Synthesis of carbocyclic NAD$^+$ containing a methylenebisphosphonate linkage for the investigation of ADP-ribosyl cyclase

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A stereospecific synthesis of carbocyclic NAD$^+$ incorporating a methylenebisphosphonatelinkage in place of the natural pyrophosphate gives an analogue 4 of the natural coenzyme NAD$^+$ 3a designed to act as an inhibitor of ADP-ribosyl cyclase and to resist non-specific phosphatase degradation.

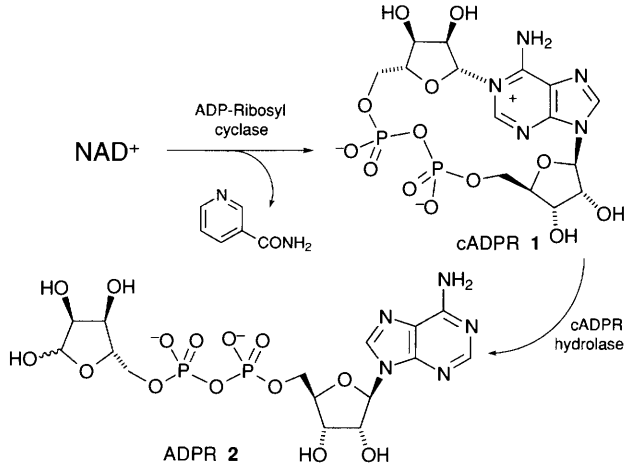
Cyclic ADP-Ribose (cADPR) **1** is a secondary messenger species involved in intracellular calcium mobilisation.[1] It activates receptors different from those used by the other known secondary messenger, inositol triphosphate.[2] cADPR is formed through cyclisation of NAD$^+$ involving displacement of nicotinamide by N-1 of the adenine with retention of configuration.[3] This transformation is catalysed by ADP-ribosyl cyclase, a protein found in various mammalian and invertebrate tissues.[1,4] The enzyme also regulates the process through degradation of cADPR by hydrolysis of the weak C$^{1''}$–N^1 bond to give adenosine diphosphate ribose, ADPR **2**.[4] Moreover, human leukocyte antigen, CD38, shows triple activity as an NAD$^+$ glycohydrolase, an ADP-ribosyl cyclase and a cADPR hydrolase.[5]

In view of the instability of cADPR to hydrolysis and the extremely low concentrations at which it is present in cells (submicromolar), we are addressing the need to generate two types of chemical species for use in intracellular examination of its detailed function. First, a stable analogue of the natural coenzyme NAD$^+$ **3a** can be used to block the formation of cADPR by competitive inhibition of ADP-ribosyl cyclase and may additionally form a stable complex with the crystalline enzyme.[6] Secondly, hydrolytically-stable analogues of cADPR **1** may act as agonists/antagonists of cADPR at the ryanodine receptor[7] and also inhibit its hydrolysis by ADP-ribosyl cyclase. The key to both of these objectives is the use of carbocyclic ribose analogues designed to inhibit glycosyl transfer and glycosyl cleavage processes, in view of their wide success as nucleotide analogue in diverse situations, especially as antiviral agents.[8] At the same time, replacement of the pyrophosphate by a methylenebisphosphonate will provide analogues stable to cleavage of the pyrophosphate moiety by non-selective, intracellular phosphohydrolases.

We here report a stereospecific synthesis of a methylenebisphosphonate carbocyclic analogue **4** of NAD$^+$. Previously, a carbocyclic NAD$^+$ analogue **3b** has been prepared from a racemic precursor by Slama and Simmons.[9] Surprisingly, the L-enantiomer of an 8-azido derivative of **3b** was claimed to be a good inhibitor of a number of NAD$^+$ glycotransferases while the D-isomer showed little or no activity.[10]

Our synthesis of carbocyclic methylene NAD$^+$ **4** uses commercially available (−)-2-azabicyclo[2.2.1]hept-5-en-3-one **5** to give the protected amine alcohol **7** in three, high-yielding steps (Scheme 2) by modification of a published synthesis:[11] *cis*-hydroxylation with catalytic osmium tetroxide occurs exclusively from the lower face to give diol **6** in 91% yield and a facile double protection then follows permitting reductive cleavage of the lactam using sodium borohydride in MeOH to give the alcohol **7**.

