Synthesis of new calix[4]arene 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 CH2Cl2 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 using spectroscopic techniques (FTIR, 1H NMR, and 13C NMR). The cytotoxic properties of the calix[4]arene superbase derivatives were investigated against different human cancer cell, including A549, DLD-1, HEPG2, and PC-3 and 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 (IC50: 4.7 µM), and the IC50 values were 18.5 µM and 74.4 µM against human prostate and lung cancer cells, respectively. Furthermore, compound 4b was found more effective on hepatocellular carcinoma cells (IC50: 210.2 µM). As a result, the synthesized calix[4]arene superbase derivatives can be developed to treat different human cancer cell. They can be considered as a preliminary result for molecular-level research.

Graphical Abstract
Keywords Calix[4]arene · Cytotoxicity · Anticancer agent · Proline

Introduction
Organic bases 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]. As the smallest ring system that complies with Hückel rules, the cyclopropenium ion (2π-electron) provides significant aromatic resonance stabilization to 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 cyclopropenamines 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 promising and simple method for preparing cyclopropenamines and recommended it for several organic synthesis applications [4–7].

In supramolecular chemistry, calix[n]arenes have solidified their position as promising 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 biomedical fields, such as they can be applied as biocatalyst and antitumor agents. The calixarene compounds have shown promising 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’ antitumor 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 polyhydroxylamine 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]. Furthermore, the cytotoxic activities of calix[4]arene derivatives on various tumor cells (MU2, MU2F, HT1080, SP6.5, 1PC227, Jurkat, MEWO, HI-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 HPV (Human papillomavirus) [31]. In our recent study, calix[4]arene derivatives bearing L-proline on their upper and lower rim were prepared and investigated their antitumor 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. In addition, 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 [33]. 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 [34]. There are a limited number of antitumor activity studies of calixarenes derivatives functionalized with different groups. Ding et al. [24, 25, 35] 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]arene were conjugated with chlorocycloprop-1-ene-1,2-diyl di-L-proline derivative obtained by reacting
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 (Schemes 1, 2), starting from compounds 1a and 1b (p-tert-butylcalix[4]arene and calix[4]arene) [36]. 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 K2CO3 in acetonitrile for 24 h as described previously [37, 38]. 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 K2CO3 in CH3CN 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-diyldi-L-proline), which is obtained by the reaction of L-proline with tetrachlorocyclopropene was reacted with compounds 3a, 3b, 6a, and 6b at room temperature in CH2Cl2 to form calix[4]arene super base derivative (4a, 4b, 7a, and 7b) in 75%, 60%, 70%, and 55% yield, respectively (Schemes 1, 2).

The synthesized compounds’ molecular structures were confirmed using 1H/13C NMR, FTIR spectroscopy, and elemental analysis. In FTIR spectra of 4a, 4b, 7a, and 7b, specific carbonyl bands (ester/amide) at 1737/1666, 1737/1674, 1740/1604, 1738/1644 cm⁻¹, respectively, approved the formation of calix[4]arene 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. L-proline units’ protons were approximately observed for -CH2 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 the 1H-NMR spectrum. The synthesis of compound 7a was also approved by the appearance of proline group signals -CH2 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 -CH2 at δ 1.83–2.37, and -CH at 4.41–4.58 ppm in the 1H-NMR spectra. The 13C-NMR spectra of 4a, 4b, 7a, and 7b showed carbon signals 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 cancer cell and healthy cell

