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First enzymatic synthesis of an N1-cyclised cADPR (cyclic-ADP ribose) analogue with a hypoxanthine partial structure: discovery of a membrane permeant cADPR agonist

Gerd K. Wagner,[a] Steven Black,[a] Andreas H. Guse[b] and Barry V. L. Potter*[a]

[a] *University of Bath, Department of Pharmacy and Pharmacology, Wolfson Laboratory of Medicinal Chemistry, Claverton Down, Bath, UK BA2 7AY. E-mail: prsbvlp@bath.ac.uk; Fax: ++44-1225-386114; Tel: ++44-1225-386639*

[b] *University Hospital Hamburg-Eppendorf, Institute of Medical Biochemistry and Molecular Biology I: Cellular Signal Transduction, Martinistr. 52, D-20246 Hamburg, Germany. E-mail: guse@uke.uni-hamburg.de; Fax: ++49-40-42803-9880; Tel: ++49-40-42803-2828*

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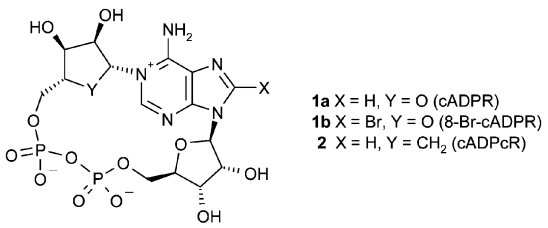
Nicotinamide 8-Br-hypoxanthine dinucleotide (8-Br-NHD$^+$) was cyclised at the N1 position by the ADP-ribosyl cyclase from *Aplysia californica* to give cyclic 8-Br-inosine diphosphoribose (8-Br-N^1-cIDPR), a novel membrane-permeant agonist of Ca^{2+} release in human T cells.

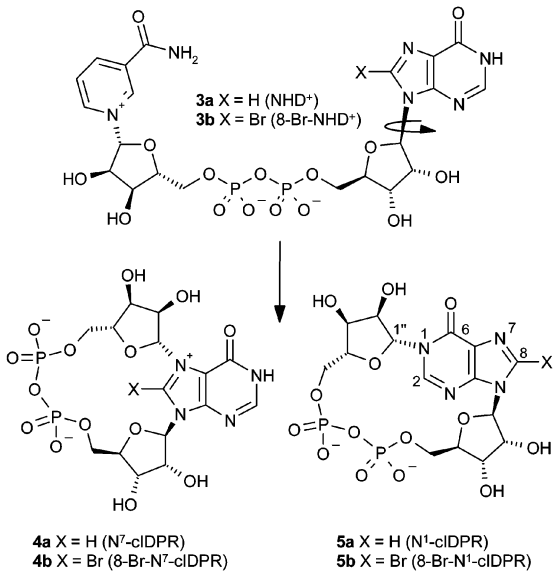
In various cell types of mammalian and invertebrate origin, the second messenger cyclic-ADP ribose (cADPR, **1a**) mediates an increase in intracellular Ca^{2+} levels.[1] In human T cells cADPR-induced Ca^{2+} entry is a key signal for cellular activation and proliferation.[2] Therefore, a more detailed understanding of the biochemical mechanisms underlying the formation and signalling of cADPR may pave the way for novel treatments of immunological disorders. There has been interest in the synthesis of various analogues of cADPR, and these efforts have been comprehensively reviewed.[3] Numerous structural modifications of cADPR described include the replacement of the adenine portion with a deaza ring system,[4,5] the modification or deletion of either one of the ribose units,[6] as well as combinations of the above structural features.[7] Moreover, the introduction of suitable substituents at the 8 position of the adenine ring system of cADPR has furnished several *antagonists* of cADPR-mediated Ca^{2+} release,[8] among which 7-deaza-8-Br-cADPR and to a lesser extent 8-Br-cADPR (**1b**) stand out as being *membrane-permeant*.[4]



