Synthesis of New Calixarene Derivatives and Evaluation of Their Cytotoxic Activity

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Abstract

Since calixarenes are more easily synthesized and functionalized than other supramolecules, they are compounds of interest in organic chemistry. In this study, the dihydrazide (**3a** and **3b**) and diamino propyl (**6a** and **6b**) derivatives of *p*-tert-butylcalix[4]arene and calix[4]arene were synthesized. Then the *L*-proline methyl ester substituted chlorocyclopropenium was reacted with the calix[4]arene derivatives (**3a**, **3b, 6a,** and **6b**) at room temperature in CH_2Cl_2 to obtain calix[4]arene superbase derivatives (**4a**, **4b**, **7a**, and **7b**) in 75%, 60%, 70% and 55% yield, respectively. The synthesized compounds' structure was elucidated by using spectroscopic techniques (FTIR, ^{1}H NMR, and ^{13}C NMR). The cytotoxic properties of the calix[4]arene superbase derivatives were investigated against different human cancerous cells, including A549, DLD-1, HEPG2, and PC-3, as well as human healthy epithelium cell line PNT1A. The cytotoxicity results showed that calix[4]arene superbase derivatives inhibited the proliferation of DLD-1, A549, HEPG2, and PC-3 cells in a dose-dependent manner. Compound **7a** had the highest toxic effect on colorectal carcinoma (IC_{50}: 4.7 μM), and the IC_{50} values were 18.5 μM and 74.4μM against human prostate and lung cancer cells, respectively. Furthermore, the compound **4b** was found more effective on hepatocellular carcinoma cells (IC_{50}: 210.2 μM). As a result, the synthesized calix[4]arene superbase derivatives can be developed to treat different human cancer cells. They can be considered as a preliminary result for molecular-level research.

Introduction

Organic bases are considered to have significant advantages over ionic bases due to milder reaction conditions and better solubility requirements in organic synthesis. In addition to this, they have various applications in functional material synthesis [1]. Among organic basis, 2,3- bis(dialkylamino)-cyclopropenimines is highly basic. The baseline of this organic molecule is the compliance of Huckel rule (2π-electron) by the smallest ring system, i.e., the cyclopropenium ion that provides aromatic resonance stabilization the conjugate acid of cyclopropenimine [2, 3]. Strong organic bases often result from a planar cyclic π-electron system through an aromatization mechanism. Cyclic π-electron system present in cyclopropeneimines gives the fundamental property that enlists it to "superbase" family [4]. The prominent proton affinity of cyclopropene is due to the presence of imino group at the three-membered cyclic ring [5]. Lambert et al. reported the excellent and simple method for preparing cyclopropenimines and recommended it for several organic synthesis applications [4–7].

In supramolecular chemistry, calix[n]arenes have solidified their position as excellent host macrocyclic compounds due to their unique structural geometry and simple preparation. They can be modified by choice at either position with several functional groups [8]. Their flexible structural property can help host different guest species, either are neutral organic molecules or ionic compounds [8–10]. Moreover, calixarene compounds have entered in biomedical fields, such as they can be applied as biocatalyst and anti-tumor agent. The calixarene compounds have shown excellent biological properties like antibacterial, antifungal, and anticancer [11–23].

