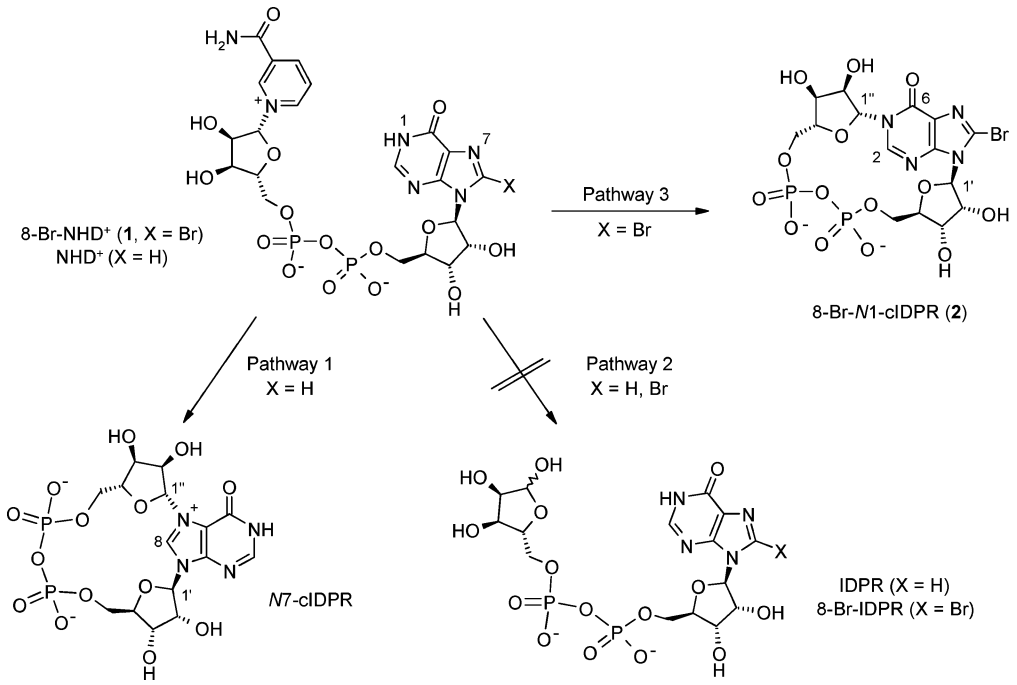


FIGURE 2. Reaction pathways for the enzymatic conversion of hypoxanthine dinucleotides.

earlier and modeled on the biosynthesis of cADPR from NAD^+ (nicotinamide adenine dinucleotide, Figure 1).[8−10] Both strategies suffer from several drawbacks. The total synthesis of cADPR analogues is a multistep process, and the chemistry chosen requires the replacement of the northern ribose with a carbocyclic surrogate (cf. Figure 1 for terminology). Therefore, this method is limited in its scope to derivatives of cADPcR (cyclic adenosine 5′-diphosphate carbocyclic ribose)[7] and cIDPcR (cyclic inosine 5′-diphosphate carbocyclic ribose)[5,6] and does not grant access to cyclic dinucleotides with an intact northern ribose, like cADPR itself or *N*1-cIDPR (cyclic inosine 5′-diphosphate ribose). In fact, it has recently been employed for the synthesis of biologically active *N*1-cIDPR analogues with an alkyl chain[11] or an ether bridge[12] in place of the northern ribose.

Numerous analogues of cADPR, modified mostly in the southern ribose and/or the nucleobase, have been prepared from their corresponding dinucleotide precursors via the chemo-enzymatic pathway, using the ADP-ribosyl cyclase (ADPRC) from *Aplysia californica* (Figure 1).[8−10] This strategy relies on the synthetic accessibility of the required NAD^+ derivatives, their recognition as substrates by the enzyme, which has a fortuitously broad substrate specificity, and their correct orientation in the active site. For example, NHD^+ (nicotinamide hypoxanthine dinucleotide), an analogue of NAD^+ with a hypoxanthine system in place of the adenine base, is indeed recognized by the *Aplysia* ADPRC. However, the enzymatically catalyzed cyclization of NHD^+ does not take place at the *N*1 but at the *N*7 position of the nucleobase (Figure 2, pathway 1), giving rise to biologically inactive *N*7-cIDPR.[14,15] Although usually shorter than the total

(8) Walseth, T. F.; Lee, H. C. *Biochim. Biophys. Acta* **1993**, *1178*, 235−242.
(9) Bailey, V. C.; Sethi, J. K.; Galione, A.; Potter, B. V. L. *Chem. Commun.* **1997**, 695−696.
(10) Ashamu, G. A.; Sethi, J. K.; Galione, A.; Potter, B. V. L. *Biochemistry* **1997**, *36*, 9509−9517.
(11) Galeone, A.; Mayol, L.; Oliviero, G.; Piccialli, G.; Varra, M. *Eur. J. Org. Chem.* **2002**, 4234−4238.
(12) Huang, L. J.; Zhao, Y. Y.; Yuan, L.; Min, J. M.; Zhang, L. H. *J. Med. Chem.* **2002**, *45*, 5340−5352.

synthesis with respect to the number of reaction steps involved, the chemo-enzymatic approach has so far not been entirely economical either. The method can provide only one analogue at a time due to the hydrolytic sensitivity of cADPR,[7] which renders any further chemical manipulation impossible once the macrocyclic framework has been assembled. However, the chemo-enzymatic approach does have the advantage that it allows the structural integrity of the northern ribose to be maintained.

Previously, we have reported in a preliminary fashion on the surprising enzymatic cyclization of 8-Br-NHD^+ (nicotinamide 8-Br-hypoxanthine dinucleotide, **1**) into 8-Br-*N*1-cIDPR (cyclic 8-Br-inosine 5′-diphosphate ribose, **2**, Figure 2, pathway 3).[16] We have now exploited the unusual chemical stability of **2** to generate a series of analogues directly from the parent compound. Herein, we describe the full synthesis of **2** in three steps from commercially available IMP (inosine 5′-monophosphate) and its subsequent functionalization at the 8 position to provide biologically active cADPR analogues. To the best of our knowledge, this is the first time that the direct modification of a cADPR analogue, identical to cADPR but for the nucleobase and with both ribose units unaltered, is reported. We believe that this strategy will considerably facilitate the preparation of further cADPR analogues as it is both fast and economical, reaching the point of structural diversification very late in the synthesis.

Results and Discussion

In agreement with earlier reports from two different groups,[14,15] we have found that incubation of an aqueous solution of NHD^+ with *Aplysia* ADPRC yields a reaction product that is fluorescent at pH >9 but nonfluorescent at lower pH. Fluorescence at basic pH is a characteristic property of *N*7-substituted nucleotides, and accordingly, the product from the enzymatic cyclization of NHD^+ had been assigned the structure of *N*7-cIDPR (Figure 2, pathway 1).[15] This structural assignment was confirmed by the HMBC spectrum of *N*7-cIDPR, which showed cross-peaks resulting from three-bond coupling of H8 with both anomeric carbons and of both anomeric protons with C8.[14] In our hands, the cyclase-catalyzed formation of *N*7-cIDPR from NHD^+ took 8 h, while under identical conditions, the cyclization of NAD^+ into nonfluorescent cADPR was complete within minutes. We reasoned that the prolonged reaction time reflected the reduced nucleophilicity at *N*7 of NHD^+ as compared to the more nucleophilic *N*1 of NAD^+.

It is well-known that nucleotides can be prompted to orient their nucleobase in a syn rather than an anti conformation through placement of a bulky substituent (e.g., bromo) at the 8 position.[17] For example, the key step in a total synthesis of cIDPcR is the intramolecular pyrophosphate bond formation from *N*1-phosphoribosyl-8-Br-IMP,[5] and it has been found that restricting the conformation of the substrate to the product-like syn form by introducing a bromo substituent at the 8 position can be useful[5] but is not critical[6] for a successful condensation reaction. In a similar vein, we wanted to explore the possibility that introduction of an 8-bromo substituent might redirect the aberrant enzymatic cyclization behavior of NHD^+. In particular, we expected 8-Br-NHD^+ (**1**, Figure 2) to preferably adopt a syn conformation, in which the hypoxanthine *N*1 is in spatial proximity to the anomeric carbon of the northern ribose. Thus, a potential cyclization at the *N*1 position would be facilitated, although this was still not necessarily anticipated given the structural differences between hypoxanthine and adenine. When **1** was subjected to the same treatment as NHD^+, a similarly slow time course was observed for the enzymatic conversion. Once the starting material had been consumed completely (RP-HPLC), the reaction product was isolated by ion-exchange chromatography and fully characterized by fluorimetry, UV, IR, FAB ms, and 1-D and 2-D ^{1}H-, ^{13}C-, and ^{31}P NMR. It is well-known that ADPRCs can act not only as cyclases but also as glycohydrolases.[18] Because of this promiscuous behavior, three possible products, i.e., 8-Br-IDPR (8-Br-inosine 5′-diphosphate ribose), 8-Br-*N*7-cIDPR, and 8-Br-*N*1-cIDPR (**2**, cf. Figure 2), had to be considered for the enzymatic conversion of **1**. Of these, only structure **2** was in comprehensive agreement with the spectroscopic data obtained.

