

**USE OF CALIX[4]ARENES TO
RECOVER THE SELF-ASSEMBLY
ABILITY OF MUTATED p53
TETRAMERIZATION DOMAINS**

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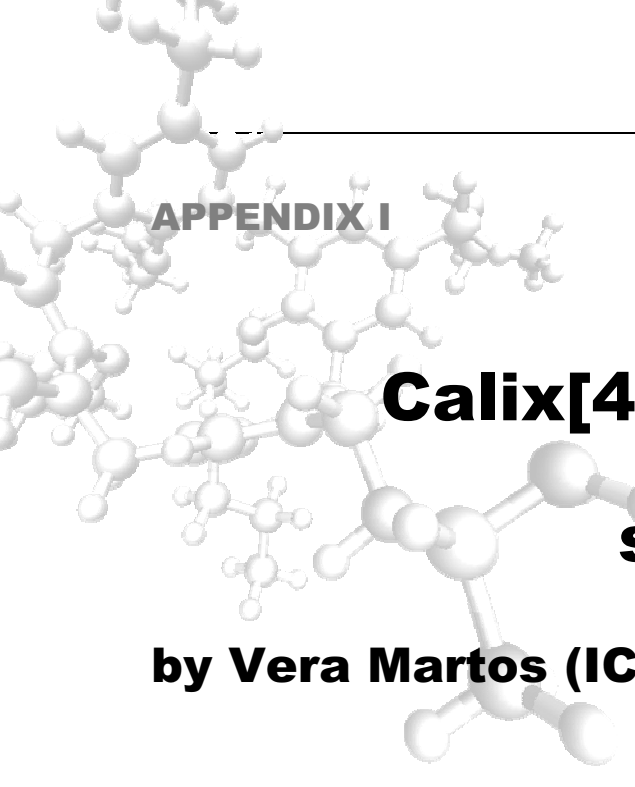
Director

Programa de Química Orgànica

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APPENDIX I



Calix[4]arenes synthesis and structural assessment

by Vera Martos (ICIQ)

General considerations.

All reactions were carried out under argon with dry solvents unless otherwise noted. All commercially available reagents were used without further purification. The solvents were dried and distilled as described in the literature (*J. Org. Chem.* 1978, **43**: 2923-5). A Dowex ion exchange resin (ACROS) was used for chloride anions exchange on the *p*-guanidinium-calixarenes.

Chromatography.

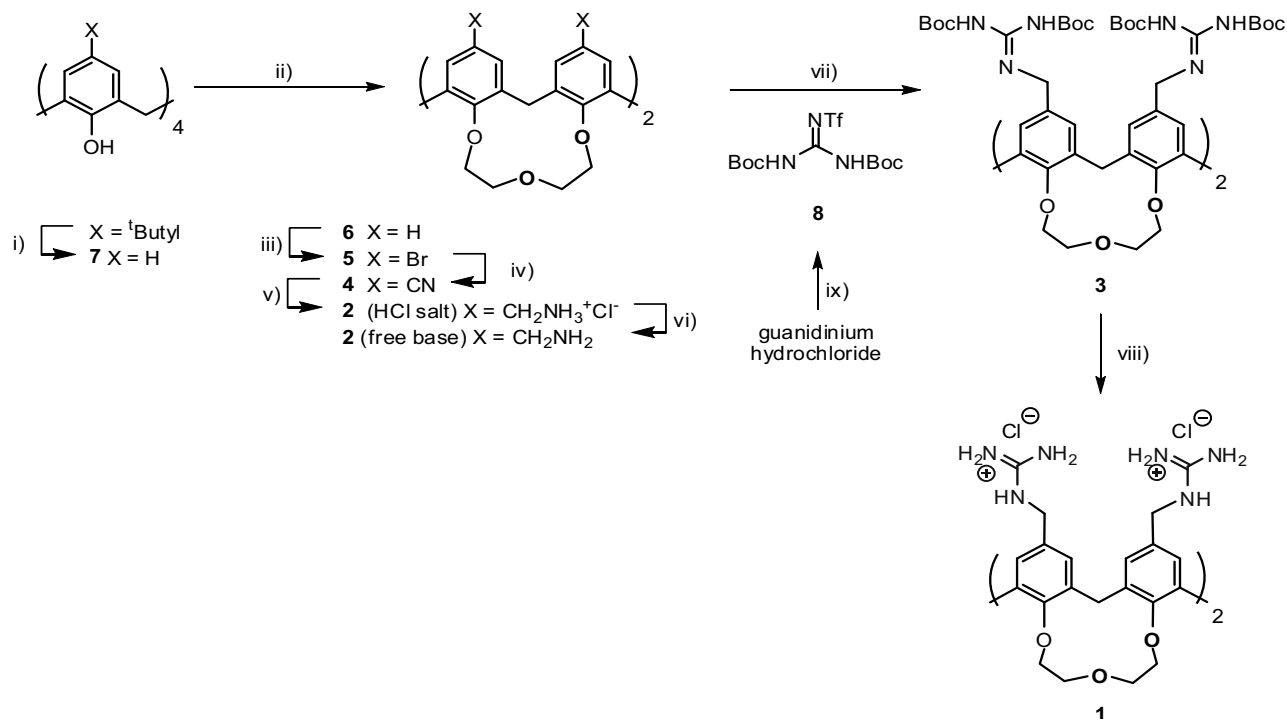
Reactions were monitored by thin layer chromatography (TLC) performed on DC-Fertigplatten SIL G-25 UV₂₅₄ (MACHEREY-NAGEL GmbH) or by analytical high performance liquid chromatography with a RP-C₁₈ column (Symmetry300™ C18 5μm 4.6x150mm) on a high performance liquid chromatograph (HPLC: Agilent Technologies Serie 1200 with UV-detection). Semi-preparative-HPLC purification was performed on a Waters HPLC 600 equipment with a quaternary pump (0.01–20mL/min). Standard column chromatography was done on silica gel by SDS (chromagel 60 ACC, 40-60mm) following the procedure described by W. C. Still (*J. Org. Chem.* 1978, **43**: 2923-5).

