Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

Design, Synthesis and Membrane Translocation Studies of Inositol-based Transporters: Novel molecular transporters show unique *in vitro* and *in vivo* spectra [**]

Kaustabh K. Maiti, Ock-Youm Jeon, Woo Sirl Lee, Dong-Chan Kim, Kyong-Tai Kim, Toshihide Takeuchi, Shiroh Futaki, and Sung-Kee Chung[*]

Synthetic Part.

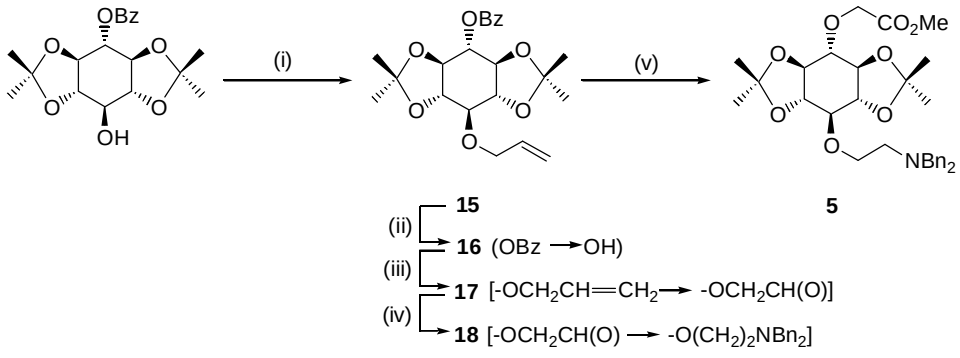
I. Materials and reagents

General Methods. All nonhydrolytic reactions were carried out in oven-dried glassware under an inert atmosphere of dry argon or nitrogen. All commercial chemicals were used as received except for solvents, which were purified and dried by standard methods prior to use. Analytical TLC was performed on a Merck 60 F254 silica gel plate (0.25mm thickness), analytical reverse-phase TLC was performed on a Merck RP-8 F254s and visualization was done with UV light both short and long range (254nm and 365nm), or by spraying with a 5% solution of phosphomolybdic acid or ninhydrine solution followed by charring with a heat gun. Column chromatography was performed on Merck 60 silica gel (70-230 or 230-400 mesh) for normal phase and MPLC was performed on Fluka 100 C_8-reversed phase silica gel. Melting points were determined on a Thomas-Hoover apparatus and uncorrected. NMR spectra were recorded on a Bruker DPX 300 (^{1}H-NMR at 300MHz; ^{13}C-NMR at 75 MHz) and Bruker DRX 500 (^{1}H-NMR at 500MHz; ^{13}C-NMR at 125MHz) spectrometers. Tetramethysilane was used as reference for ^{1}H NMR. Analytical HPLC was performed by Agilent 1100-HPLC Chemstation with an analytical column BU-300 (30nm pore size & 10μm spherical) (4.6mm ID x 25cm). Low resolution mass spectra were determined on a micromass PLATFORM ll (EI and FAB). High resolution mass spectra were determined on a JMS-700, and MALDI-TOF mass spectra on a Voyager-DE STR system at the Korea Basic Science Support Center.

II. Experimentals and spectral data

Final octa-guanidinylated transporter molecules along with doxorubicin conjugate (**1**, **2**, **3a-c**, **4**) were prepared by deprotection of Boc-groups from guanidine moieties of the corresponding protected precursors(reaction scale 25-50 mg). They were obtained as either HCl salts or TFA salts or both by treatment with saturated HCl gas containing ethyl acetate or TFA in dichloromethane (1:1) after usual purification process (**Figure 1**). For further purification of the final compounds (salts), we have used medium pressure column chromatography (MPLC) method on reverse phase silica gel (C_8 reversed phase, H_2O/CH_3CN, 1:1-1:2; all solvent mixtures containing 0.1% TFA), where the fractions containing compounds were concentrated and lyophilized (yield 76-80 % for HCl-salt and 86-94 % for TFA-salt). Final purity of the compounds after MPLC purification were evaluated by analytical HPLC with analytical column (flow rate=1.0ml/min, UV 215nm, $CH_3CN:H_2O$=40:60) Moreover, all intermediates and final target compounds were satisfactorily characterized by high resolution FAB (HRMS) and MALDI-TOF mass analyses.