In sharp contrast to the properties seen for *N*7-cIDPR, no fluorescence was detected in aqueous solution between pH 5 and 11 for the reaction product from the enzymatic conversion of **1**, which ruled out 8-Br-*N*7-cIDPR as a candidate molecule. The reduced nucleophilicity of the cyclic amide nitrogen of **1** raised the distinct possibility that enzyme-mediated hydrolysis of **1** into 8-Br-IDPR, at the C1″ of the nicotinamide ribose, might be preferred over cyclization in this position (Figure 2, pathway 2). However, the molecular ion of 619 in the FAB^+ mass spectrum for the reaction product was in accord with the molecular mass for one of the cyclic dinucleotides (8-Br-*N*7-cIDPR or 8-Br-*N*1-cIDPR) rather than the linear 8-Br-IDPR, which should differ by 17 mass units for the additional hydroxyl group. Crucially, the gHMBC spectrum of the reaction product showed cross-peaks resulting from three-bond coupling between the single heteroaromatic proton at position 2 (δ 8.70) and the anomeric carbon of the northern ribose (δ 92.0), and the anomeric proton H1″ (δ 5.85) with carbons C2 and C6 (δ 144.25 and 156.33) of the nucleobase (Figure 3). These analytical data strongly suggested the presence of a bond from *N*1 to C1″, as results from ADPRC-catalyzed cyclization of **1** at the *N*1 position (Figure 2, pathway 3). Therefore, we concluded that **1** had indeed been enzymatically converted into 8-Br-*N*1-cIDPR **2**. This structural assignment was further supported by the NOESY and ROESY spectra of **2**, which indicated a close spatial proximity of the heteroaromatic proton and H2″, H3″, H4″, and H5″. Interestingly, none of the two doublets in the ^{31}P NMR spectrum of **2** showed a pH dependent shift between pH 6.8 and 10.9 (cf. Supporting Information). In the case of

(13) Hutchinson, E. J.; Taylor, B. F.; Blackburn, G. M. *Chem. Commun.* **1997**, 1859−1860.
(14) Zhang, F. J.; Sih, C. J. *Tetrahedron Lett.* **1995**, *36*, 9289−9292.
(15) Graeff, R. M.; Walseth, T. F.; Hill, H. K.; Lee, H. C. *Biochemistry* **1996**, *35*, 379−386.
(16) Wagner, G. K.; Black, S.; Guse, A. H.; Potter, B. V. L. *Chem. Commun.* **2003**, 1944−1945.
(17) Saenger, W. *Principles of Nucleic Acid Structure*; Springer-Verlag: Berlin, 1984.
(18) Schuber, F.; Lund, F. E. *Curr. Mol. Med.* **2004**, *4*, 249−261.

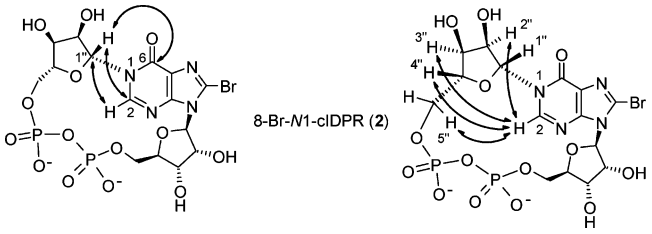


FIGURE 3. $^{1}H-^{13}C$ connectivities based on the gHMBC spectrum (left) of **2**, and proton/proton interactions derived from the NOESY spectrum (right) support the structural assignment, in particular, the presence of the *N*1/C1″ bond.

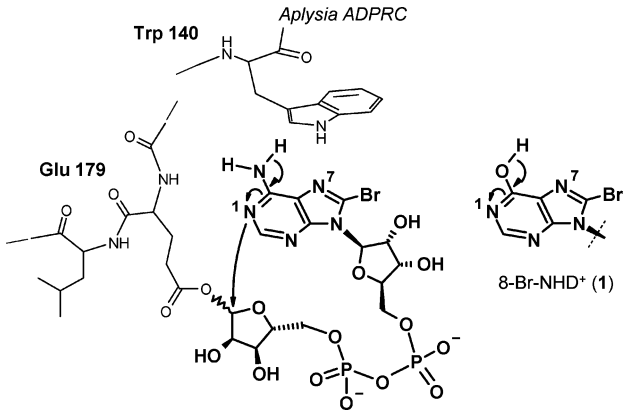


FIGURE 4. Schematic representation of the *Aplysia* ADPRC active site, with ADPR (from NAD^+) covalently bound to Glu179, prior to cyclization at *N*1, and the alignment of the nucleobase in parallel to the indole ring of Trp140 (substrate and numbering in bold, drawing based on ref 20). The analogous orientation of **1** in its enol form is indicated.

cADPR, the pH dependency of the ^{31}P NMR chemical shift value for one of the two phosphorus atoms has been attributed to the two possible protonation states of the adenine base (pK_a 8.3, cf. Figure 5).[19] As no such protonation equilibrium exists for the hypoxanthine derivative **2**, the absence of any pH dependency in the ^{31}P NMR spectrum of **2** is also in agreement with the structural assignment.

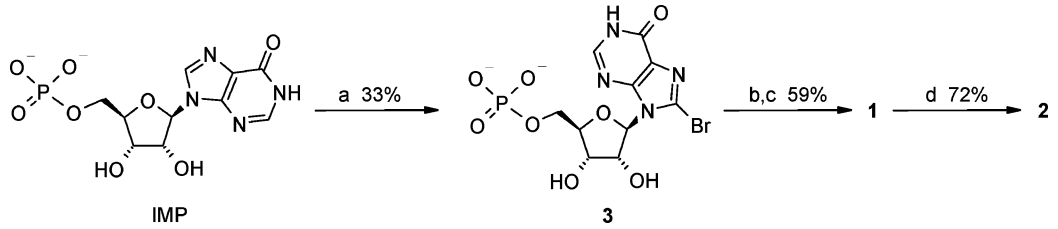
There is mounting evidence that all three types of reactions catalyzed by *Aplysia* ADPRC (i.e., *N*1- and *N*7-cyclization and hydrolysis) proceed via a common covalent intermediate.[20] According to this mechanism, at first a bond is formed from the enzyme's catalytic base Glu179 to C1″ of the dinucleotide substrate (Figure 4). In the case of the cyclization reaction, the covalently bound substrate is then aligned in a hairpin conformation that moves the nucleobase into an appropriate position for one of the ring nitrogens (*N*1 or *N*7) to cleave the enzyme–substrate bond and then subsequent release of the cyclic product.

The residue Trp140 appears to be instrumental in positioning the nucleobase correctly for cyclization.[21] However, it is evident from the different cyclization products obtained for NAD^+ (i.e., cADPR) and NHD^+ (i.e., *N*7-cIDPR) that Trp140 arranges structurally different nucleobases in different ways. Our finding that the *Aplysia* ADPRC converts **1** into **2** suggests that the enzyme orients 8-Br-NHD^+ in its active site in a manner similar to NAD^+, but clearly different from NHD^+. This result probably reflects different conformations about the glycosidic bond in both hypoxanthine derivatives (NHD^+ anti, 8-Br-NHD^+ syn) as well as different electronic properties, accounting for a different spatial orientation of the charge-transfer complexes with the indole ring of Trp140. The parallel reaction courses for the conversion of NAD^+ and **1** also imply that the enzyme is capable of stabilizing **1** in its enol form, although hypoxanthine bases normally exist predominantly in the keto form in aqueous solution.[22] However, only the enol tautomer of **1** allows a mechanistic explanation for the *N*1 cyclization of **1** that mirrors the conversion of NAD^+ into cADPR (Figure 4).

