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Cyclic aristeromycin diphosphate ribose: a potent and poorly hydrolysable Ca^{2+}-mobilising mimic of cyclic adenosine diphosphate ribose

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Abstract Cyclic aristeromycin diphosphate ribose, a carbocyclic analogue of cyclic adenosine diphosphate ribose, was synthesised using a chemo-enzymatic route involving activation of aristeromycin 5′-phosphate by diphenyl phosphochloridate. The calcium-releasing properties of this novel analogue were investigated in sea urchin egg homogenates. While cyclic aristeromycin diphosphate ribose has a calcium release profile similar to that of cyclic adenosine diphosphate ribose (EC_{50} values are 80 nM and 30 nM, respectively), it is degraded significantly more slowly ($t_{1/2}$ values are 170 min and 15 min, respectively) and may, therefore, be a useful tool to investigate the activities of cyclic adenosine diphosphate ribose.

Key words: Cyclic adenosine diphosphate ribose; Cyclic aristeromycin diphosphate ribose; Chemo-enzymatic synthesis; Calcium release; Sea urchin egg homogenate; Metabolic degradation

1. Introduction

Cyclic adenosine diphosphate ribose (cADPR (1); Fig. 1) is a novel Ca^{2+}-releasing second messenger candidate. As a naturally occurring metabolite of nicotinamide adenine dinucleotide (NAD^+, (2); Fig. 2) [1], it is known to be more effective than, and to work independently of, inositol 1,4,5-trisphosphate in mobilising internal stores of calcium in sea urchin eggs [2]. Since its discovery in 1987 [3], cADPR has been shown to be present and active in a wide variety of mammalian and invertebrate tissue [4–6] and its cyclic structure has been confirmed by X-ray crystallography [7]. The metabolic pathway of cADPR is known (Fig. 2) and evidence exists to suggest that in human lymphocytes CD38, a surface antigen, acts as a bifunctional enzyme both for synthesising and degrading cADPR [8,9].

Mounting evidence has led to the proposal that, by modulating the ryanodine receptor, cADPR is an endogenous activator of Ca^{2+}-induced Ca^{2+} release (CICR) [10]. In addition, there is intense interest in the role of cADPR as a potential second messenger and its action has been proposed to have a role in insulin release [6]. To corroborate this, cGMP has been shown to mobilise intracellular Ca^{2+} release in sea urchin eggs by stimulating cADPR synthesis [11]. The recent discovery that cADPR acts through binding to calmodulin to sensitise the CICR mechanism to Ca^{2+} ions has, for the first time, enabled these two proposed mechanisms of action to be linked. It appears that, when a large amount of cADPR is synthesised through a proposed second messenger pathway, the CICR mechanism is sensitised to such an extent that the basal level of Ca^{2+} ions alone will activate further calcium release [12].

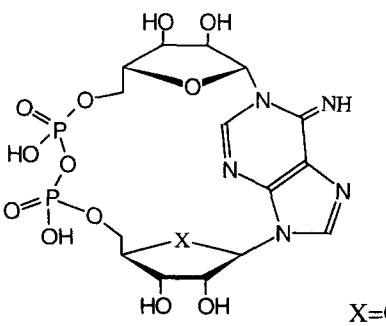
Structural analogues of cADPR are currently urgently required as pharmacological tools for the investigation of its biological properties. Since to date there is no total chemical synthesis of cADPR (although β-NAD^+ has been cyclised chemically using NaBr in dimethyl sulphoxide to yield cADPR [13]), we, like others [14], have relied upon a chemo-enzymatic approach both to prepare cADPR itself and to generate structural diversity (Fig. 3) [15]. The route involves first a chemical coupling of nucleotides resulting in NAD^+, or an appropriate analogue, and second an enzymatically mediated cyclisation to form the natural or modified cADPR, respectively. A key requirement of this approach for the synthesis of structural analogues of cADPR is the loose substrate specificity of the enzyme. Our laboratory has already shown this to be a reasonable assumption.

