Pergamon

*Bioorganic & Medicinal Chemistry Letters*, Vol. 7, No. 13, pp. 1753-1756, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00306-5

# 8-(6-AMINOHEXYL) AMINO-CYCLIC ATPR: A NEW AFFINITY PROBE FOR THE STUDY OF CYCLIC ADPR-BINDING PROTEINS

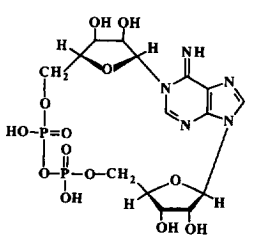
Fang-Jie Zhang and Charles J. Sih*

*School of Pharmacy, University of Wisconsin-Madison,*
*425 N. Charter Street, Madison, WI 53706-1515*

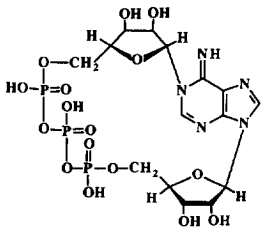
**Abstract:** 8-(6-Aminohexyl) amino-cyclic ATPR, a new affinity probe for the study of cADPR-binding proteins, was prepared in four steps with an overall yield of 21%. © 1997 Elsevier Science Ltd.

Mobilization of intracellular $Ca^{2+}$ is regulated by two major mechanisms. Activation of surface receptor stimulates the phosphoinositide pathway resulting in the formation of inositol triphosphate ($IP_3$), a second messenger for releasing $Ca^{2+}$ from the endoplasmic reticulum.[1] The second mechanism for $Ca^{2+}$ mobilization is the $Ca^{2+}$ induce $Ca^{2+}$ release mechanism (CICR), which is mediated by the ryanodine receptor.[2] Although caffeine and ryanodine have been shown to modulate CICR, the endogenous regulator of this mechanism is yet to be established definitively.

Cyclic ADP-ribose (cADPR), a metabolite of β-$NAD^+$, is an endogenous mediator of calcium mobilization in mammalian and invertebrate tissues.[3] Recent studies have shown that cADPR may function as an endogenous regulator of the CICR mechanism by activating the ryanodine receptor in a manner analogous to the activation of $IP_3$ receptor by $IP_3$.[4] To gain insights into the physiological functions of cADPR, it is necessary to isolate and characterize the cADPR-binding proteins, which in turn may shed light on the putative role of cADPR



**cADPR**

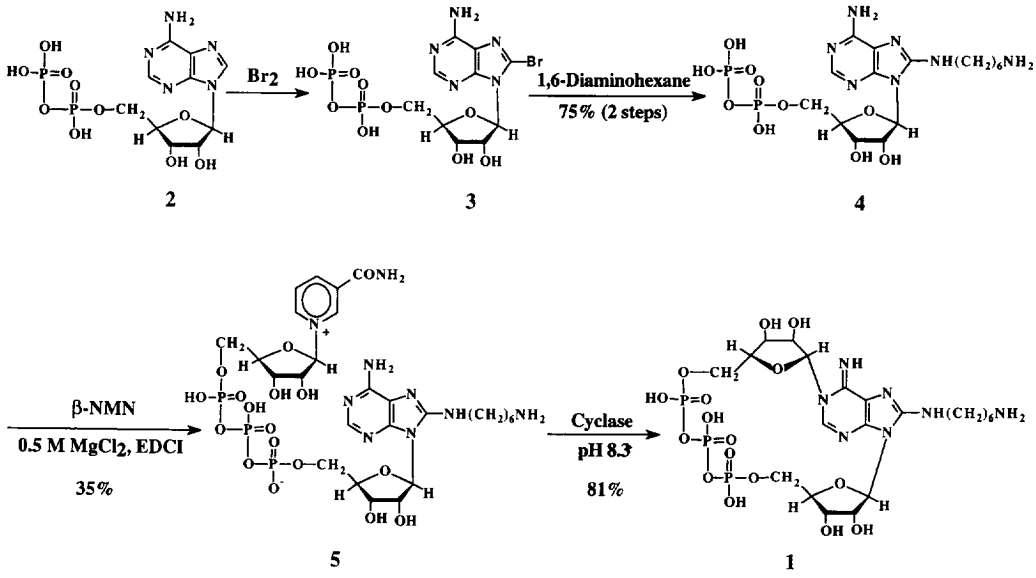


**cATPR**

as a second messenger. Although the identification of cADPR-binding proteins in sea urchin egg by photoaffinity labeling has been reported,[5] no isolation of cADPR-binding proteins from mammalian tissues has yet been achieved.

