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Chemical Synthesis and Calcium Release Activity of N^1-Ether Strand Substituted cADPR Mimic

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Abstract—8-Chloro cyclic inosine 5′-diphosphate ethoxymethyl ether **3** was synthesized by means of chemical method from protected inosine via phenylthio-type biphosphate substrate. The detection of Ca^{2+} release activity shows that **3** is a potent agonist of cADPR and has activity in intact Hela cells.

Cyclic ADP-ribose (cADPR, **1**), a metabolite of NAD^+, is of great interest because of its significant biological importance as a physiological modulator of ryanodine receptor.[1] Accordingly, many cADPR analogues have been synthesized from NAD^+ analogues by the ADP-ribosyl cyclase (ADPR-cyclase) and used for the studies on the mechanism of cADPR mediated Ca^{2+} release pathway.[2] The most structural modifications of cADPR focused on the adenosine moiety and pyrophosphate unit.[3] Due to the inherent substrate specificity of ADPR-cyclase, different groups have worked on the chemical synthesis of cADPR analogues (Fig. 1).[4,5]

The only effort related to the N^1-ribosyl-modified cADPR analogue, a carbocyclic mimic of cADPR **2a** and **2b**, was reported by Matsuda's group.[6] The biological data of **2b** showed that **2b** caused a significant release of Ca^{2+} in sea urchin eggs via micro-injection. Among the analogues of cADPR, none of the 8-substituted analogues synthesized has Ca^{2+} release activity in sea urchin egg homogenates, but most of them block cADPR from releasing Ca^{2+}.[7] On the contrary, cyclic aristeromycin-diphosphate-ribose, cyclic ATP-ribose and 7-deaza-cADPR have been shown to be agonistic analogues of cADPR.[8–10] Some analogues were found to be cell permeant and resistant to both heat and enzymatic hydrolysis.[11,12] To investigate the relationships between the structure and biological activities of cADPR, it would be interesting to synthesize the N^1-ribosyl-modified cADPR analogues.The present study introduced a facile synthesis of the N^1-ribose ring modification, that is an N^1-acyclic ether chain analogue, and it was found that the N^1-acyclic mimic **3** showed some Ca^{2+} release activity in Hela cells.

According to the synthetic strategy of cADPR, N-1 substitution and intramolecular cyclization are the key steps. To date, the most conventional preparations of acyclic nucleoside involved an N-9 substitution of purine with 2-chloromethoxyethyl acetate in the presence of a base such as K_2CO_3 or NaH as well as in situ substitution with trimethylsilyl iodide and 1,3-dioxolane.[13,14] Unfortunately, the N-1 substituted derivative **5** was not obtained by the described methods due to the weak nucleophilicity of this position. Interestingly, the reaction of protected inosine **4**[6a] with 2-chloromethoxyethyl

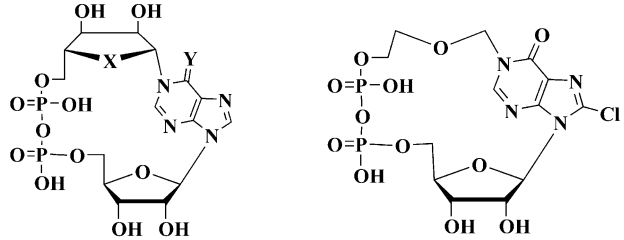

Figure 1. Structure of cADPR **1**, cyclic IDP-carbocyclic ribose **2a**, cyclic ADP-carbocyclic ribose **2b** and N^1-ether strand analogue **3**.

