Synthesis of 7-deaza-8-bromo cyclic adenosine 5′-diphosphate ribose: the first hydrolysis resistant antagonist at the cADPR receptor

Victoria C. Bailey,[a] Jaswinder K. Sethi,[b] Antony Galione[b] and Barry V. L. Potter*[a]

[a] *Department of Medicinal Chemistry, School of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath, UK BA2 7AY*

[b] *Department of Pharmacology, University of Oxford, Mansfield Road, Oxford, UK OX1 3QT*

7-Deaza-8-bromo cyclic adenosine 5′-diphosphate ribose is synthesised from 7-deazaadenosine *via* 7-deaza-8-bromo nicotinamide adenine dinucleotide; it is both a more potent antagonist than the 8-bromo derivative and has the advantage of chemical and enzymatic hydrolytic stability.

Cyclic adenosine 5′-diphosphate ribose (cADPR, **1**, Fig. 1) is a Ca^{2+}-mobilising agent known to act *via* ryanodine receptors to release internal stores of Ca^{2+} in a wide variety of cell types,[1–3] independent of the second messenger *myo*-inositol 1,4,5-trisphosphate.[4] The role of cADPR is not yet fully understood, but it may act as a second messenger for a number of receptor agonists, including nitric oxide.[5] The binding protein for cADPR is unknown, and there is doubt whether cADPR acts directly at the ryanodine receptor, although its action involves calmodulin.[6] Hence it is important to build up a structure–activity profile for cADPR in relation to Ca^{2+} release and to design molecules to probe its biological role.

Analogues substituted at the 8-position including the 8-bromo **2** and 8-amino **3** derivatives are documented as antagonists at the cADPR receptor.[7] Importantly, 8-amino-cADPR inhibits cardiac excitation–contraction coupling.[8] Recently, we reported 7-deaza-cADPR **4** as the first partial agonist, highlighting the N-7 position as an essential motif for effective Ca^{2+} release.[9] Furthermore, 7-deaza-cADPR is resistant to both chemical and enzymatic hydrolytic attack, a property desirable to confer upon an antagonist, to improve its physiological value. We have combined these two modifications and we now report 7-deaza-8-bromo-cADPR **5** as the first hydrolysis resistant cADPR antagonist.

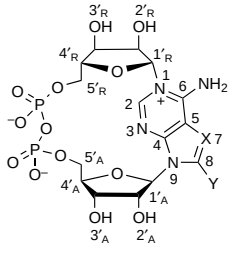
There is no effective chemical synthesis of cADPR, the only published route produces the correct stereoisomeric product in very low yield.[10] We, like others, have relied upon a chemoenzymatic route to develop structural diversity[7,11] utilising *Aplysia* ADP-ribosyl cyclase.[12] The enzyme is extremely flexible in its substrate specificity.[11–13]

7-Deazaadenosine (Tubercidin, **6**) was selectively brominated at the pseudo-8-position (6-position) using *N*-bromosuccinimde (Scheme 1). After chromatography, dry **7** was phosphorylated at the 5′-hydroxy group using essentially the general method described by Yoshikawa *et al.*[15] The novel **8**‡ was purified by ion exchange chromatography using triethylammonium hydrogen carbonate buffer (TEAB). Owing to the increased hydrophobic nature of the modified purine ring (an extra CH group and the bromo substituent), **8** eluted at a higher concentration of buffer than expected, namely 250–300 mm TEAB as opposed to the 200–250 mm observed for adenosine 5′-monophosphate, and was not contaminated by inorganic phosphate, often a problem with this method.

Dicyclohexylcarbodiimide coupled **8** to nicotinamide mononucleotide (NMN).[16] The novel pyrophosphate **9**† was purified from monophosphates and symmetrical pyrophosphates by ion exchange chromatography. 7-Deaza-8-bromo-NAD^+ eluted at 120–150 mm TEAB as its triethylammonium salt.

