Supporting Information

for

8-pCPT-2'‐O-Me-cAMP-AM: An Improved Epac-Selective cAMP Analogue

Marjolein J. Vliem, Bas Ponsioen, Frank Schwede, Willem-Jan Pannekoek, Jurgen Riedl, Matthijs R. H. Kooistra, Kees Jalink, Hans-Gottfried Genieser, Johannes L. Bos,* and Holger Rehmann*

Synthesis of 8-(4-Chlorophenylthio)-2’-O-methyladenosine-3’,5’-cyclic monophosphate, acetoxymethyl ester (8-pCPT-2’-O-Me-cAMP-AM): Synthesis was performed as described previously with some minor modifications.[S1] 360 µmol 8-pCPT-2’-O-Me-cAMP, diisopropylethylammonium salt, were suspended in 1000 µL DMF. After addition of 1800 µmol (180 µL; 5 equiv) acetoxymethyl bromide and 1080 µmol (185 µL; 3 equiv) diisopropylethylamine, the reaction mixture was stirred at ambient temperature for 30 minutes. Progress of AM-ester formation was monitored by analytical HPLC with 40% acetonitrile as eluent. The reaction was stopped by evaporation of all volatile components in a speed-vac centrifuge under reduced pressure with oil pump vacuum. The residues were re-dissolved in 0.8 mL DMF, and purified by preparative HPLC using 40% acetonitrile as eluent. The product-containing fractions were collected and evaporated. 110 µmol 8-pCPT-2’-O-Me-cAMP-AM were isolated as mixture of axial and equatorial isomers with a purity of >97% (yield: 30.6%). Small amounts of pure isomers for NMR experiments were generated by repeated analytical HPLC runs.
Formula: $C_{20}H_{21}ClN_5O_8PS$ ($M_w$: 557.9); ESI-MS pos. mode: $m/z$ 558 (M+H)$^+$, $m/z$ 486 (M-AM+H)$^+$, neg. mode: $m/z$ 556 (M-H)$^-$, $m/z$ 484 (M-AM-H)$^-$$^+$; UV/Vis (pH 7.0) $\lambda_{max}$ 282 nm ($\varepsilon = 16000$).

NMR data of the axial isomer of 8-pCPT-2’-O-Me-cAMP-AM: $^{31}$P NMR ([D$_6$]DMSO) heteronuclear decoupled: $\delta = -6.82$ ppm; $^1$H NMR ([D$_6$]DMSO): $\delta = 2.11$ (s, 3H, -CH$_3$), 3.23 (s; 2’-O-CH$_3$), 4.05 – 4.73 (m, 4H; H-2’, H-4’, H-5’, H-5’’), 5.52 – 5.77 (m, 3H; -CH$_2$-, H-3’), 6.13 (s, 1H; H-1’), 7.30 – 7.47 (m, 4H, phenyl), 7.63 (s, 2H; 6-NH$_2$), 8.17 (s, 1H; H-2) ppm.

NMR data of the equatorial isomer of 8-pCPT-2’-O-Me-cAMP-AM: $^{31}$P NMR ([D$_6$]DMSO) heteronuclear decoupled: $\delta = -4.72$ ppm; $^1$H NMR ([D$_6$]DMSO): $\delta = 2.10$ (s, 3H, -CH$_3$), 4.13 – 4.75 (m, 4H; H-2’, H-4’, H-5’, H-5’’), 5.52 – 5.70 (m, 3H; -CH$_2$-, H-3’), 6.10 (s, 1H; H-1’), 7.31 – 7.47 (m, 4H, phenyl), 7.62 (s, 2H; 6-NH$_2$), 8.22 (s, 1H; H-2) ppm.

All chromatographic experiments were performed at ambient temperature. The analytical HPLC-system consisted of a L 6200 pump, a L 4000 variable wavelength UV-detector, and a D 2500 GPC integrator (all Merck-Hitachi, Germany). The stationary phase was Kromasil™ (Eka Nobel, Sweden) C 8-100, 10 µm, in a 250 x 4.6 mm stainless steel column. Preparative HPLC was accomplished with Kromasil™ C 8-100, 5 µm material in a 250 x 16 mm stainless steel column. Mass spectra were obtained with an Esquire LC spectrometer (Bruker, Germany) in the ESI-MS mode with 50 % isopropanol / 49.9 % water / 0.1 % formic acid as matrix. UV spectra were recorded with a Helios β-spectrometer (Spectronic Unicam, United Kingdom) in aqueous phosphate buffer, pH 7.0. $^1$H NMR (internal standard (CH$_3$)$_4$Si) and $^{31}$P NMR (external standard H$_3$PO$_4$) spectra were recorded with a WH-360 spectrometer (Bruker, Germany). All reagents where of analytical grade or the best grade available from commercial suppliers. Esterase from porcine liver (Lot: 123K7033) and partially purified acetyesterase from orange peel (Lot: 78H7445) were obtained from Sigma, Germany.

