Cellular uptake of fluorescent vanadyl sulfonylcalix[4]arenes

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Supporting Information

Experimental section

All manipulations were carried out under an atmosphere of nitrogen using standard Schlenk and cannula techniques or in a conventional nitrogen-filled glove-box. Solvents were refluxed over an appropriate drying agent, and distilled and degassed prior to use. Elemental analyses were performed by the microanalytical services at the London Metropolitan University. NMR spectra were recorded on a Varian VXR 400 S spectrometer at 400 MHz or a Gemini at 300 MHz (¹H) and 105.1 MHz (⁵¹V) at 298 K; chemical shifts are referenced to the residual protio impurity of the deuterated solvent. IR spectra (Nujol mulls, KBr/CsI windows) were recorded on Perkin-Elmer 577 and 457 grating spectrophotometers. The precursor [Ph₄P][VO₂Cl₂] was prepared by the method of Fenske *et al*, but using PPh₄Cl [1] The calixarene ligands *p-tert*-butylcalix[4]areneH₄, 1,3-dialkoxy-*p-tert*-butylcalix[4]areneH₂ and methylether-*p-tert*-butylcalix[4]areneH₃ were prepared by literature methods. [2] The ligand *p-tert*-butylthiacalix[4]areneH₄ and *p-tert*-butylsulfonylcalix[4]areneH₄ were gifts from Dr Hitoshi Kumagaya of the Cosmo oil company. Complexes I, II (R' = Me), III (R' = Me), R'' = *n*Pr) and IV were prepared using published procedures. [3]

1,3-Dipentoxy-p-tert-butylcalix[4]areneH₂

This ligand was prepared using the reported procedure for 1,3-dimethoxyether-*p-tert*butylcalix[4]areneH₂, but using *n*-pentyl iodide in place of methyl iodide. ¹H NMR (told₈, 400 MHz) δ : 8.38 (s, 2H, OH), 6.64 – 6.95 (overlapping m, 8H, aryl*H*), 4.25 (d, 4H, ²J_{HH} 12.8 Hz, *endo*-CH₂), 3.55 (t, 4H, ³J_{HH} 6.8 Hz, OCH₂), 3.17 (d, 4H, ²J_{HH} 12.8 Hz, *exo*-CH₂), 1.75 (m, 4H, ³J_{HH} 6.4 Hz, CH₂), 1.41 (m, 4H, ³J_{HH} 7.6 Hz, CH₂), 1.24 (s, 18H, C(CH₃)₃), 0.82 (t, 3H, ³J_{HH} 6.4 Hz, CH₃), 0.79 (t, 3H, ³J_{HH} 7.6 Hz, CH₃), 0.65 (s, 18H, C(CH₃)₃). Anal. Calcd for C₅₄H₇₆O₄: C, 82.2 (82.2), H 9.8 (9.7). IR: 3355bm, 1486s, 1378s, 1363s, 1297m, 1261w, 1201s, 1124m, 1099s, 1040s, 1024s, 1001m, 966m, 870s, 800s. Mass Spec (Electrospray + NH₄OAc): 806.6 (M + NH₄)⁺, 789.6 (M⁺).



Figure S1: Molecular structure of **(1,3-di-***n***-pentyl)***t***-butylcalix-4-areneH**₂**·MeCN**; a molecule of MeCN and hydrogen atoms, except for H(2) and H(4) have been omitted for clarity.

II (R – *n*-pentyl) was synthesized by reacting 1,3-dipentoxy-p-*tert*-butylcalix[4]areneH₂ (1.0 mmol) with $[VO(nPrO)_3]$ (1.1 mmol) in dry toluene and the reaction mixture was refluxed for 12 h under nitrogen. The solvent was removed with reduced pressure, and the residue extracted into acetonitrile to produce crystals overnight at 0 °C. ¹H NMR (CDCl₃, 300 MHz) δ : 7.98, 7.07, 6.92

(3x s, 8H, aryl*H*), 4.32 (d, 4H, ²J_{HH} 12.8 Hz, *endo*-C*H*₂), 4.00 (t, ²J_{HH} 12.8 Hz, OC*H*₂), 3.34 (d, 4H, ²J_{HH} 12.8 Hz, *exo*-C*H*₂), 2.05 (m, 2H, ²J_{HH} 7.2 Hz, C*H*₂), 1.68 (m, 2H, ²J_{HH} 7.3 Hz, C*H*₂), 1.49 (m, 2H, 7.2 Hz, C*H*₂), 1.29 (s, 18H, C(C*H*₃)₃), 1.06 (s, 18H, C(C*H*₃)₃), 1.01 (t, 3H, ²J_{HH} 7.3 Hz, C*H*₃). Anal. Calcd for C₅₃H₆₉O₅N₂V: C, 74.1 (73.6), H 7.7 (8.0), N 3.2 (3.2). IR:1299w, 1261s, 1194m, 1093bs, 1021bs, 871w, 800s, 722w, 589w. Mass Spec (EI): 783 (M⁺ - 2MeCN), 716 (M⁺ - 2MeCN - VO), 712 (M⁺ - 2MeCN - pentyl),



Figure S2: Molecular structure of **II**·2MeCN; two molecules of MeCN and hydrogen atoms have been omitted for clarity. Selected bond lengths (Å) with estimated standard deviations: V(1)–O(1) 2.3196(14), V(1)–O(2) 1.8016(15), V(1)–O(3) 1.8068(14), V(1)–O(4) 1.7890(14), V(1)–O(5) 1.5884(14).