Ding et al. prepared hydrophilic and hydrophobic groups surfaces on calix[4] arenes. They investigated these compounds' anti-tumor activities and found that compounds to which 2-dimethylaminoethyl groups were attached to the phenolic-O position showed significant cytotoxicity [24, 25]. Because of the fascinating combination of calixarenes in chemical and structural terms, researchers began to apply calixarenes beyond the chemical field, especially in the pharmaceutical industry. In this context, the crystallization of drug molecules with calixarene derivatives helps to change the active pharmaceutical ingredients' physicochemical properties and control the drug structure [26]. p-Sulfonatocalix[4]arene increases its effectiveness by forming a complex with topoisomerase I inhibitor topotecan, which is used to treat many different types of cancer [27–29]. In connection to this application, series of polyhydroxyamine calix[n]arene derivatives were synthesized, and their application was reported in cytotoxicity study of different cancer cell lines. From their observations, it was deduced that calixarene derivatives were effectively induced cell death in human ovarian carcinoma cells [18]. The cytotoxic activities of Calix[4]arene derivatives on various tumor cells (MU2, MU2F, HT1080, SP6.5, 1PC227, Jurkat, MEWO, Hl-60, Huh7, Hep-G2, MEWO, DLM.1) were compared with standard anticancer drugs. As a result, calix[4]arene derivatives were found to be potent anticancer agents, especially in lymphoblastic leukemia and melanoma cells [30]. Previous work reported that the L- proline functionalized calix[4]arenes used against human cervical cancer to prevent L1 pentamer formation of modified (*Human papillomavirus*) HPV [31]. In our recent study, calix[4]arene derivatives bearing L-proline on their upper and lower rim were prepared and investigated their anti-tumor activity for different human cancer cells [32] and observed that these compounds have a potent cytotoxic effect against human colon cancer cells (DLD-1).

From this point of view, to obtain the calix[4]arene superbase derivative, dimethyl(3-chlorocycloprop-1-ene-1,2-diyl)di-L-prolinate derivative of the calix[4]arenes were synthesized and used in cytotoxicity studies. The compounds were investigated for the proliferation of human colorectal carcinoma, human lung cancer cells, human hepatocarcinoma, and human prostate cancer cells.

Results And Discussion

Synthesis of calix[4]arene-super base derivatives

In recent years, significant advances have been made in drug-based cancer treatment. Individualized treatment methods using drugs suitable for the specific targets of a particular cancer are being developed, which are becoming more complex than general cytotoxic chemotherapy. In addition to an increasing number of new generation antiproliferative cytotoxic drugs, anti-angiogenic agents, peptides, and therapeutic antibodies have been developed, and many of these have progressed to the clinic [33]. There are a limited number of anti-tumor activity studies of calixarenes derivatives functionalized with different groups. Dings et al. [24, 25, 34] reported that different calix[4]arene amide derivatives were highly influential in inhibiting various tumor cells.

In our previous study, considering that the L-proline derivative of calix[4]arenes showed moderate therapeutic potential, and to increase this activity, the hydrophilic faces of calix[4]aren were conjugated

with chlorocycloprop-1-ene-1,2-diyl) di-L-prolinate derivative obtained by reacting L-proline with tetrachlorocyclopropene as shown in Figure 1. From this point of view, the parent calix[4]arene derivatives (**1**, **2**, **3**, **5,** and **6** (**a-b**)) were synthesized by following the known methods (**Scheme 1** and **2**), starting from compounds **1a** and **1b** (p-tert-butylcalix[4]arene and calix[4]arene) [35]. In Scheme 1, compounds **2a** and **2b** (diester derivatives of calix[4]arene) were synthesized in good yield by refluxing compounds **1a** /**1b** with bromomethyl acetate with K_2CO_3 in acetonitrile for 24 h as described previously [36, 37]. Then, the diester derivative of calix[4]arene (**2a** /**2b**) was reacted by hydrazine in Toluene/MeOH for 24 h to give calix[4]arene hydrazine derivatives (**3a** and **3b**). In Scheme 2, the compound (**1a** and **1b**) was reacted with N-(3-bromopropyl)phthalimide in the presence of K_2CO_3 in CH_3CN under reflux conditions to furnish compound **5a** and **5b** (bis(3-phthalimidopropoxy) calix[4]arene). The phthalimido units of these compounds (**5a** and **5b**) were removed by hydrazine hydrate in EtOH to obtain compounds **6a** and **6b** (diaminopropyl derivatives of calix[4]arene). Finally, dimethyl(3 chlorocycloprop-1-ene-1,2-diyl)di-L-prolinate, which is obtained by the reaction of L-proline with tetrachlorocyclopropene was reacted with compounds **3a**, **3b**, **6a**, and **6b** at room temperature in CH_2Cl_2 to form calix[4]arene super base derivative (**4a**, **4b**, **7a**, and **7b**) in 75%, 60%, 70% and 55% yield, respectively (Scheme **1** and **2**).