Affinity column chromatography is an useful method for the isolation of ligand binding proteins. To purify cADPR-binding proteins , a cADPR affinity probe should be of the first choice. However, our previous studies[6] showed that cADPR was easily hydrolyzed by NAD-glycohydrolases (NADases), which could complicate the purification of the binding proteins. On the other hand, cyclic ATPR, an analog of cADPR was found to be twenty times more potent than cADPR in inducing $Ca^{2+}$ release and was remarkably more stable to the hydrolytic action of NADases. More importantly both cADPR and cATPR induced $Ca^{2+}$ release in the rat brain microsomal system via the same mechanism,[6] which suggested that it should be possible to isolate and characterize cADPR-binding proteins using the more stable and active analog, cATPR, as an affinity probe. Herein we report the synthesis of 8-(6-aminohexyl) amino-cATPR (**1**), a new affinity probe for the study of cADPR-binding proteins.

## Scheme 1




As shown in Scheme 1, 8-(6-aminohexyl)amino-ADP (**4**) was a key intermediate in the synthesis of compound **1**. According to the published procedure,[7] the preparation of **4** was a tedious process involving six steps and the total yield was only 30%. Moreover, in this procedure, most steps needed chromatographic purification of the products. Hence, a simpler and more efficient method was needed to synthesize **4** on a large scale. After examination of the literature method, we surmised that AMP was used as the starting material only because of the decomposition of the ADP analogs under the aminolysis condition (DMSO-$H_2O$, 80 °C) used. Therefore, we conducted the aminolysis reaction under very mild conditions and successfully developed a one-

pot two step process for the preparation of **4**, using ADP (**2**) as the starting material. Compound **2** was brominated in 0.2 M sodium acetate (pH 4.5) to furnish 8-bromo-ADP (**3**) in high yield (>95% by HPLC). The crude **3** was reacted with excess 1,6-diaminohexane in water at 23 °C. After the reaction had proceeded for 48 h, HPLC analysis showed that more than 95% of **3** had reacted and less than 8% of 8-(6-aminohexyl)amino-AMP was generated due to the decomposition of **3** and **4**. Column chromatographic purification (Dowex 1 × 8 (acetate form)) of the reaction mixture afforded **4** in 75% yield for the two step procedure.[8] The coupling of **4** to β-nicotinamide mononucleotide gave 8-(6-aminohexyl)amino-5'-triphosphopyridine nucleotide (**5**)[9] in 35% yield. Compound **5** was then cyclized by exposure to ADP-ribosylcyclase from *Aplysia californica* to give **1** in 81% yield.[10]

With compound **1** in hand, the study of the binding proteins could now be conducted. The free amino group in **1** will provide researchers with many options for conjugation to fluorescence and radiolabeled probes as well as agarose resins.[11] The purification of cADPR-binding proteins from rat brain microsomes is currently in progress in our laboratory.

**ACKNOWLEDGMENT:** This investigation was supported by National Institute of Health grant GM 331449.

## REFERENCES AND NOTES

1. Berridge, M. J.; Irvine, R. F. *Nature* **1989**, *341*, 197
2. Takeshima, T.; Nishimura, S.; Matsumoto, T.; Ishida, H.; Kangawa, K.; Minamino, N.; Matsuo, H.; Ueda, M.; Hanaoka, M.; Hirose, T.; Numa, S. *Nature* **1989**, *339*, 439
3. White, A. M.; Watson, S. P.; Galione, A. *FEBS Lett.* **1993**, *318*, 259
4. Galione, A. *Mol. and Cell. Endocrinol.* **1994**, *98*, 125
5. Walseth, T. F.; Aarhus, R.; Kerr, J. A.; Lee, H. C. *J. Biol. Chem.* **1993**, *268*, 26686
6. Zhang, F. J.; Yamada, S.; Gu, Q. M.; Sih, C. J. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 1203.
7. Tryer, I. P.; Trayer, H. R.; Small, D. A. P.; Bottomley, R. C. *Biochem. J.* **1974**, *139*, 609
8. Preparation of **4**: ADP (0.5 g, 1.1 mmol) was dissolved in 20 mL of 0.2 M sodium acetate (pH 4.5). Bromine (0.15 mL, 2.9 mmol) was added in portions and the mixture was vigorously stirred in the dark at 23 °C for 2 h. At this time the reaction was more than 95% complete as determined by HPLC. The unreacted bromine was removed by repeated extraction with $CCl_4$. The aqueous layer was lyophilized to dryness. The lyophilized powder was dissolved directly in a solution of 1,6-diaminohexane (9 g in 10 mL of water), and the mixture was stirred at 23 °C for 48 h. The solvent was then removed under reduced pressure and the residue was suspended in 40 mL of anhydrous ethanol. After centrifugation, the solid was collected and washed with 40 mL of acetone. The crude product was dissolved in 40 mL of water and applied onto a 2.5 × 25 cm column of Dowex 1 × 8 (acetate form). The column was eluted with a linear gradient of 500 mL of water and 500 mL of 1M acetic acid. Fractions of 20 mL were collected, and those containing an absorbance maximum at 280 nm (fractions 19-35) were pooled and lyophilized to give **4** as a yellowish powder (75% yield). $^1H$ NMR ($D_2O$, 300 MHz, pH 2.5) δ 1.40 (m, 4H), 1.67 (m, 4H), 2.98 (t, $J$ = 7.0 Hz, 2H), 3.45 (m, 2H), 4.23-4.38 ( m, 3H), 4.46 (m, 1H), 4.59 (m, 1H), 5.95 (brs, 1H), 8.23 (s, 1H).

