Pergamon

Tetrahedron Letters, Vol. 36, No. 51, pp. 9289-9292, 1995
Elsevier Science Ltd
Printed in Great Britain
0040-4039/95 $9.50+0.00

0040-4039(95)02004-7

NOVEL ENZYMATIC CYCLIZATIONS OF PYRIDINE NUCLEOTIDE ANALOGS: CYCLIC-GDP-RIBOSE AND CYCLIC-HDP-RIBOSE

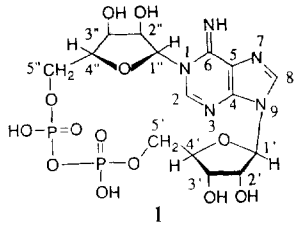
Fang-Jie Zhang and Charles J. Sih*

School of Pharmacy, University of Wisconsin,
425 N. Charter Street, Madison, Wisconsin 53706

Summary: The cyclase of *Aplysia californica* catalyzed an alternative mode of cyclization of nicotinamide guanine dinucleotide (NGD) and nicotinamide hypoxanthine dinucleotide (NHD) to form cyclic-GDP-ribose (cGDPR) and cyclic-HDP-ribose (cHDPR) respectively. In these cyclic nucleotides, the newly formed glycosyl bonds are attached onto the N-7 nitrogen of the purine rings instead of the N-1 nitrogen as in cyclic ADP-ribose (cADPR).

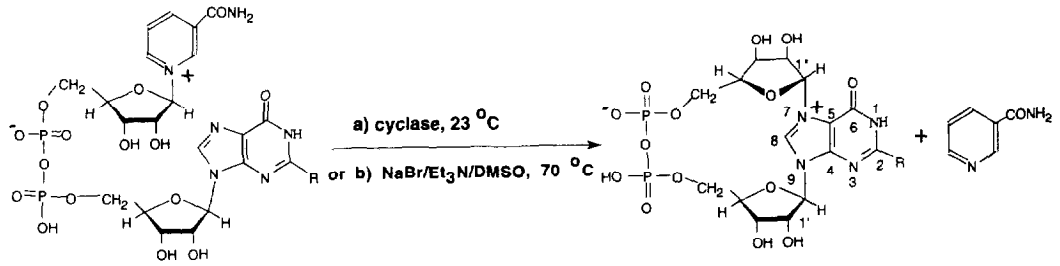
Cyclic ADP-ribose(cADPR), an endogenous metabolite of β-NAD, is a potent mediator of calcium mobilization in many mammalian and invertebrate tissues.[1] Several recent studies suggest that cADPR may play a role as a second messenger, analogous to inositol phosphate (IP3)[2] in Ca^{2+} signaling by interacting with a different intracellular Ca^{2+} channel, possibly the ryanodine receptor.[3]

The structure of cADPR was established as 1 and has now been confirmed by chemical synthesis[4] and X-ray crystallographic analysis.[5]



1

The enzyme that catalyzes the conversion of β-NAD into cADPR is widely distributed among mammalian and invertebrate tissues.[6] A uniquely rich source of cyclase activity is found in the ovotestis of *Aplysia californica*. This cyclase has been purified, and is suited for synthetic applications because it contains virtually no NADase and little cADPR hydrolase activities.[7] In examining the substrate specificity of the *Aplysia* cyclase, we discovered that this enzyme is capable of catalyzing an alternative mode of cyclization. Herein, we describe the enzymatic cyclization of nicotinamide guanine dinucleotide (NGD) and nicotinamide hypoxanthine dinucleotide (NHD) into cyclic-GDP-ribose(cGDPR) and cyclic-HDP-ribose (cHDPR) respectively. In these cyclic nucleotides, the newly formed glycosyl bonds are attached onto the N-7 nitrogen of the purine rings instead of the N-1 nitrogen of the adenine nucleus as in 1.