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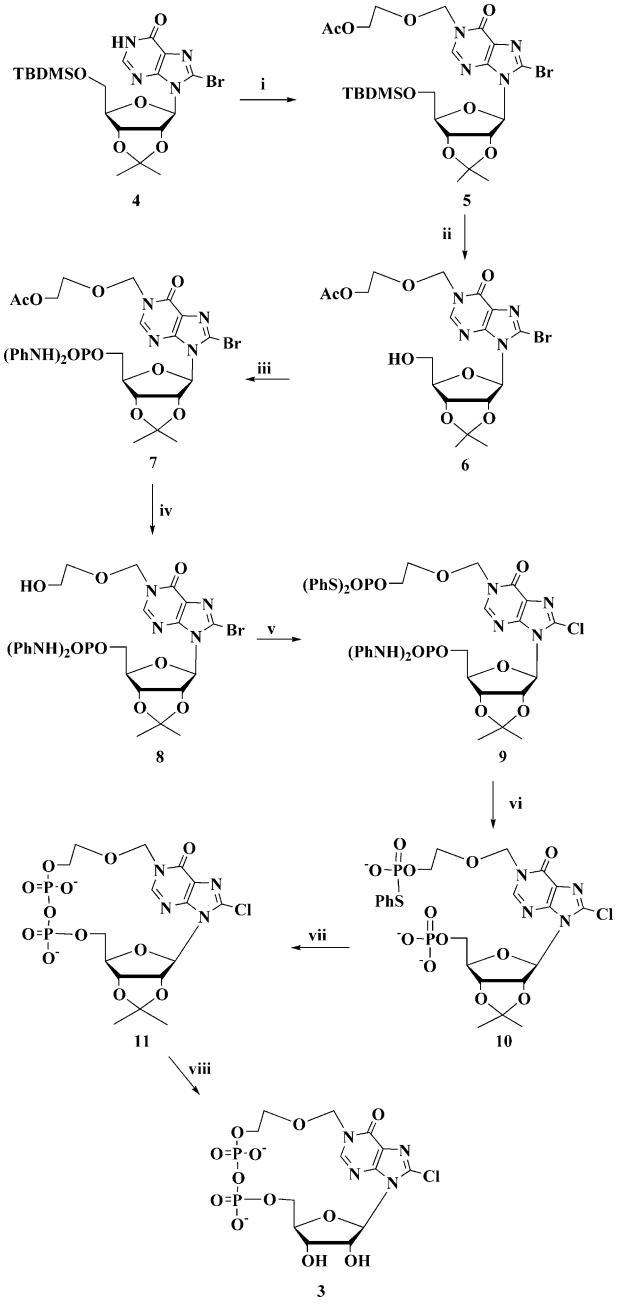

Scheme 1. Reagents and conditions: (i) DBU, $ClCH_2OCH_2CH_2OAc$, CH_2Cl_2, rt; (ii) TBAF in THF, rt; (iii) $(PhNH)_2POCl$, Py, tetrazole, rt; (iv) CH_3ONa, CH_3OH, rt; (v) PSS, TPS, tetrazole, Py, rt; (vi) (a) isoamyl nitrite, Py/AcOH/Ac_2O (2:1:1), rt; (b) H_3PO_2, Et_3N, Py, rt; (vii) I_2, 3Å MS, Py, rt; (viii) 60% aqueous HCO_2H, rt.

acetate in the presence of excess DBU afforded regioselectively the desired **5** in 82% yield. The structure of N^1-substituted ether strand was determined by the spectra of UV and ^1H NMR. The *tert*-butyldimethylsilyl (TBDMS) group of **5** was selectively removed and the resulting 5′-primary hydroxy was reacted with $(PhNH)_2POCl$ to give phosphoroamide **6** in 84% yield. Deacetylation of **7** with CH_3ONa gave the monohydroxy compound **8**, which was transformed into the corresponding biphosphate **9**[15] by treatment with cyclohexylammonium *S*,*S*-diphenylphosphorodithioate (PSS), triisopropylbenzenesulfonyl chloride (TPS) and tetrazole in pyridine in 48% yield over two steps. However, MALDI-TOF MS of **9** showed that the bromo group was replaced with chloro group during the phosphorylation reaction (Scheme 1).

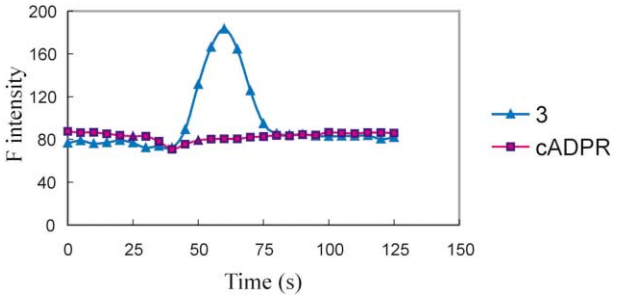

Figure 2. Effect of **3** and cADPR on intracellular Ca^{2+} dynamic changes in intact Hela cells. The data shown are typical curves for at least two experiments carried out in duplicate using different Hela cell preparations.

The successive removal of diphenylamino group and phenylthio group of **9** with isoamyl nitrite in a mixed solvent of pyridine–AcOH–Ac_2O and H_3PO_2 gave **10** for intramolecular condensation in 67% yield. The intramolecular cyclization of **10** was performed under the promotion of I_2 and 3Å MS in pyridine via a syringe pump according to Matsuda's method.[16] The desired cyclic product **11**[17] as a triethylammonium salt was obtained after purification by HPLC in 43% yield. The cyclic structure of **11** was characterized by the data of HR-FABMS and ^{31}P NMR. Deprotection of cyclic product **11** provided the compound **3**[18] in 45% yield in 60% HCO_2H solution.