9. Preparation of **5**: Compound **4** (81 mg, 0.15 mmol) and β-nicotinamide mononucleotide (72 mg, 0.22 mmol) were mixed homogeneously with 1.0 M $MgCl_2$ (370 μL, 0.37 mmol) and then 370 μL of 3.4 M Hepes-NaOH (pH 6.8) and 360 mg of EDCI were added in turn. The mixture was incubated with stirring at 37 °C for 17 h. The reaction mixture was diluted 100 times with water and applied onto a 2.5 × 70 cm column of Dowex 1 × 8 (acetate form). The column was eluted with a linear gradient formed from 800 mL of water and 800 mL of 1 M acetic acid. Fractions of 20 mL were collected, and those containing an absorbance maximum at 274 nm (fractions 25-35) were pooled and lyophilized. Compound **5** was obtained as a yellowish powder (35% yield). $^1H$ NMR ($D_2O$, 300 MHz, pH 2.5) δ 1.44 (m, 4H), 1.70 (m, 4H), 3.07 (t, $J$ = 7.0 Hz, 2H), 3.50 (m, 2H), 4.28-4.90 (m, 10H), 6.02 (d, $J$ = 7.0 Hz, 1H), 6.22 (d, $J$ = 5.4 Hz, 1H), 8.27 (s, 1H), 8.34 (m, 1H), 8.95 (d, $J$ = 8.0 Hz, 1H), 9.34 (d, $J$ = 7.0 Hz, 1H), 9.47 (s, 1H). UV $\lambda_{max}$ (pH 6.0) 274 (ε 18,000) nm. FAB MS *m/e* 858 ($[M]^+$).
10. Preparation of **1**: Compound **5** (7.0 mg, 8.1 μmol) was incubated with ADP-ribosylcyclase (5 units, Sigma) from *Aplysia californica* in 8 mL of 0.1 M $NaHCO_3$ (pH 8.3) at 23 °C for 3 h. The mixture was ultrafiltered and the filtrate was separated by HPLC on a Waters Nova-Pak $C_{18}$ column (100 × 8 mm id, 4 μm). The column was eluted isocratically with 5% acetonitrile in 3 mM TFA at a flow rate of 1 mL/min. Fractions containing **1** (retention time 6.2 min) were collected and lyophilized to give **1** as a white solid in 81% yield. $^1H$ NMR ($D_2O$, 300 MHz, pH 2.5) δ 1.45 (m, 4H), 1.69 (m, 4H), 2.98 (m, 2H), 3.39-3.62 (m, 2H), 4.15-4.90 (m, 9H), 5.54 (brs, 1H), 5.87 (brs, 1H), 6.14 (brs, 1H), 8.81 (s, 1H). UV $\lambda_{max}$ (pH 6.0) 280 (ε 15,300) nm. FAB MS *m/e* 737 ($[M+2H]^+$), 770 ($[M+2H+Na]^+$).
11. Preparation of the affinity column: Compound **1** (1.4 mg, 19 μmol) was incubated with pre-swelled cyanogen bromide activated sepharose 4B (3.5 mL) ( from Sigma) in 0.1 M sodium bicarbonate (pH 8.3, 20 mL) at 4 °C for 16 h. UV analysis of the solution after filtration revealed that 1.3 mg of **1** was attached onto the resin and HPLC analysis showed that free **1** in the solution remained unchanged. The excess reactive sites on the resin were destroyed by treatment with excess aminoethanol. Incubation of compound **1** in 0.1 M sodium bicarbonate at 4 °C to 23 °C for 24 h did not show significant decomposition from HPLC analysis.

(*Received in USA* 16 *April* 1997; *accepted* 27 *May* 1997)