myo-Inositol was converted into 1-*O*-PMB-2,3:5,6-di-*O*-isopropylidene-*myo*-inositol (PMB=p-methoxybenzyl) and 4-*O*-benzyl-2,3:5,6-di-*O*-isopropylidene-*myo*-inositol,[25] and these two intermediates were coupled employing carbonyldiimidazole and CaH_2. Subsequent treatment of the coupled product with a catalytic amount of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in toluene gave the carbonate-linked dimer. After removal of the isopropylidene protecting groups, the eight hydroxy groups of the dimeric scaffold were exhaustively acylated with N-Boc-ω-aminocaproic acid by using EDC and DMAP in DMF. The 1-*O*-PMB group was removed with ceric ammonium nitrate, and the 1-*O* position was dansylated. Removal of the N-Boc protecting groups exposed the terminal amino residues, which were guanidinylated to obtain the target transporter molecule (**1**) as its trifluoroacetate (TFA) salt, as a pair of diastereomers. Next, a suitable protection of *myo*-inositol followed by inversion of the OH group at C2 by the Mitsunobu reaction provided 1-*O*-Bz-scyllo-inositol (Bz=benzoyl),[26] which was transformed to 1-*O*-PMB-2,3:5,6-di-*O*-isopropylidene-scyllo-inositol and 1-*O*-benzyl-2,3:5,6-di-*O*-isopropylidene-scyllo-inositol. These two intermediates were then coupled under basic conditions and subsequently transformed to the transporter molecule **2** in a similar manner as described for compound **1**.



Supporting Scheme 1. Synthetic route to compound 5

(i) Allyl bromide, Ag_2O, TBAI, CH_2Cl_2, 90%; (ii) NaOMe, MeOH, 95%; (iii) O_3, $NaHCO_3$, CH_2Cl_2-MeOH (6:1), -78 ^{0}C, then PPh_3, 86%; (iv) Bn_2NH, $NaBH(OAc)_3$, DCE(dichloroethane), 76%; (v) $BrCH_2CO_2Me$, Ag_2O, TBAI, CH_2Cl_2, 94%

Compound 1: [Light brownish glassy solid (TFA salt, 16.7 mg, 89 %)]
UV: $?_{max}$ (H_2O): 336.80 nm [e =3500 ($cm^{-1}M^{-1}$)]
^{1}H-NMR (CD_3OD): d 1.25-1.35(m, 48H), 1.50(br.s., 32H), 2.17-2.37(m, 16H), 2.89(s, 6H), 3.14(br.s., 16H), 3.40(br.s., 1H), 4.64(br.s., 2H), 5.13-5.60(m, 11H), 7.21-7.26(m, 5H), 7.47-7.64(m, 3H), 8.02(d, J=7.1Hz, 1H), 8.26(br.s., 1H), 8.64(d, J=7.6Hz, 1H);
^{13}C-NMR (CD_3OD): δ 21.86, 22.97, 23.22, 25.12, 27.43, 27.58, 28.03, 31.92, 32.09, 32.50, 40.13, 43.46, 66.62, 67.18, 67.95, 68.74, 69.47, 70.92, 72.65, 73.47, 73.75, 76.28, 113.90, 114.45, 126.08, 126.29, 127.06, 127.59, 128.59, 129.90, 130.82, 130.88, 137.01, 152.23, 156.86, 159.86, 160.80, 171.24, 171.32, 171.42, 171.68.
MALDI-TOF MS: m/z calcd for $C_{88}H_{143}N_{25}O_{23}SNa$ 1973.30, found 1973.1 $[M+Na]^+$