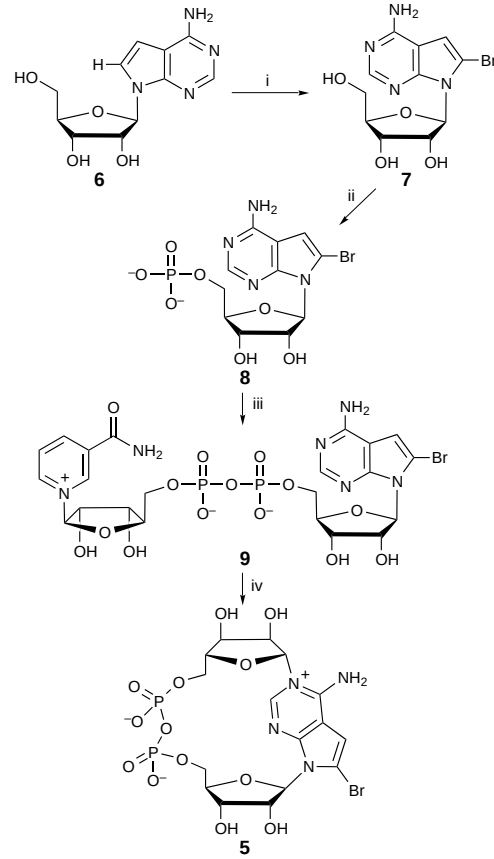
Finally, the NAD^+ analogue was cyclised using the *Aplysia* enzyme.[11] 7-Deaza-8-bromo-NAD^+ cyclised at a similar rate to cADPR. The product was purified by ion exchange chromatography, at 130–150 mm TEAB, as the triethylammonium salt.

Phosphates **8**, **9** and **5**‡ were quantified using total phosphate analysis[9,17] from which the extinction coefficients were calcu-



1 X = N, Y = H, cADPR
2 X = N, Y = Br, 8-Bromo-cADPR
3 X = N, Y = NH_2, 8-Amino-cADPR
4 X = CH, Y = H, 7-Deaza-cADPR
5 X = CH, Y = Br, 7-Deaza-8-bromo-cADPR

Fig. 1 Structures of cADPR **1**, 8-bromo-cADPR **2**, 8-amino-cADPR **3**, 7-deaza-cADPR **4** and 8-bromo-7-deaza-cADPR **5**



Scheme 1 *Reagents and conditions*: i, NBS, KOAc, DMF, 10 min, 40%; ii, $PO(OEt)_3$ 50 °C, 15 min, then $POCl_3$ (1.5 equiv.), 0 °C, 2 h, then H_2O, 44%; iii, DCC, NMN, pyridine–H_2O (4 : 1), 7 d, 28%; iv, 1.5 mm solution in 25 mm HEPES, ADP-ribosyl cyclase, 30 min, 31%

lated, and used to quantify reaction yields and solutions for the biological evaluation of **5**.

The pharmacology of **5** was investigated using sea urchin egg homogenate.[18] 7-Deaza-8-bromo-cADPR antagonised the action of 100 nm cADPR-induced Ca^{2+} release (Fig. 2), desensitised the receptor, and was apparently more potent and efficacious than 8-bromo-cADPR (IC_{50} values of 0.73 ± 0.05 and 0.97 ± 0.04 μm respectively). Furthermore, it was resistant to chemical hydrolysis and induced antagonism even after being incubated in buffer at 85 °C for 90 min. HPLC analysis of a control incubation showed that 7-deaza-8-bromo-cADPR was still present confirming that cyclic material, and not a metabolite, caused antagonism even after heat treatment. In contrast, after identical treatment, 8-bromo-cADPR was no longer able to induce antagonism and HPLC showed no cyclic material remaining. Resistance to enzymatic hydrolysis was tested by pre-incubating the antagonist with homogenate overnight, when an aliquot was removed and its ability to antagonise cADPR-induced Ca^{2+} was tested. Whilst the 8-bromo-cADPR antagonism had reduced significantly, 7-deaza-8-bromo-cADPR was still a significant antagonist. Full biological details will be reported elsewhere.

In 7-deaza analogues of adenosine the glycosidic bond is more resistant to acid catalysed hydrolysis than in adenosine.[19] Similarly, the increased hydrolytic resistance of 7-deaza analogues of cADPR is thought to arise from increased stability of the N1 ribosyl linkage. The N1 position of 7-deazaadenosine is more basic than that of adenosine (pK_a = 5.2 and 3.6 respectively[20]) and similarly this position is more basic in the 8-bromo substituted analogues than in adenosine (pK_a = 4.02 for 8-bromoadenosine[21]). The combination of modifications, therefore, makes the heterocycle substantially more basic at N1 than in adenine. Hydrolysis of the N1 ribosyl linkage probably involves participation of a ribosyl oxygen lone pair to form a carbocation which is then captured by water. The ability of the N1-ribosyl linkage to be hydrolysed should therefore depend upon the leaving group ability of the purine. The more acidic the purine, the better leaving group it will be and, conversely, with the more basic character of N1 in 7-deaza-8-bromo-cADPR, the harder it will be to cleave the N1 ribosyl bond.