In human Hela cells transfected with CD 38, a type II transmembrane glycoprotein, cADPR is shown to play a role in regulating the cell doubling time.[19] It is interesting to know if the cellular Ca^{2+} level correlates with the cADPR level in Hela cells. We measured the cellular Ca^{2+} level in Hela cells by cofocal system after incubation with cADPR and analogue **3,** respectively. In this study, the calcium level was represented by relative fluorescence intensity of Fluo-3. The suspension at a density of 2×10^5 cells/mL was attached to a 35-mm dish coated glass coverslips for 18 h. The packed cells were incubated in HEPES buffer solution containing (mM): 125 NaCl, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 2 $CaCl_2$, 6 glucose, 25 HEPES [pH 7.4 containing 20 μM Fluo-3 AM (Molecular Probe)] at 37 °C in dark for 30 min. The cells were washed twice and then incubated for another 30 min in a dye-free-HEPES solution. The Fluo-3 fluorescence measurements were carried out using a Leica TCS NT cofocal laser-scanning microscope. The results showed that compound **3** could cause the abrupt fluorescence increase in Hela cells after exposure to 100 μM **3** for about 30 s. But cADPR cannot induce an efficient increase in Fluo-3 fluorescence within 10 min under the same concentration (Fig. 2). By combining both the modifications on the N^1 and 8-position of cADPR, we found that compound **3** is a potent agonist of cADPR and has activity in intact Hela cells. This is the first study

to implicate that the N^1-ribosyl may not have a crucial role for the Ca^{2+} release activity of cADPR analogues and **3** should be a useful tool to investigate the cADPR signaling pathway.

Acknowledgements

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15. **9**: ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.26, 1.51 (each 3H, each s, $(CH_3)_2C$–), 3.81 (2H, m, H-5′), 4.06–4.36 (5H, m, –OCH_2CH_2O–, H-4′), 5.06 (1H, m, H-3′), 5.31 (1H, m, H-2′), 5.42–5.53 (2H, m, –OCH_2O–), 6.03 (1H, d, J = 1.8 Hz, H-1′), 6.72–7.52 (20H, m, arom H), 8.02 (1H, d, J = 9.9 Hz, NH), 8.10 (1H, d, J = 9.9 Hz, NH), 8.30 (1H, s, H-2). ^{13}C NMR (75 MHz, DMSO-d_6): δ 25.20, 26.00, 64.17, 67.04, 68.14, 75.12, 80.92, 82.87, 85.60, 90.62, 113.86, 117.26, 120.50, 123.75, 124.36, 125.60, 126.40, 127.26, 127.69, 128.35, 128.67, 128.82, 129.57, 129.76, 133.34, 135.12, 140.93, 141.08, 147.96, 149.12, 154.78. ^{31}P NMR (121 MHz, DMSO-d_6): δ 2.65 (s), 51.58 (s); m/z 933 $(M+Na)^+$, 949 $(M+K)^+$. Anal. calcd for $C_{40}H_{41}ClN_6O_9P_2S_2$: C, 52.72; H, 4.53; N, 9.22. Found: C, 53.12; H, 4.72; N, 9.26.
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17. **11**: ^{1}H NMR (500 MHz, D_2O): δ 1.28, 1.45 (each 3H, each s, CH_3×2), 3.61 (1H, m, H-5′a), 3.70 (2H, m, OCH_2CH_2O–), 3.75 (3H, m, CH_2, H-5′b), 4.40 (1H, m, H-4′), 5.08 (1H, d, J = 11 Hz, –OCHN–), 5.30 (1H, dd, J = 2.5, 6.5 Hz, H-3′), 5.71 (1H, d, J = 11 Hz, –OCHN–), 5.81 (1H, d, J = 2.5 Hz, H-2′), 6.22 (1H, s, H-1′). ^{31}P NMR (D_2O, 121 MHz): δ −9.04 (d, J = 15.7 Hz), −9.86 (d, J = 15.7 Hz); high resolution m/z calcd for $C_{16}H_{19}ClN_4O_{12}P_2$: 557.0247 $(M-H)^-$. Found: 557.0254.
18. **3**: ^{1}H NMR (500 MHz, D_2O): δ 3.69 (3H, m, H-5′a, CH_2), 3.80 (2H, m, CH_2), 3.92 (1H, dd, J = 3.0, 9.0 Hz, H-5′b), 4.18 (2H, m, CH_2), 4.64 (1H, m, H-4′), 4.75 (1H, m, H-3′), 5.18 (1H, d, J = 11 Hz, –OCHN–), 5.52 (1H, t, J = 5.0 Hz, H-2′), 5.85 (1H, d, J 11 Hz, –OCHN–), 5.98 (1H, d, J = 5.0 Hz, H-1′). ^{31}P NMR (D_2O, 121 MHz): δ −10.44, −10.79; high resolution m/z calcd for $C_{13}H_{15}ClN_4O_{12}P_2$: 5169934 $(M-H)^-$. Found: 516.9925.
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