Compound 2: [Light brownish glassy solid (TFA salt, 17.1 mg, 94 %)]
UV $?_{max}$(H_2O): 330.15 nm [e =3200 ($cm^{-1}M^{-1}$)]
^{1}H-NMR (CD_3OD): d 1.30-1.42(m, 58H), 2.12-2.23(m, 20H), 2.89(s, 6H), 2.96-3.08(m, 18H), 4.56(br.s., 2H,), 4.89-5.27(m, 11H), 7.12-7.22(m, 5H), 7.56-7.63(m, 3H), 8.20-8.22(m, 2H), 8.64(d, J=8.0Hz, 2H); ^{13}C-NMR (CD_3OD) δ 24.37, 25.99, 26.13, 28.48, 41.30, 60.48, 68.11, 74.21, 74.92[some inositol ring carbons are obscured], 126.28, 127.29, 128.42, 157.72, 162.51, 171.26, 172.48, 172.82, 173.06.
MALDI-TOF MS: m/z calcd for $C_{88}H_{143}N_{25}O_{23}SNa$ 1973.30, found 1973.22 $[M+Na]^+$

Compound 3a : [Light yellow foamy solid (HCl-salt, 9.6mg, 76 %), (TFA-salt, 14.8mg, 91 %)]
UV $?_{max}$(H_2O): 495.0 nm [e =15700 ($cm^{-1}M^{-1}$)]
^{1}H-NMR (CD_3OD): d 1.31(br.s., 16H), 1.86(br.s., 16H),2.14-2.16(m, 2H),2.44(br.s., 16H), 3.22-3.31(m, 23H), 3.68-4.28(m, 12H), 5.20-5.34(m, 8H),6.65-7.01(m, 6H),7.19-7.22(m, 2H), 8.30(br.s., 1H);
MALDI-TOF-MS: m/z calcd for $C_{82}H_{121}N_{27}O_{28}SNa$ 1989.08, found 1989.03 $[M+Na]^+$
Analytical HPLC (BU 300): R_t = 3.79 min (Flow rate = 1ml/min, UV 215nm, CH_3CN: H_2O = 40:60); purity 95% +

Compound 3b : [Light greenish yellow foamy solid (HCl-salt, 12.6mg, 77 %), (TFA-salt, 18.1mg, 88 %)]
UV $?_{max}$(H_2O): 490.20 nm [e =19400 ($cm^{-1}M^{-1}$)]
^{1}H-NMR (CD_3OD):d 1.39-1.61(m, 64H), 2.34(br.s., 18H),3.16-3.31(m, 24H),3.67-4.28(m, 12H), 5.28-5.44(m, 8H),6.65-6.93(m, 6H),7.20-24 (m., 2H), 8.26(br.s., 1H); ^{13}C-NMR (CD_3OD) δ 24.59, 24.89, 26.28, 27.33, 28.69, 34.07, 41.44, 61.28, 67.88, 70.21, 72.32, 126.30, 127.91,128.09,130.66,157.60, 162.13, 171.56, 172.03, 173.14, 175.79.
MALDI-TOF-MS: m/z calcd for $C_{98}H_{154}N_{27}O_{28}S$ 2190.50, found 2190.39$[M+H]^+$, m/z calcd for $C_{98}H_{153}N_{27}O_{28}SNa$ 2212.49, found 2212.53$[M+Na]^+$,
Analytical HPLC (BU 300): R_t = 3.69 min (Flow rate = 1ml/min, UV 215nm, CH_3CN: H_2O = 40:60); purity 99% +