Most analogues of cADPR have been prepared either *via* a chemo-enzymatic route or by total chemical synthesis. The total synthesis of **2**, for example, is built around the formation of the pyrophosphate bond using a suitable 1,9-disubstituted adenine precursor.[9] On the other hand, the chemo-enzymatic preparation of **1a** and **1b** relies on the ADP-ribosyl cyclase from *Aplysia californica* to cyclise nicotinamide adenine dinucleotide (NAD$^+$) or 8-Br-NAD$^+$ at the N1 position.[8] The success of this strategy depends *inter alia* on the nucleophilicity of the ring nitrogen in position 1. With the modulated nucleophilicity of the hypoxanthine system in nicotinamide hypoxanthine dinucleotide (NHD$^+$, **3a**) cyclisation occurs exclusively at the N7 position, yielding the biologically much less active N^7-cIDPR **4a** (cyclic-IDP ribose; Scheme 1) but not N^1-cIDPR **5a**.[10] So far, N1-cyclised hypoxanthine analogues of cADPR have only been obtained by total chemical synthesis.[7] The major drawbacks of this approach are that it involves difficult multistep reaction sequences and that only analogues bearing a carbo-ribose (or surrogate) at the N1 (*cf.* **2**) are accessible. Herein we describe for the first time the preparation of a N1-cyclised hypoxanthine analogue of cADPR with unusual *agonistic* bioactivity *via* an enzymatic route which avoids these limitations (Scheme 1).

It is well known that the equilibrium between *syn*- and *anti*-conformations of nucleotides can be influenced by the steric bulk of a substituent at the 8 position.[11] Therefore, we reasoned that this equilibrium might be favourably shifted towards the *syn*-conformation in 8-Br-NHD$^+$ (**3b**). In this case the hypoxanthine N1 is expected to be able to attain spatial proximity to the anomeric carbon of the nicotinamide ribose, as required for cyclisation at the N1 position. It was also hoped that the steric bulk of the bromine might prevent potential cyclisation at the N7 of the 8-Br-hypoxanthine ring and formation of **4b**.

A solution of **3b** (prepared by deamination of 8-Br-NAD$^+$) was incubated with ADP-ribosyl cyclase from *A. californica* and the reaction course was monitored by ion-pair HPLC.† For comparison NAD$^+$ and **3a** were reacted to give **1a** and **4a** respectively under the same conditions as previously described.[8,10] A considerably slower turnover was observed for the conversion of **3a** and **3b** than for the formation of **1a** from NAD$^+$. In the case of both hypoxanthine derivatives the reaction could be driven to completion only after *ca.* 8 h. These prolonged reaction times presumably reflect modulated nucleophilicity at both the N1 and N7 position in the (8-Br-)-hypoxanthine part of (8-Br-)NHD$^+$ as compared to the N1



Scheme 1 *Reagents and conditions*: ADP-ribosyl cyclase (*A. californica*), 25 mM HEPES, rt, 8 h. Cyclisation yields either compound "**a**" (*i.e.* **4a** from **3a**) or "**b**" (*i.e.* **5b** from **3b**).

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position of the adenine ring system in NAD^+, in concert with an aberrant enzymatic reaction. HPLC analysis of the crude 8-Br-NHD^+/ADP-ribosyl cyclase mixture showed the main product (retention time 10.6 min) eluting earlier than **4a** (16.2 min) and in the range of the hydrolysis product from NHD^+, the linear inosine diphosphoribose (IDPR, 8.5 min). The reaction product was isolated by ion-exchange chromatography and assigned structure **5b** based on full characterisation by UV, IR, FAB-MS, 1D and 2D ^{1}H-, ^{13}C- and ^{31}P-NMR.‡ Taking into consideration that the *Aplysia* cyclase is also capable of simply hydrolysing its substrates,[12] it was pleasing to note that the molecular ion found in the FAB^--MS correlated with the molecular mass of the product from either N1 or N7 cyclisation, but renders *hydrolysis* of **3b** to a linear product unlikely. The connectivities observed in a gHMBC spectrum for **5b** unambiguously establish N1 as the site of cyclisation (Table 1). Further support for the structural assignment of **5b** comes from the NOESY and ROESY spectra which show crosspeaks for interactions between the aromatic proton and H-2″, H-3″, H-4″, and H_a-5″.

It has been reported that the protonation state of cADPR (pK_a 8.3) affects the chemical shift value for the ^{31}P-NMR signal from one of the two phosphorus atoms.[13] Not surprisingly, the ^{31}P-NMR spectrum of **5b** showed no such pH-dependence at pH 6.8–10.9 as no such protonation equilibrium exists for the hypoxanthine system in **5b** (*cf.* NH_2 dissociation in **1a**). Cyclised at N7, **4a** has been described as being fluorescent at pH > 6.[10] While we were able to confirm these findings, we did not detect any fluorescence for **5b** at pH 5.0–11.0, which further supports its N1-cyclised structure.