Analysis.

Yields refer to chromatographically pure compounds. ¹H NMR and ¹³C NMR spectra were recorded on Bruker Avance 400 UltraShield spectrometer (¹H: 400MHz ¹³C:125MHz) and are reported in parts per million (δ) relative to the residual solvent peak. Data for ¹H are reported as follows: chemical shift (δ ppm), multiplicity (s: singlet, d: doublet, m: multiplet), coupling constant in Hz, and integration. Exact masses were measured on a Waters LCT Premier liquid chromatograph coupled time-of-flight mass spectrometer (HPLC/MS-TOF) with electrospray ionization (ESI).

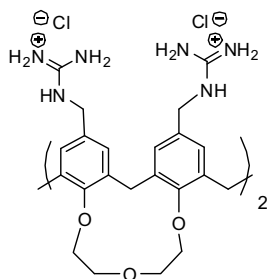
Melting points were measured with a Büchi B-540 apparatus.

Synthesis of calix4bridge (1) and NH₂-calix4bridge (2)



Reagents and conditions:

- i) AlCl_3 , phenol, toluene, 24°C, 24h (81%).
- ii) $(\text{TsOCH}_2\text{CH}_2)_2\text{O}$, NaH, DMF, 24°C, 4h (41%).
- iii) NBS, DMF, 24°C, 24h (90%).
- iv) CuCN , NMP, 200°C, MW, 15min (48%)³.
- v) 1M BH_3 -THF, sealed tube, reflux, 24h (88%) [Arduini *et al.*, *J. Org. Chem.* 1995, **60**: 1454-7]
- vi) extraction of **2** in CH_2Cl_2 from 1N NaOH solution **2** (quant.)
- vii) **8**, TEA, DCM, 24°C, 24h (45%). [Feichtinger *et al.*, *J. Org. Chem.*, 1998, **63**, 3804-5]
- viii) 4N HCl in 1,4-dioxane, 24°C, 24h,
 semipreparative HPLC purification: 0-35% CH_3CN +0.05% TFA in H_2O + 0.05% TFA in 20min (50%).
- ix) a) NaOH, $(\text{Boc})_2\text{O}$, 1,4-dioxane, 0→24°C, 24h (50%); b) Tf_2O , TEA, -78→24°C, 5h (82%).



5,11,17,23-Tetraguanidinomethyl-25,26-27,28-biscrown-3-calix[4]arene tetrachlorohydrate (1)

Boc-protected compound **3** (50mg, 0.03mmol) was dissolved in 0.6mL of 4M HCl (2.4mmol) in 1,4-dioxane (1.00mmol per Boc group), under inert atmosphere. The solution was stirred for 24h at room temperature.

