Unusual entry to the novel 8-halo-*N*1-ribosyl hypoxanthine system by degradation of a cyclic adenosine-5′-diphosphate ribose analogue

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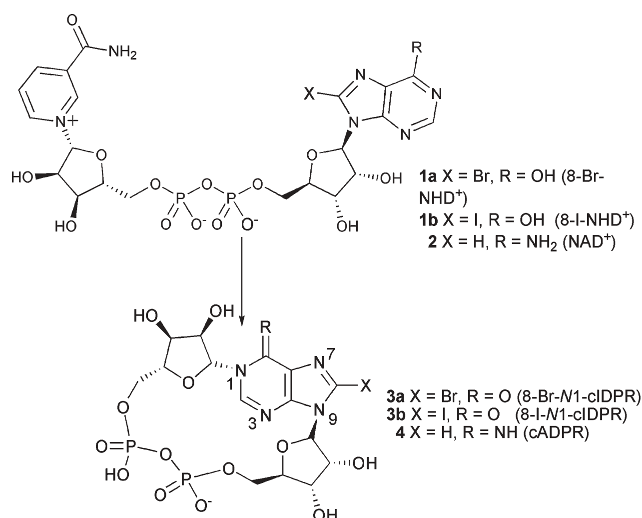
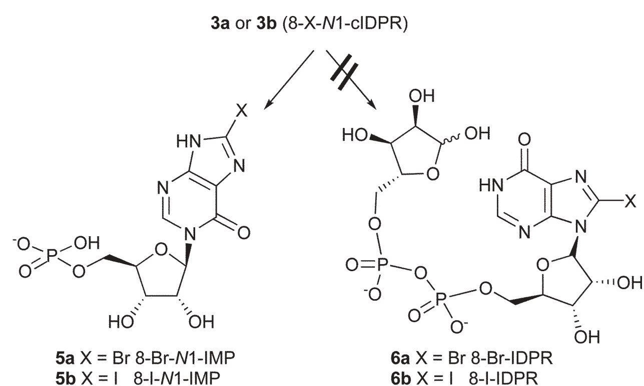
Cyclic 8-bromo-inosine-5′-diphosphate ribose (8-Br-*N*1-cIDPR) was cleanly degraded at acidic pH by *N*9 ribosyl scission and subsequent pyrophosphate cleavage to give 8-bromo-*N*1-ribosyl hypoxanthine 5′-monophosphate (8-Br-*N*1-IMP), a novel class of mononucleotide, as the sole product.

Nucleic acids and their components play an important role in a variety of fundamental biological processes. Although the synthesis of modified nucleosides has been of interest over the past four decades, the discovery of AZT (3′-azido-3′-deoxythymidine) *inter alia* as a therapeutic agent for the treatment of the human immunodeficiency virus (HIV) has triggered new developments in the synthetic chemistry of nucleosides.[1] The intense search for clinically useful nucleoside derivatives has resulted in a wealth of new approaches for their syntheses, which have been extensively reviewed.[2–6] Exploring the role of cyclic and linear dinucleotides in Ca^{2+} signalling processes mediated by cyclic ADP-ribose (cADPR) in our laboratory, we discovered and report herein a novel and facile entry to an unusual class of mononucleotide and its nucleoside counterpart.

Cyclic 8-bromo-inosine-5′-diphosphate ribose (8-Br-*N*1-cIDPR) **3a** is a novel and active analogue of cADPR (**4**), that is readily synthesised chemoenzymatically (Scheme 1).[7] **3a** was found to have remarkable stability to chemical hydrolysis at pH 7.4 and at elevated temperature in contrast to **4**, which is prone to rapid hydrolysis due to the labile nature of the *N*1 glycosydic bond.[8–10] Further examination of the stability of the compound **3a** under both acidic and basic conditions revealed that, on prolonged heating at 60 °C, degradation was observed after 50 h at pH 1 and 15 h at pH 12, while no degradation was observed at pH 7.4. Since cADPR is hydrolysed to its linear counterpart adenosine-5′-diphosphate ribose (ADPR), it seemed possible that 8-Br-cIDPR **3a** was hydrolysed into 8-Br-IDPR **6a** (Scheme 2). However, this should be substantially precluded relative to other cADPR derivatives by the *N*1-ribosyl amide linkage.



Scheme 1 *Reagents and conditions*: *Aplysia* cyclase, Hepes buffer pH 4.



Scheme 2 *Reagents and conditions*: 0.2 M HCl, pH 1, 60 °C, 1 to 6 days.

Intrigued by the stability of **3a**, we decided to investigate also the stability of the 8-iodo analogue **3b**. This was synthesised by coupling β-NMN (β-nicotinamide mononucleotide) with 8-iodo-IMP morpholidate in the presence of a Lewis acid (protocol developed by Lee *et al.*),[11] followed by enzymatic cyclisation. It was first observed that **3b** was also very stable at pH 1, pH 7.4 and pH 12 at room temperature. However, at pH 12 and 60 °C, **3b** degraded into a complex mixture of products within 3 hours, whereas at pH 1 and 60 °C a single decomposition product was observed. After purification on reverse-phase chromatography the product was isolated and identified as being the novel mononucleotide **5b** in 61% yield. As the HPLC trace of the reaction mixture did not indicate the formation of any side product, the yield obtained probably reflects loss of material during chromatographic purification. Only this structure was in comprehensive agreement with the spectroscopic data obtained.