III (R' = R'' = n-propyl) was synthesized by reacting 1,3-dipropoxy-*p*-tert-butylcalix[4]areneH₂ (1.0 mmol) with [VO(*n*PrO)₃] (2.1 mmol) in THF and the reaction mixture was refluxed for 12 h under nitrogen. The solvent was removed with reduced pressure, and the residue extracted into acetonitrile to produce crystals overnight at 0 °C. Yield 0.94 g, 89 %. A similar situation to that observed by Limberg *et al* was found, [3a] whereby the vanadium-bound alkoxides can orientate *cis* or *trans* to another. ¹H NMR (CDCl₃, 300 MHz) δ : 6.50 – 7.86 (5x m, 16H, aryl*H*), 5.27 (bm, 2H, VOC*H*₂), 5.1827 (bm, 2H, VOC*H*₂), 4.41 (overlapping d, 4H, J obscurred, *endo*-C*H*₂), 4.35 (d, 2H, ${}^{2}J_{HH}$ 12.8 Hz, *endo*-CH₂), 4.30 (d, 2H, ${}^{2}J_{HH}$ 12.8 Hz, *endo*-CH₂), 3.95 (bm, 2H, OCH₂), 3.61 (bm, 2H, OCH₂), 3.31 (overlapping d, 6H, J obscurred, *exo*-CH₂), 3.19 (d, 2H, ${}^{2}J_{HH}$ 13.1 Hz, *exo*-CH₂), 2.02 (bm, 8H, CH₂), 1.82 (bm, 4H, CH₂), 1.59 82 (bm, 4H, CH₂), 1.28 (s, 18H, C(CH₃)₃)), 1.26 (t, 12H, J not resolved, CH₃), 1.22 (s, 18H, C(CH₃)₃)), 1.05 (t, 12H, J not resolved, CH₃), 1.03 (s, 18H, C(CH₃)₃)), 1.00 (s, 9H, C(CH₃)₃)), 0.83 1.28 (s, 9H, C(CH₃)₃)). ${}^{51}V$ (C₆D₆, 105.1 MHz) δ - 534.6 (minor), -540.9 (major), ratio 1:3. Anal. Calcd for C₅₆H₈₀O₉V₂ (sample dried *in-vacuo* for 12 h, - 1.5MeCN): C, 66.75 (67.32), H 8.15 (8.07), N <0.10 (0.00). IR: 1600w, 1362m, 1301m, 1261s, 1198m 1094s, 1018s, 999s, 969w, 945w, 921w, 873m, 799s, 722w, 691w, 660w, 589w, 554w, 462w. Mass Spec (EI): 999 (M⁺ - 1.5MeCN), 940 (M⁺ - 1.5MeCN – n-propylO), 881 (M⁺ - 1.5MeCN – 2n-propylO), 798 (M⁺ - 1.5MeCN – 2n-propylO -VO), 731 (M⁺ - 1.5MeCN – 2n-propylO -2VO).



Fig S3. Packing plot of $III \cdot 1\frac{1}{2}C_2H_3N$ showing calixarene complexes and acetonitrile molecules of crystallisation arranged in layers in the a/b plane.

Complex **VI** was synthesized by reacting *p-tert*-butylsulfonylcalix[4]areneH₂ (1.0 mmol) with $[VO(nPrO)_3]$ (1.1 mmol) in dry toluene and the reaction mixture was refluxed for 12 h under nitrogen. The solvent was removed with reduced pressure, and the residue extracted into acetonitrile to produce crystals overnight at 0 °C. Yield 1.09 g, 87.9 %. ¹H NMR (CD₃CN, 300 MHz, 243 K) δ : 8.76 (bs, 2H, OH), 7.13 – 6.89 (overlapping m, 8H, arylH), 4.31 (bs, 4H, OCH₂), 3.41 (bs, 2H, CH₂), 3.27 (bs, 2H, CH₂), 1.98 (s, 1.5 H, 0.5 *Me*CN), 1.72 – 0.76 (overlapping signals, 42H, CH₃ + C(CH₃)₃). ⁵¹V (C₆D₆, 105.1 MHz) δ -361.5. Anal. Calcd for C_{50.5}H_{66.75}S₄O₁₅N_{2.25}V: C, 53.9 (53.9), H 5.9 (6.0), N 2.9 (2.8). IR: 1600w, 1534w, 1306m, 1261s, 1219w, 1203w, 1152m, 1083s, 1034s, 1000s, 910w, 854w, 805s, 744w, 620m, 596w, 552m. Mass Spec (solvent-free Maldi): 912 (M⁺ - 2.25MeCN - *n*-propylO - *n*-propylOH).