After evaporation of the solvent, the residue was triturated in ethyl acetate. The resulting solid was purified by semi-preparative HPLC (100 μ L of a 37.5mg/mL solution injected in a Waters SymmetryC₁₈ 500 \AA 10x150mm semi-preparative column, and eluted with a 5% to 35% gradient of acetonitrile in water with 0.05% TFA along 20min). Pure compound eluted at 16.2min with a final yield of 50%. Anion exchange to chloride yielded the tetrahydrochloride salt **1**.

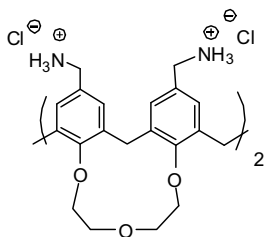
¹H-NMR (400MHz; CD₃CN): δ 7.08 (s, 8H, ArH), 5.03 (d, J = 12Hz, 2H, ArCHHaxAr), 4.48 (d, J = 13.2Hz, 2H, ArCHHaxAr), 4.28-4.23 (m, 12H, CH₂O), 4.13 (d, 8H, CH₂Gua), 3.74-3.3.69 (m, 4H, CH₂Gua), 3.28 (d, J = 13.2Hz, 2H, ArCHHaxAr), 3.21 (d, J = 12Hz, 2H, ArCHHaxAr).

¹³C-NMR (400MHz, CD₃CN) δ 157.3 (CAr ipso); 154.8 (CGua), 135.9 (CAr para), 132 (CAr ortho), 129.9, 128.1 (CHAr meta), 76.7, 74.4 (CH₂O), 43.3 (CH₂Gua), 29.7, 29 (ArCH₂Ar).

Exact Mass (ESI+) m/z of [M-Cl]⁺ calc.: 957.3867uma, found: 987.3824 100%.

M.p.: >330 $^{\circ}$ C.

5,11,17,23-Tetraminomethyl-25,26-27,28-biscrown-3-calix[4]arene tetrahydrochloride (2)



To a stirred solution of tetracyano calixarene **4** (0.60mmol) in 52mL dry THF at 0 $^{\circ}$ C, 18mL (18mmol) of a BH₃ solution (1M in dry THF) were added. The reaction mixture was heated in a sealed tube under nitrogen for 24h, then 5mL of MeOH and 15mL of 1N HCl were added (CAUTION!) and the mixture heated at 50 $^{\circ}$ C for 0.5h. THF was removed under reduced pressure. To the remaining aqueous phase was added another 10mL amount of 1N HCl and several washes with CH₂Cl₂ (3 \times 15ml) were performed. Finally the aqueous solvent was removed under reduced pressure and the remaining residue was triturated in AcOEt and filtered. The chloride salt of compound **3** was obtained in 80% yield.

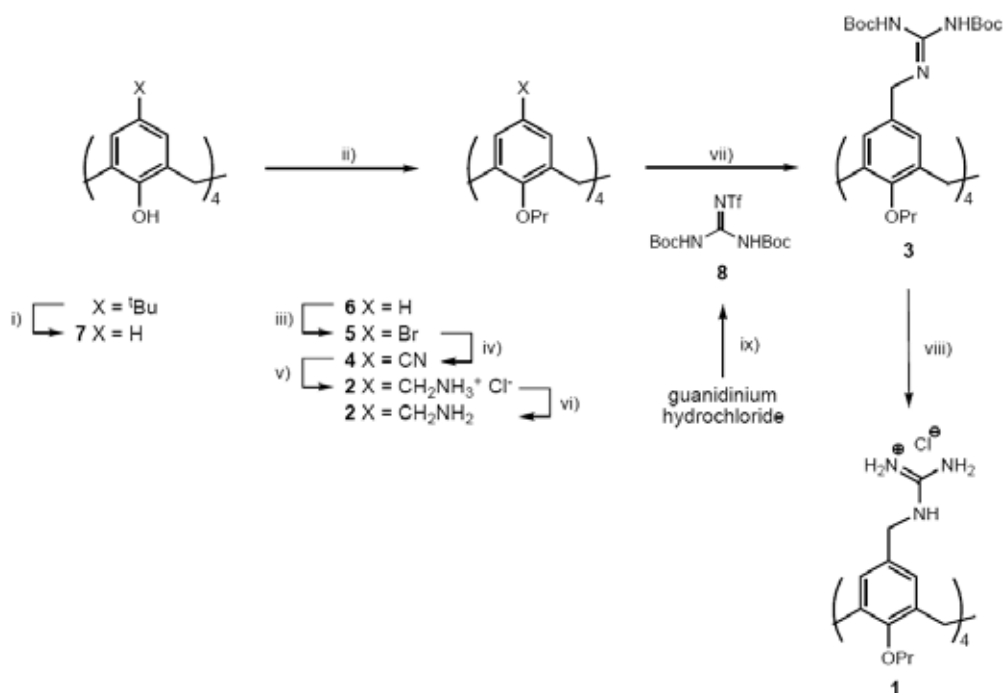
¹H NMR (400MHz; MeOD): δ 6.93 (s, 8H, ArH), 4.92 (d, 2H, ArCHHaxAr, J = 12.8Hz); 4.40 (d, 2H, ArCHHaxAr, J = 12.7Hz); 4.23-4.14 (m, 8H, OCH₂); 3.80-3.73 (m, 8H, OCH₂); 3.56 (s, 8H, CH₂N); 3.21 (d, 2H, ArCHHeqAr, J = 12.7Hz); 3.15 (d, 2H, ArCHHeqAr, J = 12.8Hz); 2.85 (bs, 8H, NH₂).