Combined modifications at the 7 and 8 positions of cADPR thus produce slightly better antagonistic properties, with the marked added advantage of hydrolytic resistance. One of the most commonly employed antagonists, 8-amino-cADPR, is unsuitable for microinjection work due to its chemical instability.[22] 7-Deaza-8-bromo-cADPR, resistant to both chemical and enzymatic hydrolysis, should be a powerful tool to investigate the cADPR signalling pathway and complements our poorly hydrolysable agonist, cyclic aristeromycin diphosphate ribose.[13]

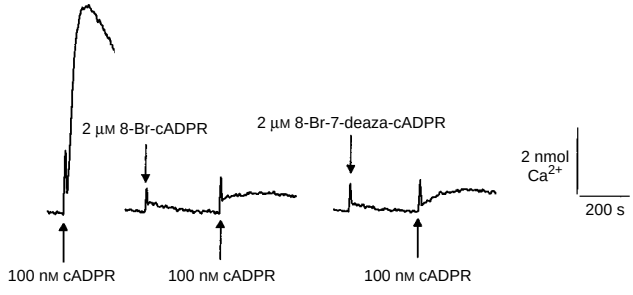


Fig. 2 *Lytechinus pictus* egg homogenates (2.5% v/v) (ref. 18) containing Fluo-3 (3 μm) were incubated at 17 °C. Homogenate (500 μl) was challenged with cADPR (final concentration 100 nm). Ca^{2+} release was monitored by a change in fluorescence. 7-Deaza-8-bromo-cADPR was tested for its antagonism by pre-treating the homogenate (500 μl) with the sample (final concentration 2 μm) 3 min prior to addition of cADPR (final concentration 100 nm). A subsequent addition of cADPR (100 nm) demonstrated the desensitisation of the Ca^{2+} release mechanism. The traces shown are representative of several such experiments.

We thank the Medical Research Council for a Project Grant (A. G., B. V. L. P.) and two studentships (V. C. B., J. K. S.). A. G. is a Wellcome Trust Fellow and B. V. L. P. is a Lister Institute Research Professor.

Footnotes

* E-mail: B.V.L.Potter@bath.ac.uk

† *Spectroscopic data* for **8**: δ_H (400 MHz, D_2O): 4.08–4.15 (3 H, m, H4′ and H5′), 4.53 (1 H, dd, *J* 5.7, 5.8 Hz, H3′), 5.07 (1 H, dd, *J* 5.3, 5.8 Hz, H2′), 6.04 (1 H, d, *J* 5.3 Hz, H1′), 6.42 (1 H, s H7), 7.92 (1 H, s, H2). δ_P (161.7 MHz, D_2O): 2.35 broadens when proton coupled. δ_C (100.4 MHz, D_2O): 65.2 (d, J_{CP} 3.7 Hz, C5′), 70.3 (C3′), 72.1 (C2′), 83.6 (d, J_{CP} 7.4 Hz, C4′), 89.8 (C1′), 104.0 (C5), 105.2 (C7), 110.4 (C8), 149.9 (C4), 150.0 (C2), 154.5 (C6). λ_{max} 276 nm, ε = 13.3 × 10^3 dm^3 mol^{-1} cm^{-1}. *m/z* (FAB^+) 425, 427 $(M + H)^+$, (FAB^-) 422, 424 $(M - H)^-$, 341 $(M - H - Br)^-$.

For **9**: δ_H (400 MHz, D_2O): 4.03–4.32 (8 H, m, H_N2', H_N3', H_A4', H_N4', H_A5' H_N5'), 4.56 (1 H, dd, *J* 5.8, 5.3 Hz, H_A3'), 5.16 (1 H, dd, *J* 5.9, 5.8 Hz, H_A2'), 5.99 (1 H, d, *J* 4.4 Hz, H_N1'), 6.06 (1 H, d, *J* 5.9 Hz, H_A1'), 6.60 (1 H, s, H_A7), 8.05 (1 H, s, H_A2), 8.14 (1 H, dd, *J* 8.3, 6.4 Hz, H_N3), 8.73 (1 H, d, *J* 8.3 Hz, H_N4), 9.09 (1 H, d, *J* 6.4 Hz, H_N6), 9.25 (1 H, s, H_N2). δ_P (161.7 MHz, D_2O): −10.5, −10.9 (2d, J_{PP} 20.1 Hz). δ_C (100.4 MHz, D_2O): 65.7 (d, *J* 3.7 Hz, C_A5'), 66.5 (C_N5'), 70.0 (C_A3'), 71.4 (C_N3'), 71.9 (C_A2'), 78.5 (C_N2'), 83.5 (d, *J* 9.2 Hz, C_A4'), 87.8 (d, *J* 9.2 Hz, C_N4'), 89.8 (C_A1'), 100.9 (C_N1'), 104.3 (C_A5), 105.0 (C_A7), 110.5 (C_A8), 129.6 (C_N4), 140.6 (C_N5), 143.1 (C_N6), 146.3 (C_N2), 150.4 (C_A2), 150.7 (C_A4), 155.5 (C_A6), 166.1 (CO). λ_{max} 270 nm, ε = 13.3 × 10^3 dm^3 mol^{-1} cm^{-1}. *m/z* (electrospray$^+$) 741, 743 $(M + H)^+$, (electrospray$^-$) 739, 741 $(M - H)^-$.