In a representative experiment, NGD (4 mg, 5.8 μmol) was incubated with 5 units of the *Aplysia* cyclase (Sigma, s.p. 10 units/mg) in 4 ml of 0.01M phosphate buffer, pH 7.0 at 23 °C for 5 h. The enzyme protein was removed by ultrafiltration and the filtrate was concentrated under reduced pressure and then subjected to HPLC on a Waters Nova-Pak C18 column (100 X 8 mm i.d. 4 μm). The column was eluted isocratically with 3 mM TFA at a flow rate of 0.8 mL/min. Fractions containing cGDPR (retention time 4.0 min) were combined and concentrated to yield 2.9 mg of cGDPR (90%) as a white solid. ^{1}H NMR (300 MHz, D_2O, pH 3.5) δ 4.00-4.11 (2H, m), 4.19-4.54 (8H, m), 5.96 (1H, d, J =3.0 Hz, H1′ or H1″), 6.19 (1H, bs, H1′ or H1″), 8.86 (1H, s, H 8); ^{13}C NMR: δ 156.7, 155.5, 151.1,133.6, 108.2, 93.8, 90.8, 84.7, 84.2, 76.8, 76.4, 69.8, 68.5, 65.5, 64.9; UV λ_{max} (pH 1 or 7) 280 nm (ϵ 8,000) and 257 nm (ϵ 13,000). FAB MS m/e 558 $(M+H)^+$.

By analogy to the transformation of NAD into **1**,[1c] we surmised that a cyclic nucleotide is similarly generated via the formation of a new glycosyl bond. This supposition is supported by its ^{1}H NMR spectrum showing the presence of the singlet at δ 8.86, corresponding to the H-8 proton of the guanine ring, and the FAB mass spectrum exhibited a (M+1) ion at m/e 558. There are four potential sites that the anomeric carbon of the ribose could conceivably attach to the guanine ring: N-1, N-3, N-7 or O-6. Although Lee and coworkers[8] recently proposed that the structure of cGDPR is similar to cADPR except with guanine replacing adenine, no experimental data was furnished to support this structural assignment. Hence, a series of NMR techniques (^{1}H,

^{13}C, H-H COSY and HMBC) were employed to deduce its chemical identity.

The 2D HMBC spectrum of cGDPR showed that the H1′ and H1″ protons (δ 5.96 and/or 6.19) showed intense cross peaks due to their respective three-bond couplings with C8 at δ 133.6. Also, H8 the only aromatic proton exhibited three-bond couplings with C1′ and C1″ (δ 94.3 and/or 91.9). These data confirm that the newly formed glycosyl bond is attached onto the N-7 position of the guanine ring and not the N-1 position. The main ^{1}H-^{13}C connectivities for cGDPR are summarized in Table 1, from which the skeletal structure of cGDPR was unambiguously assigned.

Table 1. ^{1}H-^{13}C connectivities for cGDPR established by HMBC spectrum

	^{13}C (δ)	**H8**	**H1′ or H1″**	**H1″ or H1′**
C4	151.1	HCNC		
C5	108.2	HCNC		
C8	133.6	HC	HCNC	HCNC
C1′ or C1″	93.8	HCNC	HC	
C1″ or C1′	90.8	HCNC		HC

Using the same reaction conditions, NHD was transformed by the *Aplysia* cyclase into cHDPR in 81% yield. ^{1}H NMR (300 MHZ, D_2O, pH 3.5): δ 4.00-4.13 (2H, m), 4.29 (4H, bs), 4.39-4.59 (4H, m), 6.16 (1H, d, J=3.0 Hz, H1′ or H1″), 6.36 (1H, s, H1″ or H1′), 8.30 (1H, s, H2), 9.24 (1H, s, H8); ^{13}C NMR: δ 154.7, 151.5, 148.0, 135.9, 115.0, 94.3, 91.9, 85.0, 84.2, 77.1, 76.7, 69.6, 68.4, 65.2, 64.7; UV λ_{max} (pH 7.0) 253 nm (ε 1,000). FAB MS m/e 543 $(M+H)^{+}$.

The proton-decoupled ^{13}C spectrum of cHDPR exhibited signals for C6 (δ 154.7), C5 (δ 115.0), C4 (δ 148.0), C2 and C8 (δ 151.5 and 135.9) and signals for ribosyl carbons in the region of δ 60-100. These assignments are further confirmed by its HMBC spectrum. Both C2 and C8 showed one bond couplings with the protons attached to them. The distinction of C2 and C8 was made because H8 is coupled to C5 and H2 is coupled to C6. The long-range couplings also allowed the assignment of the C4 signal since it is the only carbon with detectable coupling to both H2 and H8. Having assigned all the purine ring protons and carbons, the location of the new glycosyl bond could be clearly defined. The three-bond couplings between H8 and C1′ and C1″ and between C8 and H1′ and H1″ clearly show that the newly formed glycosyl linkage is attached onto the N-7 position of the hypoxanthine ring.