**Application of 8-pCPT-2’-O-Me-cAMP-AM**: Stock solutions of 8-pCPT-2’-O-Me-cAMP-AM were prepared in absolute DMSO to avoid hydrolysis of the ester at concentrations up to 10 µM. 8-pCPT-2’-O-Me-cAMP-AM was applied directly to the cell culture dish. When required, 8-pCPT-2’-O-Me-cAMP-AM was pre-diluted in PBS buffer immediately prior to the application. Any unnecessary exposure of 8-pCPT-2’-O-
Me-cAMP-AM to medium, or other material, which may contain esterases, was reduced to a minimum.

**Dynamic FRET monitoring**: Cells grown on coverslips and transfected with the FRET probe were placed on an inverted NIKON microscope and excited at 425 nm. Emission of CFP and YFP was detected simultaneously through 470 ± 20 and 530 ± 25 nm band-pass filters. Data were digitised, the FRET was expressed as ratio of YFP to CFP signals and the data were shown as relative change in FRET (normalised ΔFRET). Activation of Epac1 was followed using the FRET probe CFP-Epac1-YFP, and activation of PKA using the PKA regulatory subunit type II fused to CFP and the PKA catalytic subunit fused to YFP.[S2] Binding of cAMP to both probes induces a decrease in FRET.

**Rap1 activation assay**: Rap1 activation assays were performed as previously described.[S3] In brief, the assay makes use of the Ras association (RA) domain of Ral-GDS. This domain binds specifically to the active GTP but not to the inactive GDP bound form of Rap. Cell lysate was incubated with recombinant expressed RA domain of Ral-GDS fused to glutathione S-transferase immobilised on glutathione–agarose beads and bound Rap was analysed by western blotting using Rap1 antibodies (Santa Cruz Biotechnology, USA).

**PKA activation assay**: PKA activity in cells was monitored by the extent of phosphorylation of the PKA substrate VASP. This phosphorylation causes a mobility shift in SDS polyacrylamid gelelectrophoresis. Both VASP and phosphorylated VASP were visualised by western blotting using an antibody against VASP (BD Transduction Laboratories, USA).

**Transendothelial electrical resistance measurements**: Primary human umbilical vein endothelial cells (HUVEC) were seeded at 7x10⁴ cells per well (0.8 cm²) on fibronectin coated electrode arrays and grown to confluency (Applied BioPhysics Inc., Troy, USA). Measurements of transendothelial electrical resistance were performed in real time at 400Hz, 37ºC, 5% CO₂, using an electrical cell-substrate impedance sensing system (ECIS; Applied BioPhysics Inc., Troy, USA).[S4] For comparison between different samples, the resistance is plotted as a difference to the basal level of resistance.

**Adhesion assay**: Jurkat-Epac1 cells transiently transfected with pCMV-luciferase were harvested, resuspended in TSM buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl,
1 мМ CaCl₂, 2 мМ MgCl₂, 0.5% BSA) and gently rotated for 1 hour at 37 °C to allow recovery.[S5] 96-well Nunc Maxisorp plates (Corning, USA) were coated overnight at 4 °C with human serum-purified fibronectin (5 mg/L in PBS) and blocked for 1 h with 1% BSA in TSM. Subsequently, cells were added to the coated wells in the absence or presence of 8-pCPT-2′-O-Me-cAMP or 8-pCPT-2′-O-Me-cAMP-AM at the indicated concentrations, and allowed to adhere for 45 minutes at 37 °C. Non-adherent cells were removed with warmed TSM and adherent cells were lysed at 4 °C in luciferase lysis buffer (15% glycerol, 25 мМ Tris-phosphate pH 7.8, 1% Triton X-100, 8 мМ MgCl₂, 1мМ DTT). Luciferase activity was determined using a luminometer (Lumat LB9507). Samples of input cells were lysed separately to determine luciferase total input counts. Specific adhesion was determined (counts in cells bound / counts in total input x 100) and plotted directly. Error bars represent standard deviation within each experiment.

References


Figure S1. \(^{31}\)P NMR comparison of 8-pCPT-2’-O-Me-cAMP-AM isomers in [D\(_6\)]DMSO. Upper panel: Diastereomeric mixture of 8-pCPT-2’-O-Me-cAMP-AM; Middle panel: purified axial isomer; lower panel: purified equatorial isomer.