The synthesized compounds' molecular structures were confirmed by using $^1H/^{13}C$ NMR, FTIR spectroscopy, and elemental analysis. In FTIR spectra of **4a**, **4b**, **7a** and **7b**, specific carbonyl bands (ester/amide) at 1737/1666 cm^{-1}, 1737/1674 cm^{-1}, 1740/1604 cm^{-1}, 1738/1644 cm^{-1}, respectively approved the formation of calix[4]aren super base derivatives. In the ^1H-NMR spectra, the synthesis of **4a**, **4b**, **7a**, and **7b** was also approved by the existence of new protons (*L*-proline) in the aliphatic area. The protons of L-proline units was approximately observed for -CH_2 at 1.86-2.05, 2.21-2.37, 3.01-3.28, and -CH at 4.31-4.38 ppm for **4a** and 2.12-2.65, 3.47-3.76, 4.23-4.39 for **4b** in ^1H-NMR spectrum. The synthesis of compound **7a** was also approved by the appearance of proline group signals -CH_2 at δ 2.04-2.45, 3.30-3.46, and -CH at 4.33-4.56. The structure of **7b** was also approved by the presence of proline group protons -CH_2 at δ 1.83-2.37, and -CH at 4.41-4.58 ppm in the ^1H-NMR spectra. The ^{13}C-NMR spectra of **4a**, **4b**, **7a**, and **7b** showed carbon signal at δ 175.6, 171.7, 171.9, and 175.8 ppm belong to the carbonyl group, respectively. These results showed that the *L*-proline substituted tetrachlorocyclopropene subunits were successfully attached to calixarene derivatives (**see Supplementary data**).

Effects of Calix[4]arene Super Base Derivatives on Viability and Proliferation of Human Cancerous and Healthy Cells

48 h treatment of human cancerous cells with TCP compounds significantly inhibited the cell viability (Fig 2). All compounds inhibited cell viability in a dose-dependent manner (Fig 2a). As seen in Fig 2-b, the compound **7a** was found as the most potent inhibitor of the proliferation of DLD-1 cells and less inhibitory action on HEPG2 cells. The IC_{50} values against the proliferation of DLD-1, A549, HEPG2, PC-3, and PNT1A were calculated as 4.7 μM, 74.4 μM, 240.7 μM, 18.5 μM, and 153.4 μM, respectively (Fig 2c-d). Compound **7b** was found the most and less potent inhibitor against DLD-1 and HEPG2 cells. The IC_{50} values against the proliferation of DLD-1, A549, HEPG2, PC-3, and PNT1A were calculated as 16.5 μM,

70.6 μM, 223.8 μM, 89.7 μM, and 151.7 μM, respectively. Compound **4a** dose-dependently inhibited the proliferation of human cancerous cells and the IC_{50} values on DLD-1, A549, HEPG2, PC-3, and PNT1A were calculated as 18.5 μM, 90.4 μM, 288.3 μM, 70.7 μM, and 152.1 μM, respectively. Among the compounds, compound **4b** showed less inhibitory potency against all cancerous cells with IC_{50} values as 193.9 μM, 110.7 μM, 210.2 μM, 75.5 μM, and 160.6 μM, respectively.

Also, we compared the 8 (a, b) compounds synthesized in our previous studies with compounds 4 (a, b) and 7 (a, b) synthesized in this study. As shown in **Table 1**, compounds 4a and 7 (a, b) showed higher toxic effects on colon cancer cells (DLD-1) than compound 8 (a, b). Compounds 4a, 7 (a, b) showed higher toxic effects on both lung cancer cells (A549) and prostate cancer cells (PC-3) than compound 8b. Besides, compound 7a demonstrated significant cytotoxicity relative to compound 8 (a, b) in prostate cancer cells (PC-3). However, compounds 8 (a, b) showed higher toxicity in hepatocarcinoma cells (HEPG2) than compounds in this study. Finally, compounds 4 (a, b) and 7 (a, b) showed less toxicity in healthy cells (PNT1A) than compounds 8 (a, b). As a result, superbases derivatives of calix[4]arene showed much more cytotoxicity than *L*-proline derivatives.