¹³C NMR (75MHz; CDCl₃): δ 155.4 (s, Ar ipso); 136.7 (s, Ar para); 135.4 (s, Ar ortho); 129.7, 128.8 (d, Ar meta); 77.4, 75.7 (t, OCH₂); 55.4 (t, CH₂N); 30.5 (t, ArCH₂Ar).

Exact Mass: MS ESI(+) m/z [M+Cl]⁺: 717.3uma; 100%.

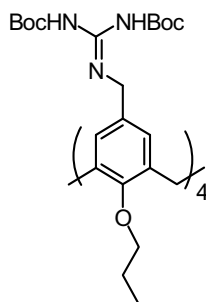
M.p.: >330 $^{\circ}$ C.

Synthesis of calix4prop (1) and NH₂-calix4prop (2)



Reagents and conditions:

- AlCl₃, phenol, toluene, 24°C, 24h (81%).
- IPr, NaH, DMF, 105°C, 7days (76%) [Casnati et al. *Supramol. Chem.* 2000, **12**, 53-65]
- NBS, DMF, 24°C, 24h (92%).
- CuCN, NMP, 200°C, MW, 15min (62%).
- 1M BH₃-THF, sealed tube, reflux, 24h (90%) [Arduini et al., *J. Org. Chem.* 1995, **60**: 1454-7]
- extraction of 2 in CH₂Cl₂ from 1N NaOH solution 2 (quant.)
- 8, TEA, DCM, 24°C, 24h (40%). [Feichtinger et al., *J. Org. Chem.*, 1998, **63**, 3804-5]
- 4N HCl in 1,4-dioxane, 24°C, 24h,
semipreparative HPLC purification: 0-30% CH₃CN + 0.05% TFA in H₂O + 0.05% TFA in 20min (94%).
- a) NaOH, (Boc)₂O, 1,4-dioxane, 0→24°C, 24h (50%); b) Tf₂O, TEA, -78→24°C, 5h (82%).



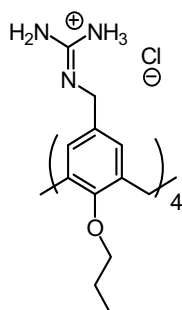
5,11,17,23-Tetradi-bocguanidinomethyl-25,26-27,28-tetrapropoxycalix[4]arene (3)

Purification was performed with flash column chromatography on silica gel by passing first CH_2Cl_2 and then $\text{Et}_2\text{O}:\text{Hexane}(1:4)$ as eluents. Finally **14a** was obtained as a pure white compound with a 40% yield.

$^1\text{H NMR}$ (400MHz; CDCl_3): δ 8.27 (s, 4H, NHBoc), 6.60 (s, 8H, ArH), 4.40 (d, $J = 13.2\text{Hz}$, 4H, ArCH*Hax*Ar), 4.28 (d, 8H, $\text{CH}_2\text{NGuaDiBoc}$), 3.97 (s, 4H, NHBoc), 3.83 (t, 8H, CH_2OAr), 3.12 (d, $J = 13.2$, 8H, ArCH*Heq*Ar), 1.93 (q, 8H, $\text{OCH}_2\text{CH}_2\text{CH}_3$), 1.48 (d, t-Butyl CH_3), 0.984 (t, 12H, $\text{OCH}_2\text{CH}_2\text{CH}_3$).