Initial synthetic studies on the conversion of **1** into **2** were carried out on a specially commissioned large batch of **1** prepared commercially.[23] Prompted by the promising bioactivity of **2**, which is an agonist of Ca^{2+} release in intact Jurkat T-lymphocytes,[16] we intensified our own efforts to develop a synthetic protocol that would grant practical access to **1** and **2**, ideally on a 100 mg scale. A first synthetic route toward **1** had been devised to provide the target dinucleotide via a bromination/phosphorylation/deamination sequence starting from adenosine. However, the selective $POCl_3$-mediated phosphorylation[24] of 8-Br adenosine at the 5′-OH promoted a concomitant bromine/chlorine exchange at the 8 position of the nucleobase, similar to what had been observed by another group under alternative phosphorylation conditions.[12,25] To avoid this complication, we modified our initial strategy. The new route obviates the need for both the phosphorylation and the deamination step, facilitating considerably the preparation of **1**. Thus, dinucleotide **1** was synthesized in three steps from commercially available IMP (Scheme 1). To obtain 8-Br-IMP (**3**) directly from IMP (disodium salt form), we successfully adapted the conditions of Ijzerman et al. for the bromination of fully protected inosine[26] to the conversion of unprotected IMP. As had already been observed in the case of inosine,[26] the direct bromination of the hypoxanthine base in IMP required a prolonged reaction time as compared to the corresponding adenine system.[27] The reaction could not be driven to completion even after 5 days when decomposition products were observed by RP-HPLC and the reaction was stopped. The target compound was separated from both residual starting material and decomposition products by ion-pair chromatography,[27] providing **3** (triethylammonium salt form) in 33% yield.

(19) Schnackerz, K. D.; Vu, C. Q.; Gani, D.; AlvarezGonzalez, R.; Jacobson, M. K. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 581–586.
(20) Love, M. L.; Szebenyi, D. M. E.; Kriksunov, I. A.; Thiel, D. J.; Munshi, C.; Graeff, R.; Lee, H. C.; Hao, Q. *Structure* **2004**, *12*, 477–486.
(21) Yamamoto-Katayama, S.; Ariyoshi, M.; Ishihara, K.; Hirano, T.; Jingami, H.; Morikawa, K. *J. Mol. Biol.* **2002**, *316*, 711–723.
(22) Chenon, M. T.; Pugmire, R. J.; Grant, D. M.; Panzica, R. P.; Townsend, L. B. *J. Am. Chem. Soc.* **1975**, *97*, 4636–4642.
(23) BIOLOG GmbH, Bremen, Germany.
(24) Ikemoto, T.; Haze, A.; Hatano, H.; Kitamoto, Y.; Ishida, M.; Nara, K. *Chem. Pharm. Bull.* **1995**, *43*, 210–215.
(25) Huang, L. J.; Zhao, Y. Y.; Yuan, L.; Min, J. M.; Zhang, L. H. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 887–889.
(26) Roelen, H.; Veldman, N.; Spek, A. L.; Kunzel, J. V. D.; Mathot, R. A. A.; Ijzerman, A. P. *J. Med. Chem.* **1996**, *39*, 1463–1471.
(27) Wlassoff, W. A.; Dobrikov, M. I.; Safronov, I. V.; Dudko, R. Y.; Bogachev, V. S.; Kandaurova, V. V.; Shishkin, G. V.; Dymshits, G. M.; Lavrik, O. I. *Bioconjugate Chem.* **1995**, *6*, 352–360.

SCHEME 1. Preparation of Key Intermediates 1 and 2[a,b]

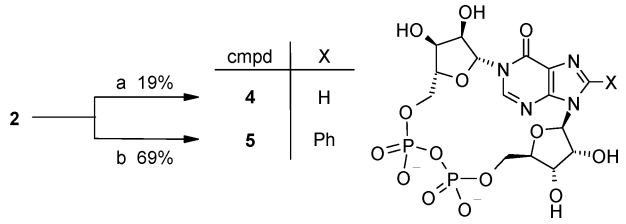


[a] Reagents and conditions: (a) aq Br_2, 10% aq Na_2HPO_4, rt; (b) morpholine, dipyridyl disulfide, PPh_3, DMSO, rt; (c) β-NMN, $MnCl_2$, $MgSO_4$, dry formamide, rt; (d) *A. californica* ADP-ribosyl cyclase, HEPES buffer (pH 7.4), rt. [b] IMP was used as the disodium salt, and **1**−**3** were isolated as triethylammonium salts.

In the following step, we sought to apply the protocol of Lee et al. for Lewis acid-catalyzed pyrophosphate bond formation[28] to the synthesis of dinucleotide **1** from mononucleotide **3** and β-NMN (β-nicotinamide mononucleotide). This approach required the activation of monophosphate **3** in the form of the corresponding phosphoromorpholidate prior to the actual coupling reaction.[29] Therefore, **3** was converted into 8-Br-IMP morpholidate through a dipyridyl disulfide/triphenylphosphine-mediated oxidation−reduction condensation with morpholine.[30] Notably, we were able to employ **3** in its triethylammonium salt form in this transformation without any detrimental effect, thus circumnavigating the time-consuming preparation of the free acid. The latter had so far been regarded as the appropriate starting point for this type of reaction[31] putatively because of the mechanism suggested by Mukaiyama and co-workers.[32] ^{31}P NMR spectroscopy was used to monitor the formation of the phosphoromorpholidate of **3**, which was indicated by the appearance of a new singlet at 5.8 ppm (d_6-DMSO). After 1 h, the reaction had gone to completion, and the 8-Br-IMP morpholidate was precipitated in the sodium salt form. Without further purification, the crude product was coupled to β-NMN in the presence of $MnCl_2 \times 4H_2O$ in formamide to give **1** in 59% yield over both steps after purification. The synthesis of key intermediate **2** was completed by the enzymatically catalyzed cyclization of the linear dinucleotide **1** as previously described.[16,33]

In aqueous medium, cADPR is prone to rapid hydrolysis because of the labile nature of the *N*1-glycosidic linkage,[7] which also precludes the use of cADPR analogues such as 8-Br-cADPR as synthetic intermediates. During our initial studies on **2**, we had observed a remarkable resistance of this cADPR congener toward chemical hydrolysis at pH 7.4 even at elevated temperatures.[16] In structural terms, the enhanced chemical stability of **2** can be explained by the replacement of the labile, *N*-substituted amidinium partial structure in cADPR with a more stable, cyclic tertiary amide in **2**. To assess the potential of **2** as a synthetic intermediate, we first examined its stability in aqueous solution at acidic and basic pH. After 24 h in either 0.1 M HCl (pH 1) or 0.2 M NaOH (pH 12) at room temperature, no decomposition of **2** was detected by HPLC. Kept at 60 °C in the same media, **2** did start to disintegrate, with a $t_{1/2}$ of 50 h at pH 1 and a $t_{1/2}$ of 15 h at pH 12. Therefore, like those hydrolysis-resistant analogues of cADPR whose improved stability results from the replacement of the northern ribose with a carbocycle,[7] **2** appears to be more stable under acidic than under basic conditions. Both acid- and base-catalyzed degradation led to the same major decomposition product that by HPLC eluted ca. 1.5 min earlier (t_r 9.9 min, λ_{max} 254 nm) than **2**. Unfortunately, all efforts to isolate this main decomposition product were unsuccessful, although it can be reasoned that just as the hydrolysis of cADPR provides the linear ADPR (adenosine 5′-diphosphate ribose),[8] **2** is predominantly hydrolyzed into 8-Br-IDPR. The intrinsic chemical stability of **2**, together with the fact that the bromine substituent in the 8 position in principle allows for a variety of structural modifications, prompted us to explore whether the direct chemical manipulation of **2**, possibly under drastic conditions, could be achieved without concomitant damage to the macrocycle. On a practical level, the stability of **2** also permitted the convenient storage of aliquots in aqueous solution at 4 °C without degradation, both for subsequent chemical reaction and biological evaluation.

SCHEME 2. Direct Synthesis of Target Compounds 4 and 5 from Intermediate 2[a,b]



[a] Reagents and conditions: (a) Pd−C 10%, H_2, 0.5 M aq $NaHCO_3$/EtOH, rt; (b) $Pd(PPh_3)_4$, phenylboronic acid, Na_2CO_3, 50% aq MeOH, reflux. [b] **4** and **5** were isolated as triethylammonium salts.