Unlike our previous study, in this study, L-proline methyl ester groups were reacted with tetrachlorocyclopropene and calix[4]arene aminopropyl and hydrazide derivatives to increase the proton binding capability of L-proline methyl ester groups. The better activity of **7a** and **7b** than **4a** and **4b** can be explained by the partial degradation of these compounds (**4a** and **4b**) in the solution phase. Superbase type compounds are relatively unstable and degradable in the solution phase [4, 7]. These compounds are also degradable in solid form in a low degree. However, the HCl salts of these compounds can be stored for quite a long time. ^{1}H-NMR spectra proved that when both **7a-b** and **4a-b** were kept in the solution phase for 24 hours, a significant proportion of cyclopropeneimines groups separated from **7a-b**(80%) and **4a-b**(65%) compounds were observed.

Table 1. IC_{50} values (μM) of calixarenes 4-8 (a,b) on various human cancerous cells.

Compd.	IC_{50} (μM)				
	DLD-1	A-549	HEPG2	PC-3	PNT1A
4a	18.5	90.4	288.3	70.7	152.1
4b	193.9	110.7	210.2	75.5	160.6
7a	4.7	74.4	240.7	18.5	153.4
7b	19.1	92.1	278.4	75.5	106.6
8a*	59.5	15.7	73.9	23.3	40.0
8b*	29.3	108.7	64.4	92.6	65.9

(*) Ref. [32]

Experimental Section

Materials and Instruments

The standard analytical grade solvents and reagents used for the study were provided by various commercial companies and can be used without further purification unless otherwise stated. The ^{1}H and ^{13}C NMR spectra were obtained on a Varian 400 NMR instrument and were used $CDCl_3$ as the deuterated solvent. Infrared spectra (FTIR) were measured using a Bruker Vertex 70 spectrometer. Elemental analyses were calculated on a Gallenkamp. Cell viability and inhibitory potential of TCP compounds were investigated using Alamar Blue reactive (Thermo Fisher Scientific, USA).

The preparing of compounds

Scheme **1** and **2** represent the synthesis of various derivatives of calix[4]arenes. The synthesis of calixarene diaminopropyl and hydrazine derivatives as precursor compounds (compound **3a, 3b, 6a,** and **6b**) was synthesized using the reported procedures with little modification [36, 37]. The target calix[4]arene super base derivatives (**4a, 4b, 7a**, and **7b**) were synthesized according to the methods given below. All compounds were characterized by ^{1}H-NMR, ^{13}C-NMR, elemental analysis, and FT-IR.

General procedure of the synthesis of calix[4]arene super base derivatives (4a, 4b, 7a, and 7b)