^{31}P NMR showed that the pyrophosphate peak at δ_P −10 ppm had disappeared with a new peak at 0.5 ppm, characteristic of a monophosphate group. This ruled out 8-I-IDPR **6b** as the potential degradation product, thus underlining the contrast with

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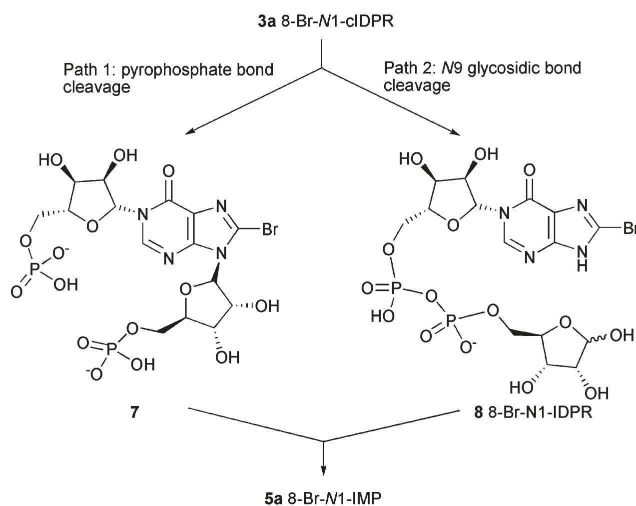
cADPR whose degradation produced its linear counterpart ADPR even in neutral aqueous conditions. The molecular ion of 473 in the ES^- mass spectrum was in accord with the molecular mass for 8-iodo-inosine-5′-monophosphate (8-I-IMP) whilst ^{1}H NMR showed the presence of only one ribose. Moreover, the resonance corresponding to H-2′ had shifted upfield from δ_H 5.3 to 4.3 ppm. Crucially, the gHMBC spectrum of the reaction product showed cross-peaks resulting from the three-bond coupling between the H-2 proton (δ_H 8.39) of the nucleobase and the anomeric carbon of the ribose (δ_C 88.1), and the anomeric proton of the ribose (δ_H 6.13) with carbons C-2 (δ_C 142.1) and C-6 (δ_C 159.3) of the nucleobase (Table 1). The ribose unit was therefore linked to the nucleobase through the *N*1 nitrogen and the product was 8-iodo-*N*1-ribosyl hypoxanthine 5′-monophosphate (8-I-*N*1-IMP **5b**) as a presumed tautomeric mixture (*N*9-(H) *vs.* *N*7-(H)).

Table 1 ^{1}H and ^{13}C connectivities based on gHMBC and gHMQC spectra of **5b**

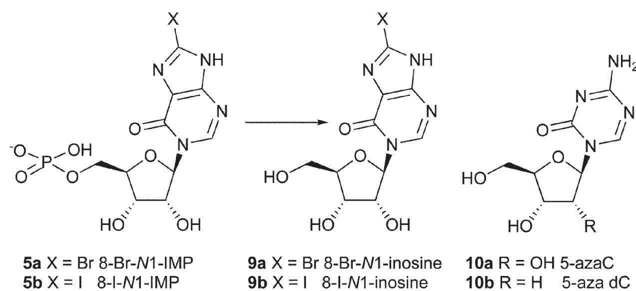
	C-2	C-6	C-1′
H-2	*H*–*C*	*H*–C–N–*C*	*H*–C–N–*C*
H-1′	*H*–C–N–*C*	*H*–C–N–*C*	*H*–*C*

This experiment was repeated with 8-Br-*N*1-cIDPR **3a** and the corresponding 8-Br-*N*1-IMP **5a** was similarly identified as being the decomposition product.†‡ This remarkable finding showed that 8-halogenated cIDPR analogues have an unstable *N*9 glycosidic bond but strong *N*1 amide linkage in sharp contrast to the case of cADPR, which has a weak *N*1 linkage.

Adenine and hypoxanthine based nucleosides are generally linked to the ribose unit through their *N*9 nitrogen atom and chemistry to prepare such analogues has been extensively reported. However, their *N*1 counteparts are not easy to synthesise—the only approach being total chemical synthesis, as classical direct ribosylation of adenine or hypoxanthine occurs preferentially at the *N*9 position. In the 1970s Imbach *et al.* reported the synthesis of *N*1-inosine and *N*1-adenosine through a rather tedious process involving 8 steps and with no stereochemical control at the glycosidic bond linkage. The α and β stereoisomers were isolated in



Scheme 3 Potential degradation pathways.