First, we prepared the parent *N*1-cIDPR **4** in 19% yield from **2** by catalytic hydrogenation under the mild conditions reported by Shuto (Scheme 2).[5] As the RP-HPLC trace of the reaction mixture did not indicate the formation of any side products, the low yield is most likely due to a loss of material during the sequence of chromato-

(28) Lee, J.; Churchil, H.; Choi, W. B.; Lynch, J. E.; Roberts, F. E.; Volante, R. P.; Reider, P. J. *Chem. Commun.* **1999**, 729−730.
(29) Moffatt, J. G.; Khorana, H. G. *J. Am. Chem. Soc.* **1961**, *83*, 649−658.
(30) Mukaiyama, T.; Hashimoto, M. *Bull. Chem. Soc. Jpn.* **1971**, *44*, 2284.
(31) Prabahar, K. J.; Cole, T. D.; Ferris, J. P. *J. Am. Chem. Soc.* **1994**, *116*, 10914−10920. Imai, J.; Torrence, P. F. *J. Org. Chem.* **1985**, *50*, 1418−1426.
(32) Mukaiyama, T.; Matsueda, R.; Suzuki, M. *Tetrahedron Lett.* **1970**, 1901−1904.
(33) We find that the turnover rate for this enzymatic conversion considerably depends on the individual batch of commercially available *Aplysia californica* ADPRC, with reaction times varying from 8 to 48 h.

graphic separations employed for the purification of **4**. The successful replacement of the bromine substituent with a hydrogen atom is indicated by the appearance of a new singlet at 8.09 ppm in the ^{1}H NMR of **4**. As expected, the spatial proximity of the aromatic proton in position 8 to the southern ribose leads to a strong H8/H1′ correlation in the NOESY spectrum of **4**. The corresponding NOE had also been observed for the structurally related cADPcR.[7]

*N*1-cIDPR **4** is identical in structure to cADPR but for the hypoxanthine nucleobase. However, unlike cADPR, **4** cannot be synthesized enzymatically from its linear precursor dinucleotide, as the cyclization of NHD$^+$ by the *Aplysia* ADPRC exclusively yields *N*7-cIDPR.[14,15] The total synthesis of **4** had been attempted in the past,[34] but while the preparation of an important synthetic intermediate toward that goal has been published,[34] to the best of our knowledge this early work has never been followed up with the completed synthesis of *N*1-cIDPR. Therefore, we believe that this is the first time the successful preparation of *N*1-cIDPR **4** is reported. Synthetic access to **4** allowed us to assess the role of the cIDPR scaffold for cADPR (ant)agonism in a cell-based model of Ca^{2+} release (cf. Biological Results). Knowledge of the bioactivity of **4** is highly significant as a starting point for the development of a structure activity relationship (SAR) for cIDPR analogues, which are modified in other parts of the molecule.

One such analogue, 8-phenyl-*N*1-cIDPR **5**, was prepared by cross-coupling of **2** and phenylboronic acid in 69% yield under Suzuki conditions adopted for the conversion of nucleosides by Amann and Wagenknecht (Scheme 2).[35] Remarkably, neither the hydroxyl groups nor the phosphates in **2** interfered with the coupling reaction that went to completion after heating to reflux for just 4 h. While unprotected nucleosides have been coupled with arylboronic acids before,[36] we believe that this is the first example where a nucleotide with unprotected hydroxyl and phosphate functionalities has been successfully employed as a substrate for a Suzuki coupling reaction.

Progress of the reaction was accompanied by a pronounced shift in RP-HPLC retention times (**2** 11.32 min and **5** 20.01 min) and UV absorbance maxima (**2** 256 nm and **5** 274 nm). The successful installation of the phenyl moiety in the 8 position was confirmed by an additional multiplet (δ 7.48−7.60 ppm) in the ^{1}H NMR spectrum of **5** and the downfield shift for the ^{13}C NMR resonance of C8 (**2** 128.6 ppm and **5** 153.3 ppm). The hypoxanthine derivative **5** represents the first example of an 8-aryl substituted cADPR analogue. Its NOESY spectrum indicates a spatial proximity of H1′ and the protons in ortho position of the phenyl ring but not of H2′ and the two meta protons, suggesting that the phenyl ring and the hypoxanthine system adopt a noncoplanar orientation.

Crucially for the applicability of our approach, the core cyclic structure in **2** remained intact during the course of the reaction despite the harsh conditions. The integrity of the cIDPR ring structure in **5** (and indeed in the other cIDPR analogues **4**, **6**, and **7** described in this paper) is supported by four main analytical parameters: first, the result for **5** from FAB mass spectroscopy (*m/z* 617) is in agreement with the molecular mass of the assigned cyclic structure. In contrast, the molecular ion for the most likely decomposition product of **5**, the linear dinucleotide 8-phenyl-IDPR resulting from hydrolytic cleavage of the *N*1-glycosidic bond, would be expected at *m/z* 635. Second, hydrolysis of the *N*1/C1″ linkage in **5** should result in additional ^{1}H NMR signals for the anomeric proton of the northern ribose (δ ca. 5−6 ppm), but no such additional peaks are found in the ^{1}H NMR spectrum of **5**. Third, several cross-peaks observed in the gHMBC spectrum of **5** can be ascribed directly to ^{1}H−^{13}C coupling along the intact *N*1/C1″ bond (Table 1). Finally, the phosphorus−phosphorus coupling constant ($J_{\alpha\beta}$) for the pyrophosphate group in **5** was recorded at 14.1 Hz, similar to a $J_{\alpha\beta}$ of 13.6 Hz reported for cADPR.[19] At the same time, phosphorus−phosphorus coupling in the linear ADPR occurs with a much higher frequency ($J_{\alpha\beta}$ 21.9 Hz), and it has been argued that this difference in $J_{\alpha\beta}$ reflects the conformational restrictions imposed on the pyrophosphate group in the cylic molecules.[19] Therefore, the value for $J_{\alpha\beta}$ serves as the fourth marker to distinguish linear and cyclic dinucleotides.

TABLE 1. Selected ^{1}H−^{13}C Connectivities from the gHMBC Spectrum of 5

	C2	C6	C1
H-2	*H*−*C*	*H*−C−N−*C*	*H*−C−N−*C*
H-1	*H*−C−N−*C*	n.d.[a]	*H*−*C*

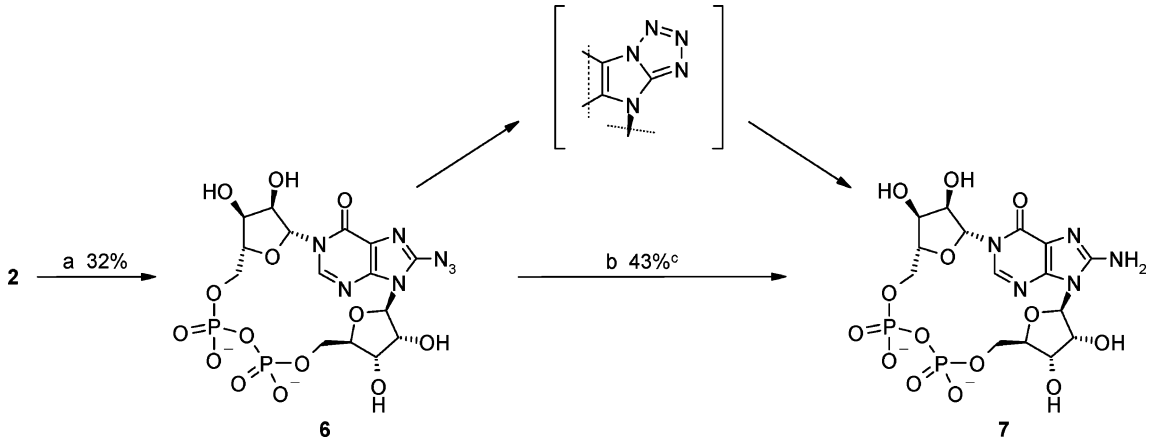
[a] Not detected.

To further explore the versatility of **2** as a synthetic intermediate, we next turned our attention to the conversion of **2** into 8-azido-*N*1-cIDPR **6** (Scheme 3). Azidonucleotides have been widely used for biological as well as chemical applications;[37] therefore, **6** was conceived as both a potentially valuable biological tool in its own right and a synthetic precursor for 8-amino-*N*1-cIDPR **7**. 8-Amino-cADPR is the most potent cADPR receptor antagonist known to date,[3] and access to its hypoxanthine congener **7** would help to elucidate the structural determinants of agonism/antagonism at the cADPR receptor. In the initial experimental setup (NaN_3, DMF, 85 °C), the starting material, **2**, in its triethylammonium salt form was consumed within 20 h under conditions routinely employed for the bromo/azido replacement reaction.[38] While by RP-HPLC only one major peak at 11.65 min was detected for the reaction product, the corresponding UV trace (λ_{max} at 258 and 277 nm) suggested the presence of at least two components. The ^{1}H NMR spectrum collected after purification pointed toward a mixture of isomers with the respective anomeric protons in a highly different electronic environment (δ H1′ 5.81 vs 6.08 ppm). At this stage, we surmised that **6** had indeed been formed because of the characteristic bathochromic shift observed in the UV spectrum but that this primary product had partly been cyclized in situ into the corresponding tetrazolo derivative (Scheme 3).