To prepare the calix [4] arene superbase derivatives, 1.10 g (6.6 mmol) of *L*-proline methyl ester hydrochloride was dissolved in anhydrous CH_2Cl_2 and an equivalent amount of NH_4OH solution was added to the solution for remove of HCl salt. The mixture stirred at room temperature for 1h, the organic phase was separated from the aqueous phase and dried over $MgSO_4$. After that, tetrachlorocyclopropene (1.10 mmol) was slowly added to the solution under nitrogen atmosphere and stirred at room temperature for overnight and then the calix[4]arene derivative (**3a**, **3b**, **6a** or **6b**) (0.5 mmol in dichloromethane) was added into the reaction mixture. After complete the reaction, the most of solvent was evoporated under vacum and then the remaining solid was extracted with $CHCl_3/H_2O$ several times, and the organic phase was dried over $MgSO_4$ in inert atmosphere. The calix[4]arene-super base derivative was formed as white solid. **4a:** Yield 75%; Mp: >190 ^{o}C (dec.). FT-IR; 3322 cm^{-1}(O-H, N-H), 2955 cm^{-1}(C-H), 1737 cm^{-1} ester(C=O), 1666 cm^{-1} (C=O) amide.^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 1.18 (s, 18H, Bu^{t}); 1.40 (s, 18H, Bu^{t});1.86-2.05 (m, 8H,$(-CH_2-)_{proline}$); 2.21-2.37 (m, 8H, $(-CH_2-)_{proline}$); 3.01-3.28 (m, 8H, $(-CH_2-)_{proline}$); 3.47 (d, 4H, *J*=13,3 Hz, Ar-CH_2-Ar); 3.77 (s, 12H, O-CH_3); 4.15 (d, 4H, *J*=13,3 Hz, Ar-CH_2-Ar); 4.31-4.38 (m, 4H, $(-CH-)_{proline}$); 4.68 (s, 4H, OCH_2); 6.98 (s, 4H, ArH); 7.12 (s, 4H, ArH); 7.86 (s, 2H, OH); 9.75 (s, 2H, NH). ^{13}C-NMR (100 MHz, $CDCl_3$):δ (ppm) 30.9, 31.5, 31.8, 33.9, 34.1, 46.9, 52.0, 59.6, 125.6, 126.1, 127.0, 132.2, 143.1, 148.2, 148.6, 149.4, 167.5, 175.6. Analytical Cal. for (%) $C_{78}H_{100}N_8O_{14}$; C, 68.20; H, 7.34; N, 8.16. Found: C, 68.26; H, 7.39; N, 8.21. **4b:** Yield 60%; Mp: >200 ^{o}C (dec.). FT-IR; 3329 cm^{-1} (O-H, N-H), 2926 cm^{-1} (C-H), 1737 cm^{-1} ester (C=O), 1674 cm^{-1} amide(C=O). ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 2.12-2.65 (m,16H, $(-CH_2-)_{proline}$); 3.47-3.76(m, 12H $-CH_2$ ve Ar-CH_2-Ar); 3.93 (s, 12H, OCH_3); 4.17 (d, 4H, *J*=13,4 Hz, Ar-

CH_2-Ar); 4.23-4.39 (m, 4H, (-CH-)$_{proline}$); 4.64 (s, 4H, OCH_2); 6.74-6.81 (m, 2H, ArH); 6.86-6.91 (m, 2H, ArH); 6.98-7.03 (m, 4H, ArH); 7.12-7.17 (m, ,4H, ArH); 8.05 (s, 2H, OH); 9.64 (s, 2H, NH). ^{13}C-NMR (100 MHz, $CDCl_3$):δ (ppm) 24.4, 30.3, 31.4, 31.5, 51.7, 52.6, 62.6, 119.8, 120.5, 126.3, 127.5, 128.7, 129.0, 129.6, 132.7, 150.2, 152.5, 171.7. Analytical Cal. for (%) $C_{62}H_{68}N_8O_{14}$; C, 64.80; H, 5.96; N, 9.75. Found: C, 64.84; H, 6.01; N, 9.78. **7a:** Yield 70%; Melting point: >200 ^oC (dec.). FT-IR; 3373 cm^{-1} (O-H, N-H), 2955 cm^{-1} (C-H), 1740 cm^{-1} ester (C=O), 1604 cm^{-1} imine(C=N). ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 1.07 (s, 18H, Bu^t); 1.36 (s, 18H, Bu^t); 2.04-2.45 (m, 16H, (-CH_2-)$_{proline}$); 2.63 (bs, 4H, -CH_2-); 3.30-3.46 (m, 8H, (-CH_2-CH_2-)$_{proline}$); 3.52 (m, 4H, -CH_2-); 3.67 (bs, 4H, Ar-CH_2-Ar); 3.81-4.06 (bs, 16H, -CH_2- ve -OCH_3); 4.22 (bs, 4H, Ar-CH_2-Ar); 4.33-4.56 (m, 4H, (-CH-) $_{proline}$); 6.89 (s, 4H, Ar-H); 7.17 (bs, 6H, Ar-H ve OH). ^{13}C-NMR (100 MHz, $CDCl_3$):δ (ppm) 24.5, 27.6, 30.4, 30.8, 31.1, 31.3, 31.6, 33.9, 38.4, 51.8, 52.6, 125.1, 125.5, 125.6, 128.0, 131.8, 142.3, 147.2, 149.1, 149.8, 171.9. Analytical Cal. for (%) $C_{80}H_{106}N_6O_{12}$; C, 71.51; H, 7.95; N, 6.25. Found: C, 71.57; H, 7.99; N, 6.28. **7b:** Yield 55%; Mp: >200 ^oC (dec.). FT-IR; 3281 cm^{-1} (O-H, N-H), 2923 cm^{-1} (C-H), 1738 cm^{-1} ester (C=O), 1644 cm^{-1} imine(C=N). ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 1.83-2.37(m, 16H, (-CH_2-)$_{proline}$); 2.55 (bs, 4H, -CH_2-); 2.99-3.32 (m, 4H, CH_2); 3.49 (bs, 4H, Ar-CH_2-Ar); 3.54-3.65 (m, 8H, -CH_2); 3.91 (s, 12H, OCH_3); 4.23 (bs, 4H, CH_2); 4.33 (bs, 4H, CH_2); 4.41-4.58 (m, 4H, (-CH-)$_{proline}$); 6.79-6.88 (m, 2H, ArH); 6.90-6.95 (m, 2H, ArH); 7.06-7.11 (m, 4H, ArH); 7.11- 7.20 (m, 6H, ArH ve OH). ^{13}C-NMR (100 MHz, $CDCl_3$):δ (ppm) 23.2, 25.5, 27.6, 30.2, 45.1, 46.9, 50.3, 52.0, 59.6, 60.5, 118.5, 119.3, 125.5, 127.8, 128.5, 129.0, 130.8, 132.9, 154.4, 166.3, 175.8. Analytical Cal. for (%) $C_{64}H_{74}N_6O_{12}$; C, 68.68; H, 6.66; N, 7.51. Found: C, 68.72; H, 6.69; N, 7.54.