Scheme 4 *Reagents and conditions*: alkaline phosphatase, Tris HCl buffer pH 8, 37 °C, o/n.

low yields by column chromatography at the end of the synthesis.[12,13] These compounds were synthesised due to their similarity to *N*1-(5′-phosphoribosyl) ATP, a key intermediate in the biosynthesis of histidine.[14] However, no further chemistry has been developed in this area. Therefore, to the best of our knowledge, this is the first time that an efficient route has been developed to obtain this class of 8-halo mononucleotide.

NMR studies were carried out at a range of pH values in order to distinguish between two mechanisms of degradation of **3a** (pyrophosphate bond—path 1—*vs.* *N*9 glycosidic bond cleavage—path 2—Scheme 3). Since the cyclic dinucleotide is stable at pH 7.4, 60 °C and fully decomposes at pH 1, 60 °C, the experiments were carried out by carefully lowering the pH at 60 °C. 8-Br-cIDPR **3a** was found to be stable at both pH 5 and 3.5; however at pH 2.5 a very slow degradation was observed. ^{1}H NMR spectroscopy showed the appearance of two doublets at δ_H 5.2 and 5.04 ppm, providing evidence for glycosidic bond cleavage (path 2)—indeed, these doublets correspond to the anomeric proton in the β and α configuration respectively. This result was further confirmed by ^{31}P NMR spectroscopy where a multiplet at δ_P −10.5 ppm was observed demonstrating that the pyrophosphate bond was intact. Lowering the pH to 1, the intermediate **8** totally degraded into 8-Br-*N*1-IMP **5a** and ribose-5′-phosphate with ^{31}P NMR spectroscopy showing disappearance of the multiplet at δ_P −10.5 ppm and formation of two singlets between δ_P 0 and 1 ppm, characteristic of monophosphates.

Simple treatment of **5a** with alkaline phosphatase in Tris HCl buffer (pH 8.0) cleaved the 5′-phosphate and generated the nucleoside 8-Br-*N*1-ribosyl hypoxanthine **9a** quantitatively (Scheme 4). This novel nucleoside bears a strong resemblance to both 5-azacytidine (5-azaC) **10a** and 5-aza-2′-deoxycytidine (5-azadC) **10b**—two potent inhibitors of DNA methylation, which have shown efficacy in clinical trials against acute myelogenous leukemia and myelodysplatic syndrome.[15–17] It is therefore worthy of future investigation which will be reported in due course. The 8-halogen motif in particular, provides a useful site for further synthetic elaboration and reduction to the formal parent nucleotide and nucleoside.

In conclusion, we present for the first time the synthesis of an *N*1-hypoxanthine nucleotide and nucleoside from the parent cyclic dinucleotide. The reaction is believed to proceed *via* an *N*1-IDPR analogue providing evidence that the *N*1 glycosidic bond of cIDPR is much stronger than that of cADPR.

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Notes and references

† *Preparation of 8-Br-N1-IMP*: solution of 8-bromo-*N*1-cIDPR (10 mg, 16.1 μmol) in 0.2 M HCl (pH 1) was stirred at 60 °C. After 24 h, HPLC analysis showed no residual starting material and the appearance of a new peak at 10.2 min ($R_{T\ (8\text{-Br-cIDPR})}$ = 11.3 min). The reaction was cooled to room temperature, neutralised by addition of 2 M NaOH and purified on a reverse-phase column eluted with a gradient of MeCN in 0.05 M TEAB. The appropriate fractions were collected and evaporated under reduced pressure; the residue was coevaporated with MeOH to remove excess triethylammonium salt and it was then treated with chelex 100 to yield 8-bromo-*N*1-IMP as a glassy solid in the sodium salt form (4.2 mg, 9.9 μmol, 61%). HPLC conditions were as follow: 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*: ^{1}H (400 MHz, D_2O) δ 8.39 (s, 1H, H-2), 6.13 (d, $J_{1',2'}$ = 4.4 Hz, 1H, H-1′), 4.31 (dd, $J_{2',3'}$ = 5.3 and $J_{2',1'}$ = 4.4 Hz, 1H, H-2′), 4.26 (dd, $J_{3',2'}$ = 5.3 and $J_{3',4'}$ = 4.9 Hz, 1H, H-3′), 4.14–4.11 (m, 1H, H-4′), 3.94–3.91 (m, 1H, H-5′a) and 3.86–3.83 (m, 1H, H-5′b). ^{13}C (100 MHz, D_2O) δ 159.3 (C-6), 156.9 (C-4), 142.1 (C-2), 138.0 (C-8), 124.3 (C-5), 88.1 (C-1′), 83.9 (C-4′, J = 8.7 Hz), 75.1 (C-2′), 69.7 (C-3′) and 62.7 (C-5′). ^{31}P (161 MHz, D_2O) δ 3.8. MS: (ES^-) *m*/*z* 425.2 [$(M - H)^-$, 100%]. HRMS (ES^-) calcd for $C_{10}H_{11}N_4O_8P^{79}Br$ 424.9503 $(M - H)^-$ found 424.9499. UV (H_2O, pH 5.6) λ_{max} 261 nm (ε 35000).

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