Using our route, but with a modified coupling procedure we have synthesised the first carbocyclic analogue of cADPR, cyclic aristeromycin diphosphate ribose (cArisDPR (3); Fig. 1), wherein the adenosine ribosyl moiety has been replaced with a carbocyclic 5-membered ring. In a comparative study with cADPR, this analogue was evaluated for its pharmacological characteristics in sea urchin egg homogenates. We report here that, while cArisDPR has a similar calcium release profile to cADPR, it is significantly longer acting and is presumably poorly hydrolysed.

2. Materials and methods

2.1. Materials

Aristeromycin ((4); Fig. 3) was a generous gift of Glaxo (Greenford, UK). ADP-ribosyl cyclase was isolated from *Aplysia californica* as described [16] and was used crude. Nicotinamide mononucleotide (NMN) and 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulphonic acid



X=O, cADPR (1)

X=CH_2, cArisDPR (3)

Fig. 1. The structure of cADPR (1) and cArisDPR (3).

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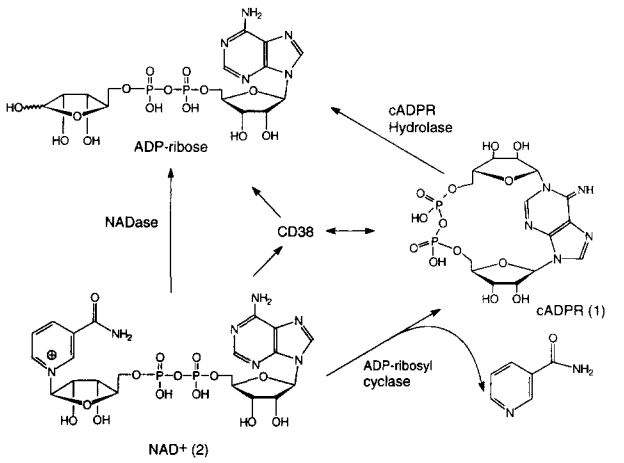

Fig. 2. The metabolic pathway of cADPR showing the structures of cADPR, NAD^+ and ADPR.

(HEPES) were purchased from Sigma (London, UK). Fluo-3 dye was purchased from Calbiochem. High-performance liquid chromatography (HPLC) was performed on a Shimadzu LC-6A with the UV detector operating at 259 nm and using a 10-μm Partisil SAX anion-exchange column isocratically eluted with 0.05 M KH_2PO_4/5% MeOH buffer. Ion-exchange chromatography was performed using an LKB Gradifrac System with the column packed with Q-Sepharose Fast Flow resin (Pharmacia) in the bicarbonate form. Gradient elution was performed using an ion-exchange buffer system of triethylammonium bicarbonate. All other chemicals were purchased from Aldrich Chemical and solvents were distilled and dried before use. ^{31}P NMR spectroscopy was performed on a Jeol EX400 spectrometer operating at 161.9 MHz. Chemical shifts are expressed as negative when downfield of external 85% H_3PO_4. UV spectra were recorded on a Perkin-Elmer Lambda-3 UV/Vis spectrometer using quartz cells (1 cm path length).