Cell lines

DLD-1 (Human colorectal carcinoma), A549 (Human lung cancer), HEPG2 (Human hepatocarcinoma), and PC-3 (Human prostate cancer) cell lines were purchased from ATCC (American Type Culture Collection, Washington, DC, USA). Human epithelial cell line PNT1A was supplied from Sigma-Aldrich (USA). The cells were cultured in recommended growth media; RPMI-1640 (DLD-1 and PNT1A), EMEM (A549 and HEPG2), and Hams' F-12 (PC-3) supplemented with 10% FBS (fetal bovine serum), 2 mM L-glutamine, 1% pen-strep (Penicillin Streptomycin, 10.000 U/mL) at 37°C, 5% CO_2 with 95% humanity.

Determination of cytotoxic potential of TCP compounds

The cell viability and cytotoxic potential of TCP compounds were carried out with Alamar Blue Assay [38]. TCP compounds were dissolved in growth media. 1 x 10^4 cells were seeded into a 96-well plate and treated with various concentrations of TCP compounds ranging from 0 to 250 µM and incubated at 37°C for 48 h. After the incubation, the media were removed, and the cells were washed with PBS and incubated with Alamar Blue (10%) for 3 h. The absorption was measured at 570 nm and 600 nm in an ELISA plate reader. Cell viability and IC_{50} values were determined from the sigmoidal plot of cell viability vs. log concentration of the TCP compounds by GraphPad Prism 8.0.2 software.

Conclusion

In conclusion, to obtain the calix[4]arene superbase derivatives, dihydrazide (**3a** and **3b**) and diamino propyl (**6a** and **6b**) derivatives of calix[4]arene were successfully functionalized with L-proline methyl ester-substituted chlorocyclopropenium. The synthesized compounds (**7a**, **7b**, and **4a**) have a potent antiproliferative effect against human colorectal carcinoma cells. Besides, compound **7a** significantly inhibited the proliferation of human prostate cancer cells. Compared with healthy cells, compound **7a** was found 32.6-fold and 8.3-fold cytotoxic against DLD-1 and PC-3's viability. Herein, the compound **7a** is an advanced candidate for the cure of human colon and prostate cancer. As a result, superbase derivatives of calix[4]arene are much more cytotoxic than *L*-proline derivatives of calix[4]arene, and these compounds have a much higher potential to be a drug candidate that can be used in human cancer treatment due to their superbase properties. Following the advanced molecular studies, superbase derivatives of calix[4]arene might be used for preclinical studies.