$^{13}\text{C NMR}$ (100MHz; CDCl_3): δ 163.59 (C Gua), 155.95 (C=O), 155.6 (C=O), 153.05 (C Ar *ipso*), 134.94 (C Ar *para*), 130.50 (C Ar *ortho*), 127.84 (CH Ar *meta*), 82.75 (C t-Butyl), 78.88 ($\text{OCH}_2\text{CH}_2\text{CH}_3$), 44.92 (CH_2Gua), 31.01 (Ar CH_2Ar), 28.38, 28.11 ($\text{CH}_3\text{t-Butyl}$), 23.19 ($\text{OCH}_2\text{CH}_2\text{CH}_3$), 10.26 ($\text{OCH}_2\text{CH}_2\text{CH}_3$).

Exact Mass (ESI+) m/z $[\text{M}+\text{H}]^+$: calc. 1678.9793uma; found: 1678.9995, 100%.



5,11,17,23-Tetra-guanidinomethyl-25,26-27,28-propoxycalix[4]arene tetrachlorohydrate (1)

To a solution of **2a** (0.036mmol, 60mg) in 7mL 1,4-dioxane, 0.3mL concentrated HCl (1.25mmol per Boc group) was added dropwise under inert atmosphere. The solution was stirred for 24 hours at room temperature.

After eliminating the solvent, **1a** was recrystallized by first dissolving the residue in 0.5mL MeOH, adding dropwise ethyl acetate and leaving the solution for 24 hours at room temperature.

$^1\text{H NMR}$ (400MHz; CDCl_3): δ 6.27 (s, 8H, ArH), 4.51 (d, $J = 13.2\text{Hz}$, 4H, ArCH*Hax*Ar), 4.12 (s, 8H, CH_2NGua), 3.83 (t, 8H, CH_2OAr), 3.22 (d, $J = 13.2\text{Hz}$, 8H, ArCH*Heq*Ar), 1.93 (m, 8H, $\text{OCH}_2\text{CH}_2\text{CH}_3$), 1.04 (t, 9H, $\text{OCH}_2\text{CH}_2\text{CH}_3$).

$^{13}\text{C NMR}$ (100MHz; CDCl_3): δ 158.41 (CGua), 157.67 (C Ar *ipso*), 136.63 (C Ar *para*), 130.82 (C Ar *ortho*), 129.04 (C Ar *meta*), 78.15 ($\text{OCH}_2\text{CH}_2\text{CH}_3$), 46.06 (CH_2Gua), 31.94 (Ar CH_2Ar), 24.45 ($\text{OCH}_2\text{CH}_2\text{CH}_3$), 10.82 ($\text{OCH}_2\text{CH}_2\text{CH}_3$).

Exact Mass (ESI+) m/z $[\text{M}-\text{Cl}]^+$ calc.: 987.4836uma; found: 987.4896; 100%.

APPENDIX II

Supplementary Material contents

Some figures from the **RESULTS SECTION** are provided as supplementary material in electronic format. The contents are listed bellow:

1. The p53 tetramerization domain and its mutants

CD unfolding curves: concentration effects
G334V precipitation events in CD unfolding curves
R337H DSC curves: effects of pH on ΔH_m
DSC curves: mathematical adjustment

2. Calix4bridge: a designed ligand for the p53 tetramerization domain

DSC curves p53wt + calix4bridge: mathematical adjustment
 ^1H -STD for NH_2 -calix4bridge
L344P negative control: HSQC
L344P negative control: ESI-MS

3. Calix4prop: an unexpected ligand for the p53 tetramerization domain

DSC mathematical adjustments with 6eq of calix4prop
HSQC titration of p53wt + calix4prop
HSQC titration of p53wt + NH_2 - calix4prop
HSQC titration of R337H + calix4prop (pH 7)
HSQC titration of R337H + NH_2 - calix4prop
HSQC titration of R337H + calix4prop (pH 5)
HSQC titration of R337H + calix4prop (pH 9)
HSQC titration of G334V + calix4prop
HSQC titration of G334V + NH_2 - calix4prop
HSQC titration of p53wt + 4G4Pr-cone
HSQC titration of R337H + 4G4Pr-cone
HSQC titration of p53wt + COOH - calix4prop
HSQC titration of R337H + COOH - calix4prop
NOESY p53wt + calix4prop
L344P negative control: HSQC
L344P negative control: CD
L344P negative control: ESI-MS

MS-ESI. Tables of masses for the free proteins, and the proteins bound to one and two molecules of ligand (for both calix4prop and calix4bridge).