2.2. Chemistry

Aristeromycin (4) was phosphorylated selectively at the 5′ position using $POCl_3$ in a similar fashion to the method developed by Yoshikawa et al. [17] (2 equivalents $POCl_3$ in $PO(OEt)_3$, 6 h at 0°C, then quench with an excess of iced water) and the resulting phosphate purified by ion-exchange chromatography. This nucleotide was activated for coupling using diphenylphosphochloridate (DPPC, 1.5 equivalents, a 2:1 ratio of dioxane and DMF, 2 h at 20°C) and was coupled without isolation of the intermediate (5) by addition of 2′, 3′-diacetyl NMN (0.5 equivalents, a 1:1 ratio of pyridine and DMF, 18 h at 20°C). NMN itself was acetylated with acetic anhydride (pyridine, 18 h at 4°C) and used immediately [18]. The resulting pyrophosphate coupled product was deacetylated with methanolic ammonia (1:1 methanol/conc NH_3, 6 h at 0°C) to give (6) which was purified by ion-exchange chromatography. Cyclisation of (6) to cArisDPR (3) was performed enzymatically using ADP-ribosyl cyclase from *Aplysia californica* and the conversion was monitored by HPLC (1.5 mM cArisDPR, 25 mM HEPES, 10 μl enzyme, 0.5 h at 20°C). The product was purified by ion-exchange chromatography (40% yield) and quantified using UV analysis assuming the extinction coefficient to be the same as that of cADPR. This assumption was made based on the knowledge that the extinction coefficient of aristeromycin is the same as for adenosine [19]. The UV data for the final product was λ_{max} = 260 nm with $\varepsilon = 14.3 \times 10^3 M^{-1}$. Full synthetic details will be reported elsewhere.

2.3. Calcium release measurements

Lytechinus pictus homogenate (2.5%), as previously described [3], was incubated at 17°C in an intracellular-like medium (Im) containing an ATP-regenerating system, mitochondrial inhibitors and Fluo-3 (3 μM). Extramicrosomal Ca^{2+} was measured by monitoring Fluo-3 fluorescence (excitation 490 nm and emission 535 nm) in a Perkin-Elmer LS-50B fluorimeter. Additions to the cuvette were between 5 and 10 μl in Im with 10 μM EGTA.

2.4. Monitoring the metabolism of cADPR and cArisDPR

Degradation of cADPR and cArisDPR (500 nM) in egg homogenate was determined using the Ca^{2+} release bioassay as above. At appropriate time intervals, 50-μl samples were taken and assayed for remaining cXDPR (where X = A or Aris). Ca^{2+} release, following addition of the test sample to 500 μl of assay homogenate, was related to cXDPR concentration from a standard curve. Caffeine (1 mM) was added prior to the sample to increase sensitivity of the bioassay by approximately 10-fold, allowing measurement of cXDPR in the sample in the range 20–500 nM.

3. Results and discussion

Aristeromycin 5′-monophosphate was prepared by selective phosphorylation of the 5′ hydroxyl group using established methodology. The crude product was precipitated from the reaction residue using acetone and then purified by ion-exchange chromatography. If necessary, further precipitations of the free acid form of the nucleotide using acetone/water were carried out until a ratio of less than 5:1 nucleotide: inorganic phosphate was seen by ^{31}P NMR spectroscopy. The product showed a broad single peak in the ^{31}P NMR spectrum, with δ_P = 0.06 ppm which sharpened on broad band proton irradiation. In our experience, these precipitation steps were essential in order to minimise complications in the subsequent phosphate-coupling step arising from contaminating inorganic phosphate.

NAD^+ analogues have previously been synthesised by coupling adenosine monophosphate analogues to NMN using dicyclohexylcarbodiimide [20] and 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide-HCl [14]. However, we favoured the more chemically flexible method of Michelson [21]. The nucleotide was activated using DPPC in dry dioxane and dry DMF to produce a mixed anhydride intermediate. The residue after evaporation was dissolved in a mixture of dry DMF and dry pyridine and treated with the phosphate monoanion of NMN (2′, 3′-diacetyl form which had much greater solubility than the unprotected form), which displaced the acidic diphenyl phosphate moiety to give a pyrophosphate. Treatment of this pyrophosphate with methanolic ammonia and subsequent purification of the resulting product by ion-exchange chromatography led to nicotinamide aristeromycin dinucleotide ($NArisD^+$ (6)).