Declarations

Conflict of Interest

There are no conflicts to declare.

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Figures

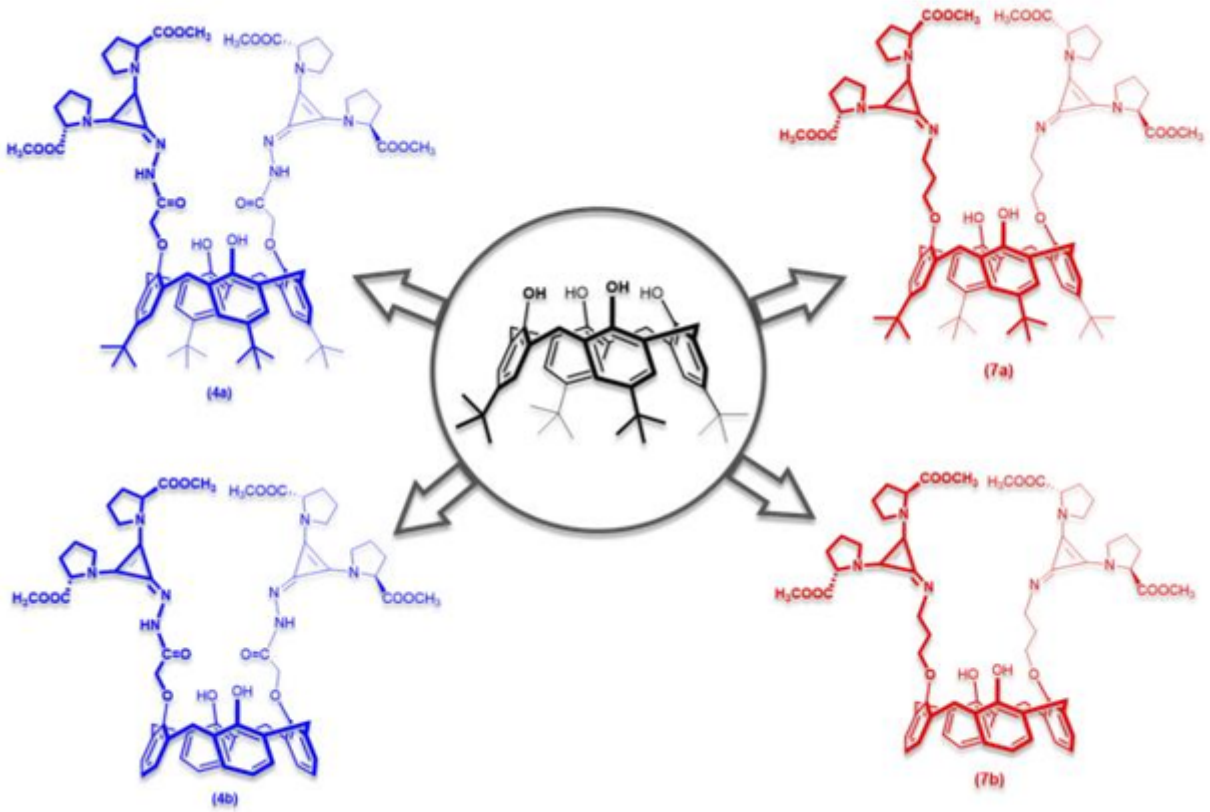

Figure 1

Calix[4]arene super base derivatives as antiproliferative agents (4a, 4b, 7a, and 7b)