It is interesting to note that these novel cyclic nucleotides are structurally identical to the products obtained by the non-enzymatic cyclization of NHD and NGD reported by Yamada and coworkers.[4] Since alkylation of adenosine with methylating agents afforded 1-methyladenosine,[9] it is not surprising that both enzymatic and nonenzymatic cyclization occurred with the N-1 nitrogen of the adenine ring to give cADPR, **1**,

as the only cyclized product. In the case of guanine and hypoxanthine, it is the N-7 position of these bases that is the most nucleophilic site towards alkylation.[9] Hence, it is reasonable to expect that the putative oxocarbenium ion intermediates are intercepted by the N-7 nitrogen of their respective heterocyclic bases to generate novel cyclic nucleotides.

In conclusion, we have shown that the *Aplysia* cyclase catalyzed the conversion of NGD and NHD into the novel cyclic nucleotides, cGDPR and cHDPR respectively. The newly formed glycosyl linkages are attached onto the N-7 position of the purine rings and not to the N-1 position of the guanine ring as was proposed.[8] This alternative mode of cyclization provides one with more flexibility for chemoenzymatic synthesis of novel analogs of cADPR. While both cGDPR and cHDPR are much less active than cADPR in inducing calcium release in the rat microsomal system,[10] their novel ring structures are more stable than cADPR towards heat and enzymatic (NADase) hydrolysis. Moreover, their unique fluorescent properties provide investigators with a useful tool for monitoring the cyclase reaction in crude homogenates.

Acknowledgments: This work was supported by National Institutes of Health grant GM 331449.

References and Notes

1. (a) Clapper, D. L.; Walseth, T. F..; Dargie, P. J.; Lee, H. C. *J. Biol. Chem.* **1987**, *262*, 9561-9568. (b) Lee, H. C.; Walseth, T. F.; Bratt, G. T.; Hayes, R. N.; Clapper, D. L. *J. Biol. Chem.* **1989**, *264*, 16008-1615. (c) Gu, Q. M.; Sih, C. J. *J. Amer. Chem. Soc.* **1994**, *116*, 7481-7486. (d) Lee, H. C. *Mol. & Cell. Biochem.* **1994**, *138*, 229-235.

2. Berridge, M. J. *Nature* **1993**, *361*, 315-325.

3. (a) Putney, J. W., Jr. *Science* **1993**, *262*, 676-678. (b) White, A. M.; Watson, S. P.; Galione, A. *FEBS* **1993**, *318*, 259-263. (c) Lee, H. C. *J. Biol. Chem.* **1993**, *268*, 293-299.

4. Yamada, S.; Gu, G. M.; Sih, C. J. *J. Amer. Chem. Soc.* **1994**, *116*, 10787-10788.

5. Lee, H. C.; Aarhus, R.; Levitt, D. *Structural Biol.* **1994**, *1*, 143.

6. (a) Walseth, F. T.; Aarhus, R.; Zelenikar, R. J., Jr.; Lee, H. C. *Biochim. Biophys. Acta* **1991**, *1094*, 113-120. (b) Rusinko, N.; Lee, H. C. *J. Biol. Chem.* **1989**, *264*, 11725-11731.

7. Glick, D. L.; Hellmich, M. R.; Beushansen, S.; Tempst, P.; Bayley, H.; Strumwasser, F. *Cell Regul.* **1991**, *2*, 211-218.

8. Graeff, R. M.; Walseth, T. F.; Fryxell, K.; Branton, W. D.; Lee, H. C. *J. Biol. Chem.* **1994**, *269*, 300260-30267.

9. Jones, J. W.; Robins, R. K. *J. Am. Chem. Soc.* **1963**, *85*, 193-201.

10. White, A. M.; Watson, S. P.; Galione, A. *FEBS Letters* **1993**, *318*, 259-263.

(*Received in USA* 20 *September* 1995; *revised* 18 *October* 1995; *accepted* 20 *October* 1995)