Tosylation of **7** using toluene-*p*-sulfonyl chloride in pyridine gives the key precursor **8** which we have used in two approaches to the required target **4**. The first involves coupling **8** with adenosine 5′-methylenebisphosphonate, AMPPCP **9**, using typical conditions described by Poulter[12] (Scheme 3). Yields for the coupling are low and we are currently seeking to improve this stage of the synthesis. Removal of both isopropylidene and Boc protecting groups by refluxing in distilled water gives quantitative conversion into and allows easy isolation of the amine **11**. It is interesting to note that when compound **7** is



Scheme 1 Enzymic cyclisation of NAD$^+$ and hydrolysis of the cyclic ADP ribose product

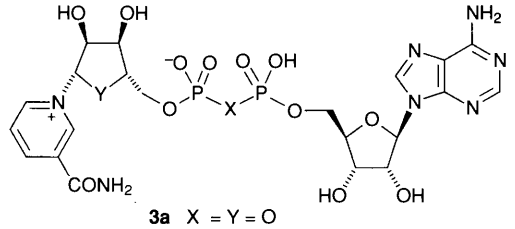
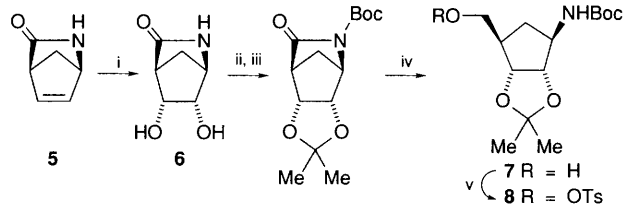
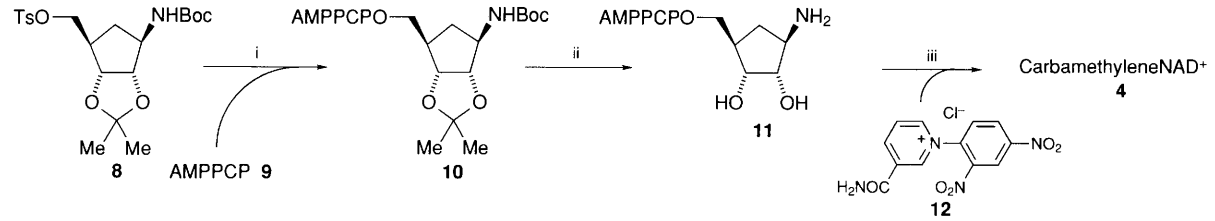


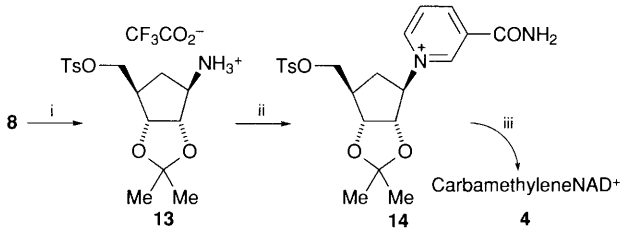
Fig. 1



Scheme 2 *Reagents and conditions*: i, OsO_4, NMO, Me_2CO, room temp., 3 h, 91%; ii, 2,2-DMP, TsOH, DMF, room temp., 20 h; iii, Boc_2O, DMAP, MeCN, room temp., 15 h, 85%; iv, $NaBH_4$, MeOH, 0 °C to room temp., 2 h, 85%; v, TsCl, pyridine, room temp., 15 h, 88%



Scheme 3 *Reagents and conditions*: i, MeCN, room temp., 10 d, 35%; ii, H_2O, heat, 6 h, 100%; iii, MeOH, heat, 24 h, 7%



Scheme 4 *Reagents and conditions*: i, excess TFA, CH_2Cl_2, heat, 6 h, 100%; ii, Pr^i_2NEt, **12**, MeOH, room temp., 2 h, 100%; iii, $AMPPCH_2P$ **9**, MeCN, room temp., 3 d, 25%

subjected to the same hydrolysing conditions, selective deprotection of the NHBoc group occurs leaving the isopropylidene group intact.

We have found that reaction of free amine **11** with *N*-(2,4-dinitrophenyl)-3′-carbamoylpyridinium chloride **12** in a Zincke reaction[13] is very slow in MeOH at room temp. and the vast majority of starting material is recovered after 3 d reaction. We thus refluxed **11** with **12** in MeOH overnight[14] to effect only partial conversion into the desired carbocyclic NAD^+ compound **4**. While sufficient material has been isolated from this route to allow confirmation of the structure of the product, we sought to improve the coupling steps, in particular of the Zincke reaction.

Our second route for the conversion of tosylate **8** into carbocyclic NAD^+ **4** consists of removal of both the isopropylidene and *tert*-butoxycarbonyl protecting groups in one step by heating with excess TFA in CH_2Cl_2 (Scheme 4). Following removal of solvent, the crude amine salt **13** is dried and used directly in the coupling reaction. Diisopropylethylamine is added to a stirred mixture of salt **13** and Zincke reagent **12** in MeOH at room temp. Immediately on addition of the base, the solution turns deep red showing production of dinitroaniline. Reaction is complete after 2 h and the dinitroaniline is easily removed by washing with copious CH_2Cl_2. ^1H NMR and mass spectrometry identified the sole products in the aqueous phase to be the required pyridinium salt **14** and salts of diisopropylethylamine. Synthesis of **4** is completed by Poulter coupling of **14** with AMPPCP **8** in 25% yield.