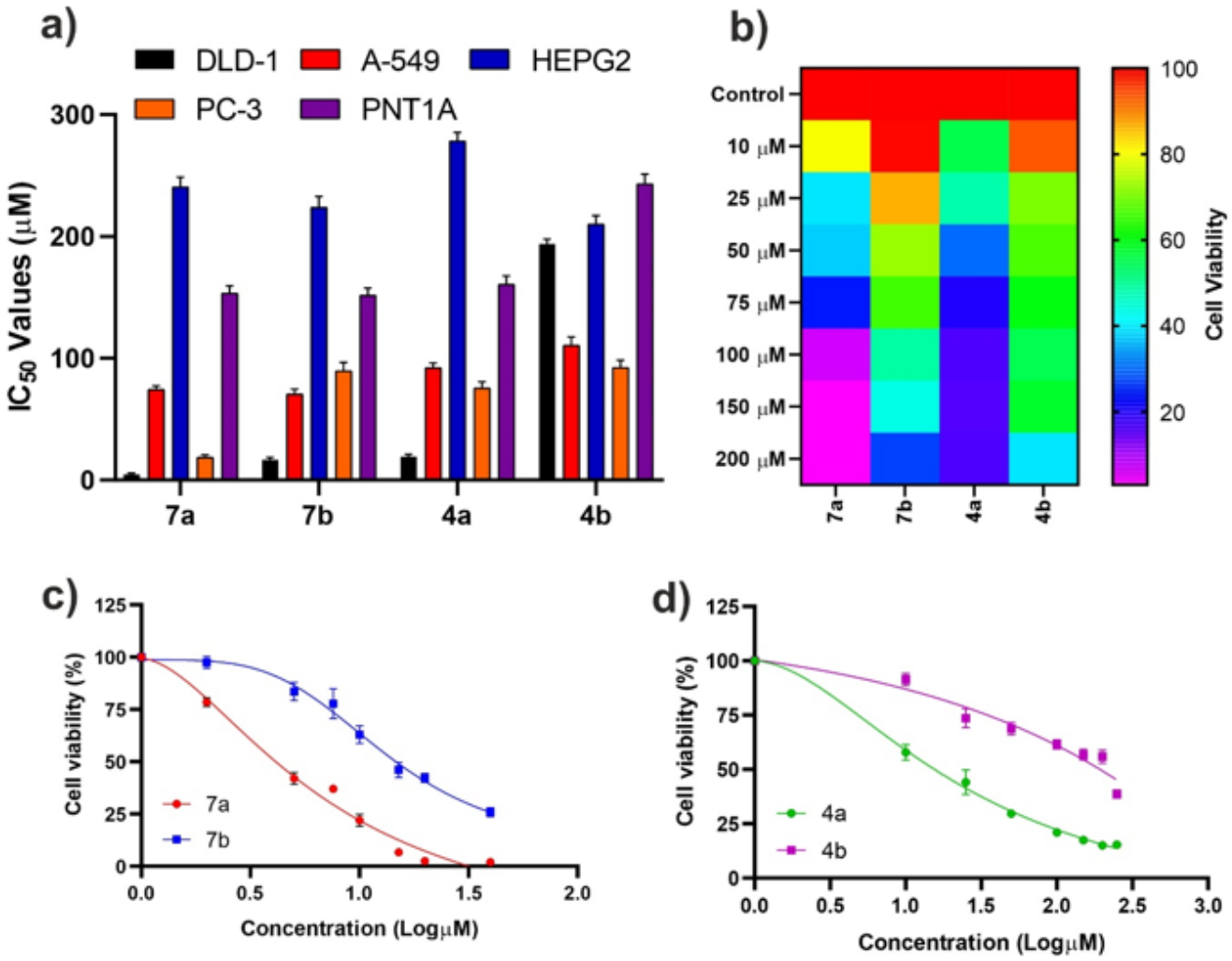

Figure 2

Cytotoxic potential of synthesized TCP compounds. a) The IC50 values of TCP compounds on DLD-1, A-549, HEPG2, PC-3, and PNT1A cells. b) Representative Heat-map analyses of various concentrations of the compounds on DLD-1 cells. c) and d) Sigmoidal plot of compound 7a, 7b, 4a, and 4b to calculate IC50 values. The results are presented the mean ± SD, $p<0.05$ was set the limit of significance, n=6.

Supplementary Files

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- Scheme2.png