Compound 3c : [Light yellowish foamy solid (HCl-salt, 13.2mg, 80%), (TFA-salt, 17.5mg, 86%)]
UV $?_{max}$(H_2O): 488.31 nm [e =16600 ($cm^{-1}M^{-1}$)]
^{1}H-NMR (CD_3OD): d 1.30-1.61(m, 96H), 2.30-2.43(m, 16H), 2.86(br.s., 2H), 3.20-3.39(m, 14H), 3.67-4.18(m, 10H), 5.20-5.34(m, 8H), 6.65-6.79(m, 6H),7.11(br.s., 2H), 8.30(br.s., 1H); ^{13}C-NMR (CD_3OD) δ 25.11, 25.35, 26.23, 27.03, 27.89, 28.66, 29.42, 34.80, 42.15, 44.58, 46.19, 62.11, 68.46, 69.04, 70.69, 72.42, 121.31, 121.92, 126.29, 126.58,127.22, 128.30, 130.40, 157.63, 162.13, 162.59, 171.02, 172.09, 172.48, 173.19.
MALDI-TOF-MS: m/z calcd for $C_{114}H_{186}N_{27}O_{28}S$ 2414.92, found 2414.72 $[M+H]^+$
Analytical HPLC (BU 300): R_t = 3.57 min (Flow rate = 1ml/min, UV 215nm, CH_3CN: H_2O = 40:60); purity 95% +

Compound 4 : [Light reddish glassy solid (HCl-salt, 11.6mg, 76%)]
UV $?_{max}$(H_2O): 490.0 nm [e = 10000 ($cm^{-1}M^{-1}$)]
^{1}H-NMR (CD_3OD): d 1.30-1.60(m, 80H), 2.38(br.s., 16H), 3.19(br.s., 16H), 3.21- 342(m, 14H), 3.53- 3.72(m, 18H), 3.78(br.s., 5H), 3.89-4.21(m, 10H), 5.18-5.34(m, 8H), 7.31-7.59(m, 3H);
MALDI-TOF-MS: m/z calcd for $C_{132}H_{213}N_{28}O_{39}$ 2814.56, found 2815.93 $[M+H]^+$, m/z calcd for $C_{132}H_{212}N_{28}O_{39}Na$ 2836.55, found 2835.67 $[M+Na]^+$,
Analytical HPLC (BU 300): R_t = 3.77 min (Flow rate = 1ml/min, UV 215nm, CH_3CN: H_2O = 40:60); purity 95% +

Peptide Synthesis and Fluorescence Labeling. R8-Fl and Rhodamine-Tat were chemically synthesized by Fmoc (9-fluorenylmethyloxycarbonyl) solid-phase peptide synthesis using Rink amide resin followed by deprotection using trifluoroacetic acid in the presence of ethanedithiol as reported.[10] For the preparation of R8-Fl, Gly-Cys residues are attached to the C-terminus of the R8 peptide. Fluorescent labeling was conducted by the treatment of the peptide with 5-(Iodoacetamido) Fluorescein (Sigma) (1.5 eq) in dimethylformamide/methanol (1:1) for 1.5 h followed by high-performance liquid chromatography (HPLC) purification. For the preparation of Rhodamine-Tat, the rhodamine moiety was introduced on the N-terminus of the peptide resin using 5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester (Molecular Probes) prior to the trifluoroacetic acid/ethanedithiol treatment and HPLC purification. The fidelity of the products was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS). Actual sequences of the synthesized peptides: R8-Fl, RRRRRRRRGC(Fluorescein)-amide; Rhodamine-Tat, Rhodamine-GRKKRRQRRRPPQ-amide.

Bioassay Part

Cell Culture. Human cervical cancer-derived HeLa cells were cultured as exponentially growing subconfluent monolayers on 60-mm dishes in alpha-minimum essential medium (α-MEM) supplemented with 10% (v/v) calf serum. A subculture was performed every 3-4 days. COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplanted with 10% (v/v) fetal bovine serum without antibiotics. The subculture was conducted every 3-4 days using the cells grown to subconfluence. RAW264.7 cells were cultivated as previously described.[17]