This notion was based on previous investigations into such azido/tetrazolo equilibria, which had also estab-

(34) Aritomo, K.; Urashima, C.; Wada, T.; Sekine, M. *Nucleosides Nucleotides* **1996**, *15*, 1−16.
(35) Amann, N.; Wagenknecht, H. A. *Synlett* **2002**, 687−691.
(36) Western, E. C.; Daft, J. R.; Johnson, E. M.; Gannett, P. M.; Shaughnessy, K. H. *J. Org. Chem.* **2003**, *68*, 6767−6774.
(37) Pathak, T. *Chem. Rev.* **2002**, *102*, 1623−1667.
(38) Buenger, G. S.; Nair, V. *Synthesis* **1990**, 962−966.

SCHEME 3. Conversion of 2 into 7 via the 8-Azido Analogue 6 and/or Its Putative Tetrazolo Isomer[a,b]



[a] Reagents and conditions: (a) NaN_3, DMF, 85 °C; (b) dithiothreitol, 0.05 M TEAB (pH 8.5), rt. [b] **6** and **7** were isolated as triethylammonium salts. [c] Yield over both steps.

TABLE 2. Properties Reported for the Azido/Tetrazolo Equilibrium[39] and Experimental Data Collected for 6 and Its Putative Tetrazolo Isomer

	azido[39]	tetrazolo[39]	**6**	putative tetrazolo isomer
IR (cm^{-1})	2100−2200	1000−1100	2164	n.a.[a]
^1H NMR (ppm)	upfield shift	downfield shift	5.81 (H-1), 8.81 (H-2)	6.08 (H-1), 8.88 (H-2)

[a] Not available.

lished characteristically different ^1H NMR resonances and IR bands for corresponding azido and tetrazolo isomers (cf. Table 2).[39] To substantiate our structural assignment, we set out to obtain pure samples of **6** and its putative tetrazolo isomer for collection of unambiguous ^1H NMR and IR spectra.

As our attempts to separate the two isomers chromatographically had not met with any success, we directed our efforts at the modification of reaction conditions to obtain pure samples of both isomers. Neither changes in solvent (dry vs wet DMF) nor in reaction temperature (60−120°) significantly altered the preference for one isomer or the other. It was, however, observed that the ratio of both isomers was affected by the addition of triethylamine to an aqueous solution of the mixture. To avoid the possibility of base-induced conversion of **6** into the supposed tetrazolo isomer during the course of the bromo/azido substitution, **6** was used not in its triethylammonium salt form but as the free acid during the next attempt. Under otherwise identical conditions, this sole alteration led to the formation of only one single product whose IR spectrum did indeed display the bands characteristic for the azido group (Table 2). While this result, taken together with ^1H NMR data, corroborates the structural assignment for **6**, we can only speculate at this stage about the nature of the second isomer, which we have not managed to isolate in pure form. While those resonances seen in the ^1H NMR of the isomeric mixture that can now clearly be attributed to the nonazido isomer are in very good agreement with the suggested fused tetrazolo structure, it is interesting to note that in previous accounts on 8-azido-cADPR,[8] 8-azido-cADPcR,[40] and 8-azido-cIDPRE,[41] the formation of the corresponding fused tetrazolo isomer through cyclization at *N*7 under standard conditions has never been described. Moreover, as all previous accounts of the azido/tetrazolo equilibrium in the adenosine series concern 2- and 6-azido adenosines, further experimental evidence is clearly required before the question can be answered conclusively as to whether 8-azido adenosines/inosines show an analogous behavior and whether the elusive isomer of **6** is in fact the fused tetrazole, as we suspect.

However, further support for our tentative structural assignment came from the observation that under mild reducing conditions,[42] the mixture of **6** and its putative tetrazolo isomer was converted into one single product, 8-amino-*N*1-cIDPR **7** (Scheme 3). Isolation and purification of the intermediate **6** was indeed not required for the preparation of **7** from **2**, and the overall yield of 43% for **7** under one-pot conditions exceeded the isolated yield of 32% for intermediate **6**. The lower isolated yield for **6** is probably due to retention of this highly polar azidonucleotide on the ion-exchange resin during chromatographic purification. The successful reduction of the azido group of **6** to the amino group of **7** was most clearly visible from the hypsochromic shift in the UV spectrum (λ_{max} **6** 274 nm and **7** 261 nm) and from the downfield shift of the ^{13}C NMR resonance of C8 (**6** 146.69 ppm and **7** 153.15 ppm). 8-Amino-*N*1-cIDPR **7** is the hypoxanthine analogue of the most potent antagonist of cADPR to date, 8-amino-cADPR. To clarify whether **7** displays the agonist profile

(39) Temple, C.; Kussner, C. L.; Montgomery, J. A. *J. Org. Chem.* **1966**, *31*, 2210−2215. Temple, C.; Thorpe, M. C.; Coburn, W. C.; Montgomery, J. A. *J. Org. Chem.* **1966**, *31*, 935−938.

(40) Shuto, S.; Fukuoka, M.; Kudoh, T.; Garnham, C.; Galione, A.; Potter, B. V. L.; Matsuda, A. *J. Med. Chem.* **2003**, *46*, 4741−4749.

(41) Gu, X. F.; Yang, Z.; Zhang, L.; Kunerth, S.; Fliegert, R.; Weber, K.; Guse, A. H. *J. Med. Chem.* **2004**, *47*, 5674−5682.

(42) Cartwright, I. L.; Hutchinson, D. W.; Armstrong, V. W. *Nucleic Acids Res.* **1976**, *3*, 2331−2339.

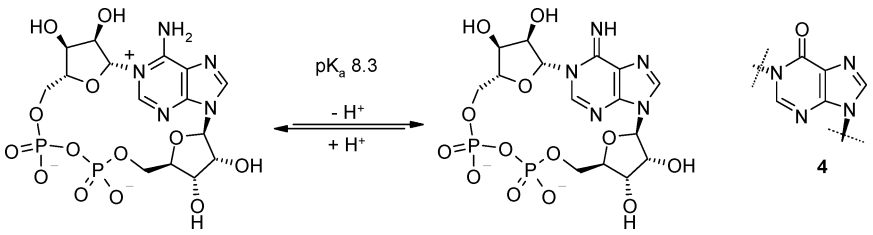


FIGURE 5. Amino/imino equilibrium for cADPR. Also indicated is the nucleobase fragment of *N*1-cIDPR **4** for direct comparison.

of its parent **2** or acts as an antagonist like 8-amino-cADPR will significantly improve our understanding of the molecular requirements that determine cADPR agonism/antagonism. To this end, biological studies using **7** as well as the other new biological tools described herein are currently underway. While these results will be reported comprehensively in due course, some preliminary biological data are already available.

Biological Results. Under physiological conditions, cADPR exists in an amino/imino equilibrium (Figure 5), the relevance of which for biological activity has been unclear.[43] *N*1-cIDPR **4** is now the closest structural analogue to cADPR available. As **4** can be seen as a congener of cADPR stabilized in the imino form, its effect on cADPR-mediated Ca^{2+} release was expected to provide clues about the contribution of the imino form of cADPR to bioactivity.

Since 8-Br-*N*1-cIDPR (**2**) is biologically active in intact Jurkat T-lymphocytes,[16] *N*1-cIDPR **4** was tested for its biological effects in the same cellular system. In contrast to **2**, its parent **4** is not membrane-permeant, due to its more hydrophilic character. Thus, permeabilized cells were used as the test system of choice as described.[43−45] Addition of **4** resulted in a rapid release of Ca^{2+} ions from intracellular stores previously loaded with Ca^{2+} by endoplasmic/sarcoplasmic Ca^{2+} ATPases (Figure 6A). Ca^{2+} release induced by **4** at 100 μM was almost indistinguishable from Ca^{2+} release induced by naturally occurring cADPR, both in its kinetic profile and amplitude (Figure 6A). The concentration−response relationships of **4** and cADPR also were almost identical (Figure 6B), indicating that **4** is a novel synthetic mimic of the natural second messenger cADPR. As **4** and its derivatives are expected to be very stable biologically, they can be useful as molecular tools. Moreover, pursuit of an analogue program based around **4** will likely yield interesting bioactivity. Regarding the development of SAR for cADPR agonism in T cells, the discovery of near identical biological profiles for cADPR and **4** is of obvious significance. Another cyclic inosine compound, *N*7-cIDPR, which is formed from NHD$^+$ by the *Aplysia* ADPRC, had been found to be inactive in a model of Ca^{2+} release in sea urchin egg homogenates.[15] Therefore, it can be concluded that a global change of the orientation of the (hypoxanthine) base within the cyclic structure is not tolerated without loss of activity. The agonist profile of *N*1-cIDPR **4** now shows that replacement of the amino/