The usefulness of cADPR as a pharmacological tool is limited by its rapid hydrolysis.[6] Conflicting results have been published with regard to the hydrolytic stability of **4a**.[10] At pH 7.4 we found that only after refluxing for 7 h is **4a** hydrolysed to IDPR. Under the same conditions **5b** is still intact to more than 96% after 10 h. This enhanced resistance towards hydrolysis can be attributed to the inherent stability of the uncharged secondary amide structure resulting from N1 cyclisation and this should be usefully mirrored *in vivo*.

Upon incubation of Jurkat T cells with different external concentrations of **5b** a concentration-dependent increase in *intracellular* Ca^{2+} levels was observed (Fig. 1), illustrating that **5b** is a membrane permeant agonist of cADPR-induced Ca^{2+} release. Together with its hydrolytic stability, this biological profile makes **5b** a potentially valuable tool for the investigation of cADPR signalling in human T cells. The full biological characterisation of **5b** will be reported in due course.

In conclusion, we present for the first time the enzymatic synthesis of a N1-cyclised hypoxanthine analogue of cADPR with useful biological properties. Compared to the complex chemical syntheses published so far for structurally related compounds this new route, if proved versatile, greatly facilitates the preparation of these derivatives with all other structural motifs intact. The 8-bromo substituent also allows for further modifications at this position. Very notably, a single chemical change in the 8-Br-cADPR structure causes an activity switch from antagonist to agonist (**1b** *vs.* **5b**) worthy of future investigation. We expect **5b** to find general application in exploring the role of cADPR in cell signalling.

Table 1 Selected ^{1}H–^{13}C connectivities from the gHMBC spectrum of **5b**

	C2	C6	C1″
C2–H	*H–C*	*H*–C–N–*C*	*H*–C–N–*C*
C1″–H	*H*–C–N–*C*	*H*–C–N–*C*	*H–C*

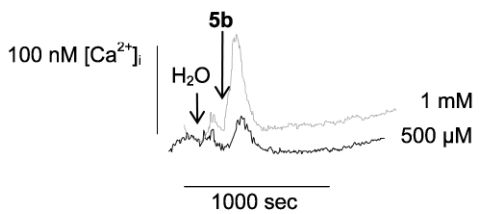

Fig. 1 Intracellular Ca^{2+} release in Jurkat T cells is stimulated by **5b**.

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Notes and references

† *Preparation* of **5b**: A solution of 8-Br-NHD^+ (Na^+ salt, 30.3 mg, 40 µmol [BIOLOG GmbH, Bremen, Germany]) and ADP-ribosyl cyclase from *A. californica* (approx. 15 µg) in 25 mM HEPES buffer (pH 7.4, 90 mL) was stirred at rt for 8 h. The aqueous solution was diluted with MilliQ water (conductivity 50 µS), and the mixture was separated on a Q Sepharose column (10 × 3 cm) using a gradient (0 to 50%) of MilliQ water against 1 M TEAB (pH 7.3). At a flow rate of 5 mL min^{-1}, 95 fractions of 10 mL each were collected. Fractions 55–60 (29–32% TEAB) were combined and the solvent was evaporated. The residue was repeatedly coevaporated with methanol until a constant mass was achieved. The glassy product was lyophilised to give 24.0 mg (75%) of **5b** in the triethylammonium salt form. HPLC conditions were as follows: Phenomenex RP-18 (4 µm), 150 × 4.60 mm; 0.17% (m/v) Cetrimide and 45% (v/v) phosphate buffer (pH 6.4) in methanol.

‡ *Spectral characterisation* of **5b** (^{1}H- and ^{13}C-NMR assignments are based on gCOSY, gHMBC and gHMQC experiments): UV (H_2O, pH 4.9) 255 (λ_{max}, 11145), 273 nm (sh, 6137); IR (KBr) 3500, 2936, 2738, 2674, 2491, 1694 (C=O), 1548, 1476 cm^{-1}; FAB^- $[M - H]^-$ 620.9435, 618.9456. $C_{15}H_{19}BrN_4O_{14}P_2$ requires $[M - H]^-$ 620.9458, 618.9478. δ_H (D_2O) 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); δ_C (D_2O) 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); δ_P (10% D_2O in 50 mM HEPES, pH 6.8–10.9) 10.18 ± 0.01 (d, 12.5 ± 0.55 Hz), 9.14 ± 0.01 (d, 12.5 ± 0.55 Hz).

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