Purification of **4** is achieved by Sephadex A-25 chromatography using TEAB buffer followed by reverse-phase HPLC.† The structure of **4** has been confirmed by ultraviolet absorption, NMR (^1H, ^{31}P, ^{13}C spectra, ^1H–^{13}C-correlation NMR spectroscopy and specific decoupling experiments) and high resolution mass spectrometry.‡

Enzyme evaluation and CD38 inhibition studies using **4** are in progress and results will be reported elsewhere.

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Footnotes

† μBondapak™ RP-18 10 μm (19 × 300 mm) column (Waters) eluted with a gradient of 5–40% *v/v* MeCN in 0.1 mol dm^{-3} TEAB; retention time 13.8 min.

‡ *Selected data*: FAB^+, Found for **4** $[M + H]^+$ 660.1592; $C_{23}H_{32}N_7O_{12}P_2$ requires [M + H] 660.1584. λ_{max} 260, ε 17600 (H_2O); δ_P (162 MHz, D_2O) 17.63 (bd, J = 19 Hz); δ_H (400 MHz, D_2O) 9.21 (1 H, dd, J = 1 and 1 Hz, –NC*H*CCONH$_2$), 8.98 (1 H, ddd, J 6, 1 and 1 Hz, PyN–C*H*), 8.64 (1 H, ddd, J = 8, 1 and 1 Hz, *p*-C*H* Py), 8.33 (1 H, s, H_8 Ade), 8.04 (1 H, s, H_2 Ade), 7.88 (1 H, dd, J = 8 and 6 Hz, *m*-C*H* Py), 5.89 (1 H, d, J = 5.5 Hz, O–CH–Ade), 4.86 (1 H, ddd, J = 8.5, 8.5 and 8.5 Hz, CH_2CH–Py), 4.62 [1 H, dd, J = 5.5 and 5 Hz, C*H*(OH)CH–Ade], 4.38 [1 H, dd, J = 8.5 and 5.5 Hz, C*H*(OH)CH–Py], 4.35 [1 H, dd, J = 5 and 3.5 Hz, C*H*(OH)CH(OH)CH–Ade], 4.22 (1 H, ddd, J = 3.5, 3.5 and 3 Hz, C*H*–O–CH–Ade), 4.09 [1 H, dd, J = 12 and 3.5 Hz, O–C*H*(H)–O–CH–Ade], 4.05 [1 H, dd, J = 5.5 and 2.5 Hz, C*H*(OH)CH(OH)CH–Py], 4.03 [1 H, dd, J = 12 and 3 Hz, O–CH(*H*)–O–CH–Ade], 3.97–3.91 [1 H, m, C*H*(H)CHCH$_2$CH–Py], 3.86–3.81 [1 H, m, CH(*H*)CHCH$_2$CH–Py], 2.46 [1 H, ddd, J = 13, 8.5 and 8.5 Hz, C*H*(H)CH–Py], 2.28–2.21 (1 H, m, C*H*CH$_2$CH–Py], 2.12 (2 H, t, J = 19 Hz, PCH_2P), 2.06–2.00 [1 H, m, CH(*H*)CH–Py]; δ_C (100.6 MHz, D_2O) 164.9 (C=O), 155.4 (C^6 Ade), 152.8 (C^2 Ade), 148.8 (C^4 Ade), 145.6 (N–*C*H Py), 144.6 (*p*-*C*H Py), 142.5 [N–*C*H–C(CONH$_2$) Py], 139.8 (C^8 Ade), 133.5 [*m*-*C*(CONH$_2$) Py], 128.4 (*m*-*C*H Py), 118.3 (C^5 Ade), 86.7 (O–*C*H–Ade), 84.0 (*C*H–O–CH–Ade), 77.0 [*C*H(OH)CH–Py], 76.0 (*C*H–Py), 73.9 [*C*H(OH)CH–Ade], 72.2 [*C*H(OH)CH(OH)CH–Py], 70.1 [*C*H(OH)-CH(OH)CH–Ade], 64.6 [d, J_{CP} = 4 Hz, *C*H$_2$CHCH$_2$CH–Py], 63.8 (d, J_{CP} = 4 Hz, *C*H$_2$CH–O–CH–Ade), 43.1 (*C*HCH$_2$CH–Py), 28.8 (*C*H$_2$CH–Py), 26.1 (t, J_{CP} = 128 Hz, PCH_2P).

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