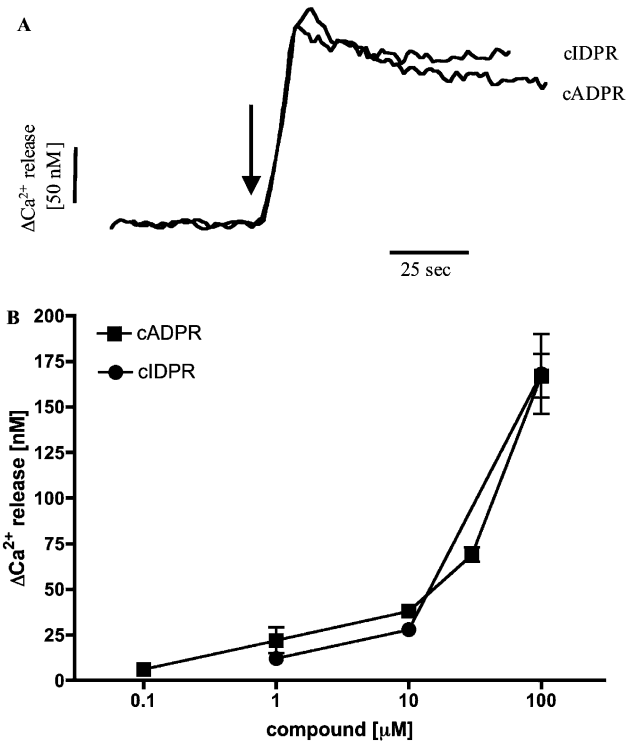


FIGURE 6. Effect of *N*1-cIDPR (**4**) on Ca^{2+} release in permeabilized Jurkat T cells. Jurkat T cells were permeabilized and transferred into an intracellular buffer as described in the General Experimental Procedures. Ca^{2+} pools were loaded by the addition of ATP and an ATP regenerating system consisting of creatine kinase and creatine phosphate. Fura2/free acid was present to monitor the [Ca^{2+}] by ratiometric fluorescence. Then, **4** or cADPR was added (panel A: arrow, 100 μM). The concentration−response relationship is presented in panel B; data are mean ± SEM (n = 3−6).

imino moiety at the 6 position of cADPR with a keto group alone does not interfere with binding of the ligand to its receptor. This result indicates that, at least in Jurkat T-lymphocytes, cADPR itself may be active predominantly in its imino form.

Conclusion

In summary, we have presented a novel and unexpected synthetic route to 8-bromo-*N*1-cIDPR **2**, a stable and robust intermediate that is itself biologically active and can be directly converted into other 8-substituted cADPR analogues. We demonstrate that *N*1-cIDPR **4** is as potent as cADPR in mobilizing Ca^{2+} in permeabilized T-lymphocytes, thus illustrating the potential promise afforded by further structural modification of *N*1-cIDPR. The activity of **4** also suggests that the imino,

(43) Guse, A. H.; Cakir-Kiefer, C.; Fukuoka, M.; Shuto, S.; Weber, K.; Bailey, V. C.; Matsuda, A.; Mayr, G. W.; Oppenheimer, N.; Schuber, F.; Potter, B. V. L. *Biochemistry* **2002**, *41*, 6744−6751.

(44) Guse, A. H.; Roth, E.; Emmrich, F. *Biochem. J.* **1993**, *291*, 447−451.

(45) Guse, A. H.; da Silva, C. P.; Emmrich, F.; Ashamu, G. A.; Potter, B. V. L.; Mayr, G. W. *J. Immunol.* **1995**, *155*, 3353−3359.

not the amino, form of cADPR may be the active species biochemically since **4** is the closest possible analogue of cADPR in the crucial *N*1-ribosyl area yet synthesized. It is anticipated that **4**, with its amide linkage at the normal site of both biological and chemical breakdown, will be highly stable biologically and that it and analogues thereof may become useful tools to unravel the cADPR-mediated pathway of cellular signaling.

Experimental Procedures

Nicotinamide 8-Br-Hypoxanthine 5′-Dinucleotide (8-Br-NHD$^+$, 1). A solution of **3** (60 mg, 114 μmol) in dry DMSO (2 mL) was coevaporated with dry DMF (4 mL). The procedure was repeated four times, and the residue was dissolved in dry DMSO (0.5 mL). Distilled morpholine (64 μL, 733 μmol, dried over KOH) was added, and the mixture was stirred for 5 min at room temperature. To the resulting slurry, dipyridyl disulfide (77 mg, 351 μmol) was added, and stirring continued for another 5 min at room temperature. To the clear, slightly yellow solution thoroughly ground triphenylphosphine (92 mg, 351 μmol) was added, and the reaction was stirred for 45 min at room temperature. Initially, a deep yellow precipitate was formed, which disappeared after 5 min. To monitor the reaction, a small trace was diluted with deuterated DMSO for collection of a ^{31}P NMR spectrum. Upon formation of the morpholidate and simultaneous oxidation of PPh_3, two singlets at 5.84 ppm (8-Br-IMP morpholidate) and 26.19 ($OPPh_3$) appeared. Once the starting material had been consumed completely, 0.1 M NaI in acetone was added dropwise until a light yellow precipitate formed. The precipitate was filtered off, washed with acetone until the yellow color had gone, and used in the next step without further purification. A small trace was retained for NMR spectroscopic characterization: ^{1}H NMR (D_2O) δ 8.10 (s, 1H), 6.03 (d, 1H, 4.2 Hz), 5.27 (t, 1H, 5.0 Hz), 4.69 (t, 1H, 5.4/6.4 Hz), 4.23−4.17 (m, 1H), 4.09−3.92 (m, 2H), 3.47 (s, 4H), 2.83 (s, 4H); ^{31}P NMR (D_2O) δ 8.29 (s). The crude 8-Br-IMP morpholidate, β-NMN (35 mg, 105 μmol), and anhydrous $MgSO_4$ (25 mg, 200 μmol) were suspended in 0.2 M $MnCl_2$ in formamide (0.74 mL, 148 μmol), which had been dried over 4 Å MS for 3 days. Under an atmosphere of argon, the reaction was stirred for 60 h at room temperature. Crude **3** was precipitated through dropwise addition of MeCN. The supernatant was removed with a Pasteur pipet. The gum-like residue was dissolved in a small volume of 0.05 M TEAB buffer (pH 6.1) and purified using CSA.[46] Fractions 30−33 were combined and evaporated. The excess TEAB was destroyed by coevaporation of the solid residue with MeOH (5×) to give 50 mg (59% over both steps) of **1** in the triethylammonium form as a glassy residue, and the material was lyophilized for storage: UV (H_2O, pH 5.6) λ_{max} 254 nm (ϵ 14700), 273 (sh); HPLC 4.35 min (λ_{max} 255 nm); ^{1}H NMR (D_2O) δ 9.32 (s, 1H), 9.14 (d, 1H, 5.9 Hz), 8.88 (d, 1H, 8.2 Hz), 8.23 (t, 1H, 6.2/7.7 Hz), 8.14 (s, 1H), 6.04 (d, 1H, 5.2 Hz), 5.96 (d, 1H, 5.2 Hz), 5.18 (t, 1H, 5.4/5.7 Hz), 4.63−4.59 (m, 1H), 4.45−4.36 (m, 3H), 4.29−4.15 (m, 5H), 3.16 (t, 6H, 7.2 Hz), 1.23 (t, 9H, 7.2 Hz); ^{13}C NMR (D_2O) δ 165.25, 157.03, 149.85, 146.00, 145.95, 142.48, 139.69, 133.87, 128.78, 99.95, 90.13, 87.00 (d, 8.4 Hz), 83.51 (d, 8.4 Hz), 77.51, 71.34, 70.54, 69.61, 46.54, 8.12; ^{31}P NMR (D_2O) δ −10.73 (bs), −10.95 (bs); HRMS (FAB, negative) calcd. for $C_{21}H_{24}BrN_6O_{15}P_2$ 740.9958, 742.9937 (monoanion), found 740.9969, 742.9956.