For **5**: δ_H (400 MHz, D_2O): 3.89–3.93 (1 H, m, H_A5'), 3.98–4.03 (1 H, m, H_R5'), 4.15–4.20 (1 H, m, H_A4'), 4.23–4.27 (1 H, m, H_R5'), 4.30–4.40 (2 H, m, H_A5', H_R3'), 5.44 (1 H, dd, *J* 5.2, 6.1 Hz, H_A2'), 5.97 (1 H, d, *J* 4.0 Hz, H_R1'), 6.03 (1 H, d, *J* 6.1 Hz, H_A1'), 6.91 (1 H, s, H_A7), 8.75 (1 H, s, H_A2), all other protons are obscured by the water peak at δ 4.8. δ_P (161.7 MHz, D_2O): −10.8, −11.6 (2d, J_{PP} 18 Hz). λ_{max} = 277 nm, ε = 10.85 × 10^3 dm^3 mol^{-1} cm^{-1}. *m/z* (electrospray$^+$) 619, 621 $(M + H)^+$, (electrospray$^-$) 617, 619 $(M - H)^-$, 79, 81 $(Br)^-$.

References

1 H. C. Lee and R. Aarhus, *Cell Reg.*, 1991, **2**, 203.
2 S. Takasawa, K. Nata, H. Yonekura and H. Okamoto, *Science*, 1993, **259**, 370.
3 A. H. Guse, C. P. DaSilva, F. Emmrich, G. A. Ashamu, B. V. L. Potter and G. W. Mayr, *J. Immunol.*, 1995, **155**, 3353.
4 A. Galione, *Trends Pharmacol. Sci.*, 1992, **13**, 304.
5 N. J. Willmott, J. K. Sethi, T. F. Walseth, H. C. Lee, A. M. White and A. Galione, *J. Biol. Chem.*, 1996, **271**, 3699.
6 H. C. Lee, R. Aarhus, R. M. Graeff, M. E. Gurnack and T. F. Walseth, *Nature*, 1994, **370**, 307.
7 T. F. Walseth and H. C. Lee, *Biochim. Biophys. Acta*, 1993, **1178**, 235.
8 S. Rakovic, A. Galione, G. A. Ashamu, B. V. L. Potter and D. A. Terrar, *Curr. Biol.*, 1996, **6**, 989.
9 V. C. Bailey, J. K. Sethi, S. M. Fortt, A. Galione and B. V. L. Potter, *Chem. Biol.*, 1997, **4**, 51.
10 S. Yamada, Q.-M. Gu and C. J. Sih, *J. Am. Chem. Soc.*, 1994, **116**, 10 787.
11 G. A. Ashamu, A. Galione and B. V. L. Potter, *J. Chem. Soc., Chem. Commun.*, 1995, 1359; 1929.
12 H. C. Lee and R. Aarhus, *Cell Reg.*, 1991, **2**, 203.
13 V. C. Bailey, S. M. Fortt, R. J. Summerhill, A. Galione and B. V. L. Potter, *FEBS Lett.*, 1996, **379**, 227.
14 D. E. Bergstrom and A. Brattesani, *Nucleic Acids Res.*, 1980, **8**, 6213.
15 M. Yoshikawa, T. Kato and T. Takenishi, *Bull. Chem. Soc. Jpn.*, 1969, **42**, 3505.
16 N. H. Hughes, G. W. Kenner and A. Todd, *J. Chem. Soc.*, 1957, 3733.
17 A. Briggs, *J. Biol. Chem.*, 1922, **53**, 13.
18 D. L. Clapper, T. F. Walseth, P. J. Dargie and H. C. Lee, *J. Biol. Chem.*, 1987, **262**, 9561.
19 J. E. Pike, L. Slechta and P. F. Weley, *J. Heterocycl. Chem.*, 1964, **1**, 159.
20 L. Ji, N. A. Corfu and H. Sigel, *J. Chem. Soc., Dalton Trans.*, 1991, 1367.
21 E. R. Garrett and P. J. Mehta, *J. Am. Chem. Soc.*, 1972, **94**, 8532.
22 C. M. Perez-Terzic, E. N. Chini, S. S. Shen, T. D. Dousa and D. E. Clapham, *Biochem. J.*, 1995, **312**, 955.

Received, 14th January 1997; Com. 7/00336F