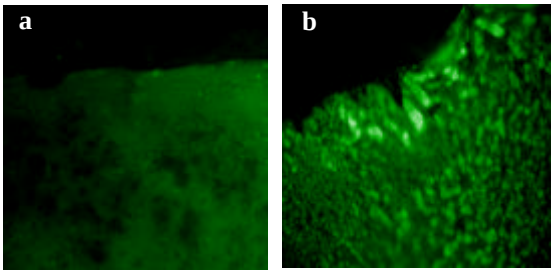
Confocal Laser Scanning Microscopy (CLSM). 2×10^5 HeLa cells were plated into 35-mm glass-bottomed dishes (Iwaki) and cultured for 48 h. After removing the medium, the cells were washed twice with PBS. The cells were incubated with compound **3c** in serum-containing α-MEM. After incubation, the cells were washed with PBS (×5). Distribution of the fluorescently labeled peptides was analyzed ***without fixing*** using a confocal scanning laser microscope (Olympus FV300) equipped with a 40× objective lens. For 4 °C experiments, cells were preincubated in refrigerator (4 °C) for 1 h. Washing and incubation of the cells were then conducted using cold PBS and a 4 °C-refrigerator, respectively, prior to observation of the cells in cold PBS. Rhodamine-transferrin, Rhodamine-Tat, MitoTracker, and LysoTracker are simultaneously added to the cultured media with the vectors (**Fig. 2**). HeLa cells were plated and cultured for 48 h. After removing the medium, the cells were washed with PBS (phosphate buffer solution). The cells were incubated for 15 min at 37 °C in the presence of doxorubicin (control) and compound (**4)** at the concentrations indicated in DMEM. After incubation, the cells were washed with cold PBS (×3), and ***without fixing*** CLSM was performed using a Carl Zeiss LSM 510 Meta Confocal Microscope with a 40× objective lens. FITC fluorescence was excited with the 488 nm line of an argon laser (**Fig. 4A**).

Flow Cytometry. 1.5×10^5 HeLa cells were plated into 12-well microplates (Iwaki) and cultured for 48 h. After removing the medium, the cells were incubated with compound (**3c)** in serum-containing α-MEM. After incubation, the cells were washed three times with PBS containing heparin (0.5 mg/ml), and then incubated with 0.01% trypsin (400 μl) for 10 min at 37 °C. After addition of 600 μl of PBS, the cells were centrifuged at 2,000 rpm for 5 min. The cell pellet was suspended and washed twice with 1 ml of PBS, and finally re-suspended in 1 ml of PBS to perform fluorescence analysis with a FACSCalibur (BD Biosciences) flow cytometer using a 488-nm laser excitation and a 515-545-nm emission filter. Each sample was analyzed 10,000 events.

Organ Biodistribution Study.. The transporter (**3c)** was dissolved in sterile distilled water (1 mL) and injected into 8-week-old mice (C57BL/6) intraperitoneally at the dosage level of 77 mg/kg. The treated mice were perfused after 20 min with 4 % paraformaldehyde in PBS (pH 7.4). The organs were incubated overnight in 0.5 M sucrose in PBS. Placed in cryoprotectant, they were cut into 15-μm sections with cryostat, and transferred to coated glass slides. After drying at 37 °C, the sections were washed with PBS and treated with 0.3 % Triton X-100 for 15 minutes at room temperature and analyzed by CLSM.

Brain uptake experiments were carried out as follows. Each of three mice (C57BL/6) was injected intraperitoneally with sterile distilled water (500 μL; control), doxorubicin (21.3 mg/kg; 500 μL of water), and compound (**4**, 115.8 mg/kg; 500 μL of water), respectively. After 20 min, the mice were perfused with 4 % paraformaldehyde in PBS (pH 7.4), and the brains were incubated overnight in 0.5 M sucrose in PBS, cut into 15-μm sections with a cryostat. After drying at 37 °C, the sections were washed with PBS and treated with 0.3 % Triton X-100 at room temperature, and analyzed by CLSM.

MTT Test is a rapid colorimetric method for measuring cellular growth and survival, and it is based on the cleavage of a water-soluble tetrazolium dye (MTT) by succinate dehydrogenase [ref.: M. Pooga, A. Elmquist, U. Langel in *Cell-penetrating peptides* (Ed. U. Langel), CRC Press, Boca Raton, **2002**, pp. 245-261].



Supporting Figure 1. Fluorescence microscope images of mouse brain cortex. [**a**:control (water), **b**: compound (**3c**, HCl salt, 57mg/kg), 20 minutes after *ip* injection.