Cyclic 8-Bromo-inosine 5′-Diphosphate Ribose (8-Br-*N*1-cIDPR, 2). An aqueous solution (20 mL) of **1** (43 μmol) was diluted to 90 mL with 25 mM HEPES buffer (pH 7.4). The solution was incubated with ADP-ribosyl cyclase from *A. californica* (130 μL) at room temperature, and progress of the reaction was monitored by HPLC. Once the peak for the starting material had disappeared,[33] the reaction (κ = 550 μS/cm) was diluted with H_2O to bring the conductivity to 80 μS/cm. The aqueous solution was purified using CSB,[46] and fractions 43−47 were combined and evaporated. The excess TEAB was destroyed by coevaporation of the solid residue with MeOH (5×) to give **2** (31 μmol, 72%) in the triethylammonium form as a glassy residue: UV (H_2O, pH 4.9) λ_{max} 255 nm (11100), 273 (sh); HPLC 11.32 min (λ_{max} 256 nm); IR (KBr) 3414 (s, OH), 2678 (s), 1696 (s, C=O), 1231 (s, P=O), 1120 (s), 1060 (s), 1036 (s); ^{1}H NMR (D_2O) δ 8.70 (s, 1H, H-2), 5.97 (d, 5.6 Hz, 1H, H-1′), 5.85 (s, 1H, H-1″), 5.17 (t, 5.4 Hz, 1H, H-2′), 4.58 (dd, 3.6/4.8 Hz, H-3′), 4.41−4.37 (m, 1H, H_a-5′), 4.30 (dd, 12.0/3.0 Hz, 1H, H_a-5″), 4.25−4.23 (m, 2H, H-3″ and H-4″), 4.22−4.19 (m, 2H, H-4′ and H-2″), 4.03 (dd, 12.0/3.6 Hz, 1H, H_b-5″), 3.95−3.93 (m, 1H, H_b-5′), 3.03 (q, 7.6 Hz, TEA CH_2), 1.11 (t, 7.3 Hz, TEA CH_3); ^{13}C NMR (D_2O) δ 156.33 (C-6), 149.14 (C-4), 144.25 (C-2), 128.64 (C-8), 123.83 (C-5), 92.00 (C-1″), 91.19 (C-1′), 84.91 (d, 10.0 Hz, C-4′), 83.29 (d, 10.0 Hz, C-4″), 75.66 (C-2″), 72.76 (C-2′), 70.64 (C-3′), 67.65 (C-3″), 64.92 (m, C-5′), 62.42 (m, C-5″), 46.89 (TEA CH_2), 8.58 (TEA CH_3); ^{31}P NMR (10% D_2O in 50 mM HEPES, pH 6.8−10.9) δ 10.18 ± 0.01 (d, 12.5 ± 0.55 Hz), 9.14 ± 0.01 (d, 12.5 ± 0.55 Hz). HRMS (FAB, negative) calcd. for $C_{15}H_{19}BrN_4O_{14}P_2$ 618.9456, 620.9435 (monoanion), found 618.9478, 620.9458.

8-Br-inosine 5′-Monophosphate (8-Br-IMP, 3).[47] Freshly prepared bromine water (ca. 3.5%, 20 mL) was added dropwise to a solution of IMP × $8H_2O$ (disodium salt, 0.5 g, 0.93 mmol) in 10% aq Na_2HPO_4 (15 mL, pH 6.2). The reaction was stirred at room temperature and in the dark for 5 days. The slightly yellow color was discharged by addition of saturated aq $Na_2S_2O_3$ (two drops). The aqueous solution (pH 5.9) was evaporated, and the residue was dissolved in 0.05 M TEAB buffer (pH 6.2) and purified twice using CSA.[46] Fractions 26−34 of the second separation were combined and evaporated. After the excess TEAB had been destroyed by coevaporation of the solid residue with MeOH (5×), 162 mg (33%) of **3** in the triethylammonium form (1 equiv by NMR) were obtained as a glassy residue: UV (H_2O, pH 5.2) λ_{max} 253 nm; HPLC 10.34 min (λ_{max} 255 nm); ^{1}H NMR (D_2O) δ 8.13 (s, 1H), 6.08 (d, 1H, 5.4 Hz), 5.24 (t, 1H, 5.7 Hz), 4.62 (t, 1H, 5.0/5.4 Hz), 4.29−4.23 (m, 1H), 4.19−4.05 (m, 2H), 3.16 (q, 6H, 7.4 Hz), 1.24 (t, 9H, 7.4 Hz); ^{31}P NMR (D_2O) δ 2.75 (s); HRMS (FAB, negative) calcd. for $C_{10}H_{11}BrN_4O_8P$ 424.9498, 426.9477 (monoanion), found 424.9484, 426.9469.

Cyclic Inosine 5′-Diphosphate Ribose (*N*1-cIDPR, 4). $NaHCO_3$ (510 mg) was dissolved in an aqueous solution (12 mL) of **2** (59 μmol), and the aqueous solution was diluted with EtOH (6 mL). Pd−C (10%, 22 mg) was added, and the reaction vessel was flushed with H_2 (10×). The reaction was stirred at room temperature for 13 h under atmospheric pressure of H_2. The catalyst was filtered off through Celite and washed with H_2O (100 mL) and MeOH (100 mL). The combined filtrate was evaporated, and the residue was dissolved in H_2O (50 mL). The aqueous solution was treated with Dowex 50WX2-100 (H^+ form) for 15 min at room temperature to remove the sodium ions. The resin was filtered off and washed with H_2O (500 mL). The aqueous filtrate was purified using CSB,[46] and fractions 53−59 were combined and evaporated. The excess TEAB was destroyed by coevaporation of the solid residue with MeOH (5×). The crude product was dissolved in 0.05 M TEAB buffer (pH 6.2) and purified twice using CSA. Fractions 30−32 of the second separation were combined and evaporated. The excess TEAB was destroyed by coevaporation of the solid residue with MeOH (5×) to give **4** (11 μmol, 19%) in the triethylammonium form as a glassy residue: UV (H_2O, pH 5.3) λ_{max} 248 nm (ϵ 8000), 267 (sh); HPLC 8.28 min (λ_{max} 250 nm); ^{1}H NMR (D_2O) 8.82 (s, 1H, H-2), 8.09 (bs, 1H, H-8), 5.96 (s, 1H, H-1″), 5.89 (d, 1H, 6.1 Hz, H-1′), 5.18 (t, 1H, 5.6 Hz, H-2′), 4.57−4.56 (m, 1H, H-3′), 4.51−4.48 (m, 1H, H_a-5′), 4.36−4.32 (m, 1H, 2.8/11.9 Hz, H_a-5″), 4.29−4.28 (m, 3H, H-2″, H-3″, and H-4″),

(46) CSA, CSB: chromatographic system A/B. For details cf. the general Experimental Section in the Supporting Information.

(47) Skibo, E. B.; Meyer, R. B. *J. Med. Chem.* **1981**, *24*, 1155−1161.

4.27−4.25 (m, 1H, H-4′), 4.08−4.06 (m, 1H, H_b-5″), 4.03−3.98 (m, 1H, H_b-5′), 3.08 (q, 7.3 Hz, TEA CH_2), 1.15 (t, 7.3 Hz, TEA CH_3); ^{13}C NMR (D_2O) δ 157.84 (C-6), 148.21 (C-4), 144.19 (C-2), 143.09 (C-8), 124.16 (C-5), 91.87 (C-1″), 90.49 (C-1′), 84.78 (d, 10.4 Hz, C-4′), 83.17 (d, 9.2 Hz, C-4″), 75.62 (C-2″), 72.72 (C-2′), 70.86 (C-3′), 67.52 (C-3″), 65.08 (C-5′), 62.15 (C-5″), 46.73 (TEA CH_2), 8.26 (TEA CH_3); ^{31}P NMR (D_2O) δ −9.38 (d, 14.1 Hz), −10.43 (d, 14.1 Hz); HRMS (FAB, negative) calcd. for $C_{15}H_{19}N_4O_{14}P_2$ 541.0373 (monoanion), found 541.0364.

Cyclic 8-Phenyl-inosine 5′-Diphosphate Ribose (8-Ph-*N*1-cIDPR, 5). To a solution of **2** (29 μmol), anhydrous Na_2CO_3 (32 mg, 310 μmol), and phenylboronic acid (5.5 mg, 45 μmol) in 50% aqueous MeOH (3 mL) was added $Pd(PPh_3)_4$ (3.4 mg, 2.9 μmol). Under an atmosphere of argon, the reaction was heated to reflux for 4 h. The green−brown mixture was cooled to room temperature, and the solvents were removed in vacuo. The residue was suspended in H_2O, and the slurry was filtered through Celite. The filtrate was purified using CSB,[46] and fractions 62−73 were combined and evaporated. The excess TEAB was destroyed by coevaporation of the solid residue with MeOH (5×) to give **5** (20 μmol, 69%) in the triethylammonium form as a glassy residue: UV (H_2O, pH 6.0) λ_{max} 270 nm (ϵ 11400); HPLC 20.01 min (λ_{max} 274 nm); ^{1}H NMR (D_2O) δ 8.80 (s, 1H, H-2 hyp), 7.60−7.58 (m, 2H, H-2 and H-6 ph), 7.54−7.48 (m, 3H, H-3, H-4, and H-5 ph), 5.96 (s, 1H, H-1″), 5.91 (d, 1H, 6.4 Hz, H-1′), 5.38 (t, 1H, 5.5/6.1 Hz, H-2′), 4.61 (dd, 1H, 2.6/5.1 Hz, H-3′), 4.54−4.49 (m, 1H, H_a-5′), 4.37 (dd, 1H, 2.8/11.9 Hz, H_a-5″), 4.31 (bs, 2H, H-3″ and H-4″), 4.28−4.27 (m, 1H, H-2″), 4.19−4.17 (m, 1H, H-4′), 4.11 (dd, 1H, 3.6/11.9 Hz, H_b-5″), 4.06−4.01 (m, 1H, H_b-5′), 3.10 (q, 7.2 Hz, TEA CH_2), 1.17 (t, 7.2 Hz, TEA CH_3); ^{13}C NMR (D_2O) δ 157.31 (C-6 hyp), 153.28 (C-8), 149.08 (C-4 hyp), 143.67 (C-2 hyp), 131.02 (C-4 ph), 129.70 (C-2/C-6 ph), 129.03 (C-3/C-5 ph), 127.27 (C-1 ph), 122.85 (C-5 hyp), 91.80 (C-1″), 89.74 (C-1′), 84.51 (d, 10.0 Hz, C-4′), 83.05 (d, 9.2 Hz, C-4″), 75.60 (C-2″), 72.31 (C-2′), 70.61 (C-3′), 67.49 (C-3″), 64.92 (d, 4.6 Hz, C-5′), 62.21 (C-5″), 46.64 (TEA CH_2), 8.22 (TEA CH_3); ^{31}P NMR (D_2O) δ −9.51 (d, 14.1 Hz), −10.41 (d, 14.1 Hz); HRMS (FAB, negative) calcd. for $C_{21}H_{23}N_4O_{14}P_2$ 617.0686 (monoanion), found 617.0697.