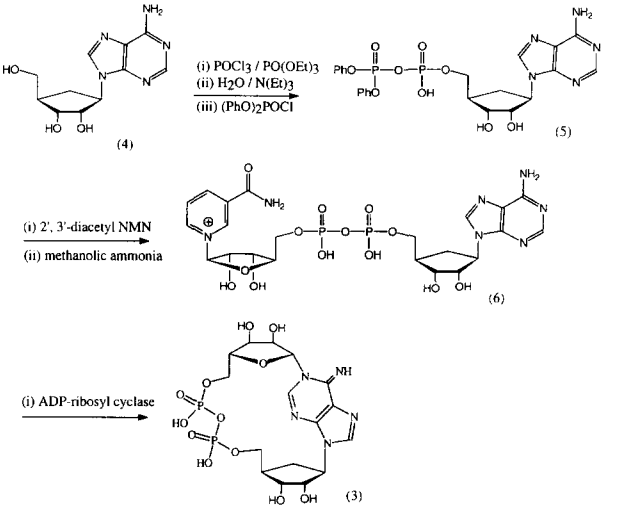

Fig. 3. The chemo-enzymatic route used to synthesise analogues of cADPR showing the structures of $NArisD^+$ and cArisDPR.

The ^{31}P NMR spectrum of this compound exhibited an AB quartet with $\delta_P = -11.08$ ppm and -11.86 ppm and $J_{PP} = 20.8$ Hz.

Enzymatic cyclisation of NArisD$^+$ (6) was performed according to the protocol previously detailed from this laboratory [15] and the formation of the cyclised product, cArisDPR (3), was monitored by HPLC (Fig. 4). The retention times for NArisD$^+$, cArisDPR and nicotinamide were 2.49 min, 3.84 min and 1.80 min, respectively, and the reaction was assumed to be complete when the peak assigned to nicotinamide in the sample was no longer increasing. The cyclisation mixture was quenched by excessive dilution after 30 min, a similar reaction time to that required for cADPR itself, and cArisDPR was easily purified from a small amount of unreacted starting material and nicotinamide by ion-exchange chromatography.

cArisDPR, together with the parent compound cADPR, was assayed for concentration-dependent Ca^{2+}-mobilising activity in sea urchin egg microsomes (Fig. 5a). A maximally effective concentration of 1 μM cADPR elicited 2.7 nmol Ca^{2+} release when measured in absolute terms. In comparison, the same concentration of cArisDPR caused only a slight reduction in this value at 2.5 nmol Ca^{2+} release. The EC_{50} values were 30 nM for cADPR and 80 nM for cArisDPR.

A time course for the degradation of cArisDPR was measured straightforwardly by incubating a sample with homogenate and then testing aliquots for calcium-releasing ability (Fig. 5b). cADPR was seen to degrade rapidly in this system with its Ca^{2+}-releasing ability falling to near-negligible values of over 25 min. This was presumably a result of enzymatic cleavage of the N^1-ribosyl bond by cADPR hydrolase. There was also a signifcant decrease in releasing potency of over 70% in the first 10 min. In contrast, the time course for the degradation of cArisDPR was markedly slower. In the first 10 min, there was only a 10% reduction in response and Ca^{2+} release was still observable after 5 h of incubation.

Carbocyclic analogues of nucleosides and nucleotides have proven to be good mimics of their parent compounds although often with additional (e.g. antiviral) properties [22]. The replacement of the adenosine ribose moiety of cADPR with a carbocyclic ring as in cArisDPR leads to surprising hydrolytic stability towards the degrading enzyme even though it is the remote N^1-ribosyl linkage which is cleaved in the catabolism of cADPR. We propose that this could be due to the compound binding to the enzyme active site in a similar, but subtly different, conformation to that of cADPR making the hydrolytic attack of a water molecule more difficult, although clearly not impossible. While cADPR-induced Ca^{2+} mobilisation has been observed in many tissues and cell lines [23] (and recently also in T cells [24]), the possibility that its presence in many others remains undetected due to rapid metabolism of endogenously added cADPR cannot be excluded. The advent of poorly hydrolysable analogues, such as cArisDPR, and ultimately completely non-hydrolysable compounds will allow the potentially wider role of cADPR in signalling to be more easily explored. The synthesis of cArisDPR is an important step in this direction and this compound will not only be a useful biological tool, but