Fig. S4. Packing plot of $VI \cdot 2\frac{1}{4}C_2H_3N$ showing the anti, bi-layer motif with weak S=O···H–C and MeCN···H–C interactions.

X-Ray Crystallography

For each sample, a crystal was mounted in oil on a glass fibre and fixed in the cold nitrogen stream on an automated CCD diffactometer equipped with Mo K α radiation and a graphite monochromator, except for III ·1½(C₂H₃N) which was measured at Daresbury Laboratory SRS Station 9.8. Intensity data were measured by ω -scans [II·2(C₂H₃N), ligand] or thin-slice ω -scans [III·1½(C₂H₃N), VI·2¼(C₂H₃N)]. Data were processed using the CrysAlis-RED [II·2(C₂H₃N), ligand] [4] or SAINT [III·1½(C₂H₃N), VI·2¼(C₂H₃N)] [5] programs. The structures were determined by the direct methods routines in the SIR-92 [II·2(C₂H₃N), ligand] [6] or SHELXS [III·1½(C₂H₃N), VI·2¼(C₂H₃N)] [7] programs and refined by full-matrix least-squares methods, on F^2 , in SHELXL.[7] The non-hydrogen atoms were refined with anisotropic thermal parameters, except in some disordered groups/solvent molecules of crystallisation. Disorder was modelling in in both *n*-Pr chains, one *t*-Bu group and one MeCN molecule for III·1½(C₂H₃N), three *t*-Bu groups and in both *n*-Pr chains for VI·2¼(C₂H₃N).

Biological Methods.

Cell Viability Studies. MTS assays were performed using a CellTiter 96[®] AQ_{ueous} Non-Radioactive Cell Proliferation Assay (Promega). All cell lines used have been described earlier [8]. All cells were incubated at 37 °C. Freshly harvested CHO or HeLa cells were suspended in DMEM (Invitrogen) with 10% Foetal Calf serum and 2mM glutamine, THP-1 and HL-60 cells in RPMI (Invitrogen) with 10% Foetal Calf serum and 2mM glutamine, at a concentration of 1×10^5 cells /mL. The cells were incubated in experimental media consisting of DMEMor RPMI supplemented with the test compounds at different concentrations for 24 to 72 hrs at 37 °C in a humidified atmosphere and 5% CO₂. Cell viability was measured by MTS assay at 24, 48 or 72 h. After incubation, cell viability was assessed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-2H-tetrazolium (MTS) tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium [9]. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells [10]. Aliquots of 20 μ l of the CellTiter 96 Aqueous One Solution Reagent were added directly to the wells and the plates were incubated for 4 h at 37 °C in a humidified atmosphere, 5% CO₂ and then absorbance at 490 nm was read with a 96-well plate reader. The quantity of formazan product as measured by the absorbance at 490 nm is directly proportional to the number of living cells in culture [11].

Image acquisition. CHO cells were grown on coverslips overnight, incubated with calixarene ligands for different time points at 37°C. Cells were washed three times in ice-cold PBS and either mounted on glass-slides or used imaged as live cells. Inihibtion of cellular endocytosis was achieved by incubating the cells for at least 60 minutes before addition of calixarene ligands with 0.4M sucrose, (25 μ g/mL), filipin (5 μ g/mL), monensin (50 μ M), nystatin (50 μ g/mL) or methyl-β-cyclodextrin (10mM) as described earlier [8,11]. The actin polymerisation stain was performed with AlexaFluor488-phalloidin purchased from Invitrogen according to the manufacturer's guidelines. Pictures were acquired using a Leica DMII inverted fluorescence microscope.

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Figure S5: Representative cell viability assays (THP-1, HL60 cells tested for proliferation after 72 hours using an MTS assay). Phosphate Buffered Saline used as a control in all studies, data represent at least mean ±SEM of at least three independent experiments done in duplicate.



Figure S6: Representative cell viability assays (HeLa cells tested for proliferation after 24, 48, 72 hours using an MTS assay, HL60 cells tested for 72 hours). Phosphate Buffered Saline used as a control in all studies, data represent at least mean ±SEM of at least three independent experiments done in duplicate.



Figure S7: Control fluorescence with **VI** on its own. Images of CHO cells 1 hour after preincubation with inhibitors with **VI** added for 4 hours at 100 μ M concentration, filipin (5 μ g/ml), β methyl- cyclodextrin (0.1 M), monensin (50 μ M), nystatin (50 μ g/ml) and sucrose (0.4 M). Figure shows representative images acquired in living cells with a Leica fluorescence microscope.



Figure S8: CHO cells were incubated with **VI** (blue) for 4 hours and then stained with AlexaFluor488 phalloidin (green). Actin polymerization in CHO cells is not influenced by the presence of **VI**. Figure shows representative images acquired in living cells with a Leica fluorescence microscope.

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