Cyclic 8-Azido-inosine 5′-Diphosphate Ribose (8-N_3-*N*1-cIDPR, 6). Preparation of a stock solution of the free acid of **2** in dry DMF: a solution of **2** (49 μmol) in H_2O (5 mL) was treated with Dowex 50WX2-100 (H^+ form) at room temperature for 30 min to convert the triethylammonium salt into the free acid. The resin was filtered off and washed with H_2O (400 mL), and the combined filtrate was evaporated. The glassy residue was dried in vacuo and dissolved in dry DMF (5.0 mL).

A total of 2.0 mL of the DMF stock solution of **2** (19 μmol of 8-Br-*N*1-cIDPR) was evaporated to dryness. The residue was coevaporated with dry DMF (3 × 4 mL) to remove residual H_2O and finally dissolved in dry DMF (2 mL). NaN_3 (10 mg, 154 μmol) was added, and the reaction was stirred under an atmosphere of argon and in the dark for 32 h at 85 °C. The solvent was removed in vacuo, and the cream-colored residue was dissolved in H_2O. The aqueous solution was filtered through Celite, and the filtrate was diluted to 400 mL with H_2O. The aqueous solution was purified using CSB,[46] and fractions 39−41 were combined and evaporated. The excess TEAB was destroyed by coevaporation of the solid residue with ice-cold MeOH (5×) to give **6** (6 μmol, 32%) in the triethylammonium form as a glassy residue. The material was lyophilized for collection of the IR spectrum: UV (H_2O, pH 6.0) λ_{max} 274 nm (ϵ 10 000); HPLC 11.65 min (λ_{max} 277 nm); IR (KBr) 3416 (s, OH), 2678 (s), 2164 (m, N_3), 1696 (s, C=O), 1517 (m), 1227 (s, P=O), 1119 (s), 1060 (s), 1036 (s); ^{1}H NMR (D_2O) δ 8.68 (s, 1H, H-2), 5.90 (s, 1H, H-1″), 5.72 (d, 1H, 6.1 Hz, H-1′), 5.10 (t, 1H, 5.0/5.8 Hz, H-2′), 4.57 (dd, 1H, 3.0/ 4.6 Hz, H-3′), 4.42−4.37 (m, 1H, H_a-5′), 4.32 (dd, 1H, 2.6/11.6 Hz, H_a-5″), 4.27−4.24 (m, 3H, H-2″, H-3″, and H-4″), 4.19−4.17 (m, 1H, H-4′), 4.05 (dd, 1H, 2.9/11.9 Hz, H_b-5″), 3.95−3.92 (m, 1H, H_b-5′), 3.05 (q, 7.4 Hz, TEA CH_2), 1.13 (t, 7.4 Hz, TEA CH_3); ^{13}C NMR (D_2O) δ 156.28 (C-6), 147.93 (C-4), 146.69 (C-8), 143.27 (C-2), 121.31 (C-5), 91.87 (C-1″), 88.02 (C-1′), 84.39 (d, 10.7 Hz, C-4′), 83.04 (d, 9.2 Hz, C-4″), 75.42 (C-2″), 72.33 (C-2′), 70.38 (C-3′), 67.34 (C-3″), 64.65 (C-5′), 62.06 (C-5″), 46.54 (TEA CH_2), 8.12 (TEA CH_3); ^{31}P NMR (D_2O) δ −9.55 (d, 14.1 Hz), −10.32 (d, 14.1 Hz); HRMS (FAB, negative) calcd. for $C_{15}H_{18}N_7O_{14}P_2$ 582.0387 (monoanion), found 582.0374.

Cyclic 8-Amino-inosine 5′-Diphosphate Ribose (8-NH_2-*N*1-cIDPR, 7). A total of 3.0 mL of the DMF stock solution of **2** (29 μmol of 8-Br-*N*1-cIDPR) was evaporated to dryness. The residue was coevaporated with dry DMF (3 × 4 mL) to remove residual H_2O and finally dissolved in dry DMF (4 mL). NaN_3 (15 mg, 231 μmol) was added, and the reaction was stirred under an atmosphere of argon and in the dark for 19 h at 85 °C. The solvent was removed in vacuo, and the cream-colored residue was dissolved in H_2O (2 mL). The aqueous solution was filtered through Celite, and the Celite was washed with H_2O (2 × 2 mL). The combined aqueous filtrate was diluted with 0.05 M TEAB buffer (20 mL, pH 8.5). Dithiothreitol (45 mg, 292 μmol) was added, and the reaction was stirred under an atmosphere of argon and in the dark for 32 h at room temperature. The solvent was removed in vacuo, and the residue was dissolved in a small volume of 0.05 M TEAB buffer (pH 6.2). The aqueous solution was purified using CSA,[46] and fractions 25−27 were combined and evaporated. The excess TEAB was destroyed by coevaporation of the solid residue with MeOH (5×), and the material was purified using CSB. Fractions 43−47 were combined and evaporated. The excess TEAB was destroyed by coevaporation of the solid residue with MeOH (5×) to give **7** (13 μmol, 46% over both steps) in the triethylammonium form as a glassy residue: UV (H_2O, pH 5.0) λ_{max} 261 nm (ϵ 10 400), 290 (sh); HPLC 10.30 min (λ_{max} 263 nm); ^{1}H NMR (D_2O) δ 8.59 (s, 1H, H-2), 5.89 (s, 1H, H-1″), 5.73 (d, 1H, 6.0 Hz, H-1′), 5.23 (t, 1H, 5.5/5.7 Hz, H-2′), 4.56 (dd, 1H, 2.6/5.0 Hz, H-3′), 4.42−4.36 (m, 1H, H_a-5′), 4.32 (dd, 1H, 3.7/12.0 Hz, H_a-5″), 4.25 (bs, 2H, H-3″ and H-4″), 4.22 (bs, 1H, H-2″), 4.19−4.16 (m, 1H, H-4′), 4.05 (dd, 1H, 4.3/11.9 Hz, H_b-5″), 3.97−3.93 (m, 1H, H_b-5′), 3.06 (q, 7.4 Hz, TEA CH_2), 1.13 (t, 7.3 Hz, TEA CH_3); ^{13}C NMR (D_2O) δ 155.90 (C-6), 153.15 (C-8), 147.53 (C-4), 141.31 (C-2), 120.12 (C-5), 91.82 (C-1″), 87.89 (C-1′), 84.12 (d, 10.7 Hz, C-4′), 82.96 (d, 9.2 Hz, C-4″), 75.54 (C-2″), 71.95 (C-2′), 70.45 (C-3′), 67.35 (C-3″), 64.80 (d, 4.6 Hz, C-5′), 62.13 (d, 3.1 Hz, C-5″), 46.55 (TEA CH_2), 8.14 (TEA CH_3); ^{31}P NMR (D_2O) δ −9.51 (d, 14.1 Hz), −10.41 (d, 14.1 Hz); HRMS (FAB, negative) calcd. for $C_{15}H_{20}N_5O_{14}P_2$ 556.0482 (monoanion), found 556.0496.

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Supporting Information Available: General experimental procedures, 1-D ^{1}H, ^{13}C, and ^{31}P NMR and 2-D NMR spectra for compounds **1**−**7** and spectral data on the azido/tetrazolo isomerism. This material is available free of charge via the Internet at http://pubs.acs.org.

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