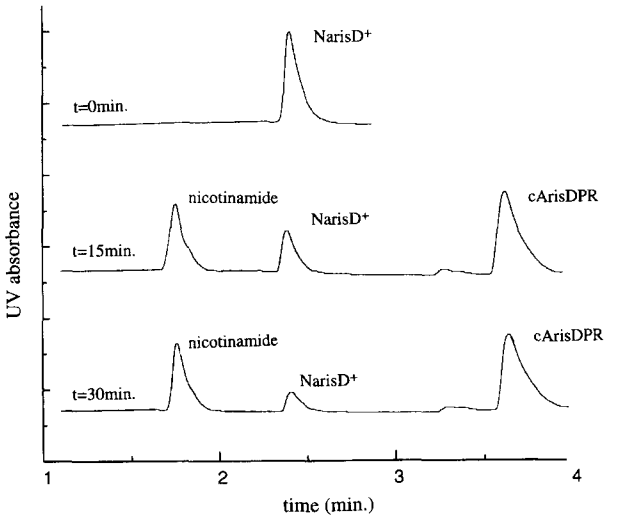

Fig. 4. Monitoring the cyclisation of NArisD$^+$ by HPLC. The enzymatic cyclisation was carried out as described in section 2. (a) $t = 0$ min, NArisD$^+$ 100% at 2.49 min. (b) $t = 15$ min, cArisDPR is seen at 3.84 min and the by-product nicotinamide is at 1.80 min. (c) $t = 30$ min, the % of cArisDPR had increased only nominally so the reaction was quenched immediately.

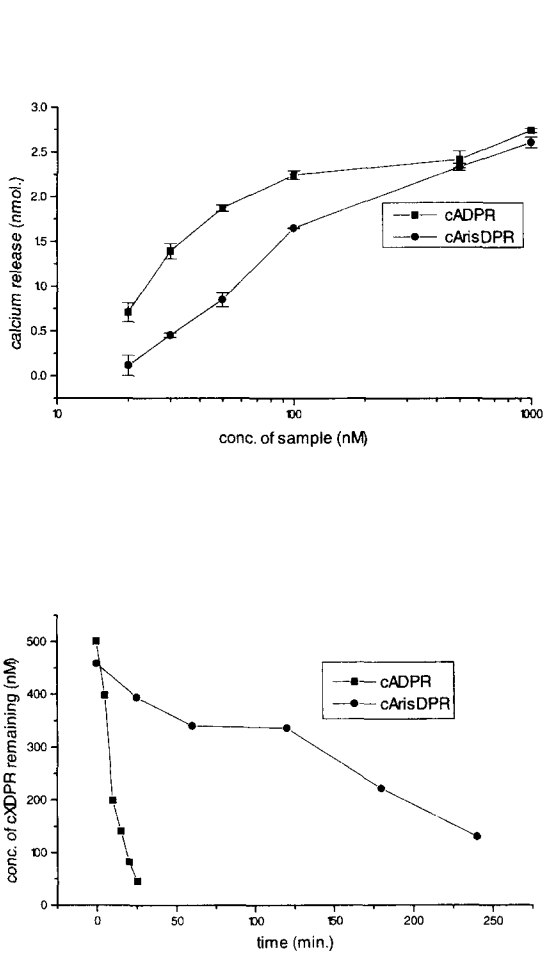

Fig. 5. Stimulation of Ca^{2+} wrelease from sea urchin egg homogenates by activation with cArisDPR. (a) Concentration dependence of cADPR (■) and cArisDPR (●). cADPR and cArisDPR were assayed for calcium-mobilising activity as described in section 2.3. The EC_{50} values for cADPR and cArisDPR are 30 nM and 80 nM, respectively. (b) Degradation of cADPR (■) and cArisDPR (●). Degradation of sample in egg homogenate was measured as described in section 2.4, after addition of an initial dose sufficient to give a concentration of 500 nM sample. X in cXDPR is either A or Aris.

also a key prototype for the design of a fully metabolically stable cADPR mimic or antagonist.

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