Forty-eight hours treatment of human cancer cell 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. 2b, compound 7a was found to be the most potent inhibitor of the proliferation of DLD-1 cells and least inhibitory action on HEPG2 cells. The IC₅₀ values against the proliferation of DLD-1, A549, HEPG2, PC-3, and PNT1A were calculated as 4.7, 74.4, 240.7, 18.5, and 153.4 µM, respectively (Fig. 2c, d). Compound 7b was found to be the most potent inhibitor against DLD-1 and HEPG2 cells, respectively. The IC₅₀ values against the proliferation of DLD-1, A549, HEPG2, PC-3, and PNT1A were calculated as 16.5, 70.6, 223.8, 89.7, and 151.7 µM, respectively. Compound 4a dose-dependently inhibited the proliferation of human cancer cell, and the IC₅₀ values on DLD-1, A549, HEPG2, PC-3, and PNT1A were calculated as 18.5, 90.4, 288.3, 70.7, and 152.1, respectively. Among the compounds, compound 4b showed less inhibitory potency against all cancer cell with IC₅₀ values as 193.9, 110.7, 210.2, 75.5, 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*). Furthermore, 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 [32].

In this study, L-proline methyl ester groups were reacted with tetrachlorocyclopropane 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.

![Scheme 1](image-url)
in the solution phase \[4, 7\]. These compounds are also degradable in solid form to 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 h, a significant proportion of cyclopropeneimines groups were separated from \(7a-b\) (80%) \(4a-b\) (65%) compounds were observed.

Fig. 2 Cytotoxic potential of synthesized TCP compounds. a The IC\(_{50}\) values of TCP compounds on DLD-1, A-549, HEPG2, PC-3, and PNT1A cells. b Representative Heatmap analyses of various concentrations of the compounds on DLD-1 cells. c, d Sigmoidal plot of compounds \(7a, 7b, 4a,\) and \(4b\) to calculate IC\(_{50}\) values. The results presented the mean ± SD, \(p < 0.05\), set the limit of significance, \(n = 6\). **\(p < 0.001\), ***\(p < 0.0001\).
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

Schemes 1 and 2 represent the synthesis of various derivatives of calix[4]arenes. The synthesis of calixarene diamino-propyl and hydrazine derivatives were synthesized as precursor compounds (compound 3a, 3b, 6a, and 6b) using the reported procedures with slight modification [37, 38]. Then, 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.

The 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$_2$Cl$_2$ and an equivalent amount (6.6 mmol) of L to prepare the calix [4] arene super base derivatives, 1.10 g (1.10 mmol) was slowly added to the solution under nitro-gen atmosphere and stirred at room temperature for over-night 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 evaporated under vacuum and then the remaining solid was extracted with CHCl$_3$/H$_2$O several times, and the organic phase was dried over MgSO$_4$. After that, tetrachlorocyclopentene (1.10 mmol) was slowly added to the solution under nitrogen atmosphere and stirred at room temperature of overnight then the calix[4]arene-super base derivative was formed as white solid. 4a: Yield 75%; Mp: >190°C (dec.). FT-IR: 3322 cm$^{-1}$ (O-H, N-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); 1.40 (s, 18H, Bu)$^1$; 1.86–2.05 (m, 8H,-(CH$_2$)$_2$proline) 2.21–2.37 (m, 8H, -(CH$_2$)$_2$proline); 3.01–3.28 (m, 8H, -(CH$_2$)$_2$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-)$^1$

| 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]
CDCl3; δ (ppm) 1.83–2.37 (m, 16H, -CH2-proline); 2.55 (bs, 4H, -CH2-); 2.99–3.32 (m, 4H, CH2); 3.49 (bs, 4H, Ar-CH2-Ar); 3.54–3.65 (m, 8H, -CH2); 3.91 (s, 12H, OCH3); 4.23 (bs, 4H, CH2); 4.33 (bs, 4H, CH2); 4.41–4.58 (m, 4H, -CH2-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 vs OH). 13C-NMR (100 MHz, CDCl3): 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, 175.8 (C=O-proline). Analytical Cal. for (%) C64H72N2O12: 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% CO2 with 95% humidity.

Determination of cytotoxic potential of TCP compounds

The cell viability and cytotoxic potential of TCP compounds were carried out with Alamar Blue assay [39]. TCP compounds were dissolved in growth media. 1 × 104 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 IC50 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 chlorocycloprenium. 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, 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. Therefore, 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.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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