Scientific paper

Design, synthesis, and biological evaluation of *N*-propionylcysteamine-functionalized calix[n]arenes: antioxidant and enzyme inhibitory activities

Mürüvvet Kurt, Murat Özer*, Gülderen Uysal Akkuş

Department of Chemistry, Faculty of Science and Arts, Afyon Kocatepe University, 03204, Afyonkarahisar, Türkiye

** Corresponding author: E-mail: mozer@aku.edu.tr*

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Abstract

In this study, calix[n]arenes were functionalized with *N*-propionylcysteamine *via* phenolic moieties, followed by ipso-sulfonation to improve water solubility. Sulfonated derivatives calix[4]arene (**C4–4**) and calix[8]arene (**C8–4**) were characterized by FT-IR, ^{1}H NMR, and ^{13}C NMR. Antioxidant activities were determined using DPPH, ABTS$^{\bullet}$ radical scavenging, and Fe(II) chelation assays. At a concentration of 1 mM, DPPH scavenging was 36.45% (**C4–4**) and, 36.02% (**C8–4**); Fe(II) chelation 33.33% and, 32.65%; ABTS$^{\bullet}$ scavenging 33.41% and, 39.54%. Both compounds moderately inhibited acetylcholinesterase and α-glucosidase. These results indicate that sulfonated cysteamine-calixarenes possess notable antioxidant potential and moderate enzyme inhibition, making them promising scaffolds for multifunctional bioactive molecule development.

Keywords: Calixarene, Cysteamine, Antioxidant Activity, Enzyme Inhibition

1. Introduction

Calixarenes serve as an important platform for biological applications such as molecular recognition and enzyme inhibition, owing to their unique structures and functional groups. These compounds are considered versatile host molecules in supramolecular chemistry, forming stronger complexes than other macrocyclic compounds like cyclodextrins and crown ethers. Due to their easily functionalizable structures at both the upper and lower rims, calixarenes are capable of interacting with a wide range of molecules, including anions, cations, and neutral species, exhibiting a variety of biological and chemical activities.[1] These properties make calixarenes valuable for applications in biological processes, such as enzyme inhibition.

In recent years, attention has shifted towards calix[n]arenes functionalized with thiol containing groups, such as cysteamine, due to their significant biological potential. Cysteamine, a naturally occurring amino thiol, has shown potent antioxidant activity and is employed in therapeutic applications to mitigate oxidative stress a key contributor to various diseases, including neurodegenerative disorders and cancer.[2] By functionalizing calixarenes with cysteamine derivatives, researchers aim to create compounds with enhanced antioxidant properties, which could be particularly beneficial for protecting cells from oxidative damage and inflammation. Among these, *N*-propionylcysteamine-functionalized calixarenes are especially intriguing, as this modification may provide a platform for additional bioactive properties and enzyme inhibition.

Reactive oxygen species (ROS) are major contributors to oxidative stress in living cells and are involved in the development of chronic diseases such as cardiovascular disease and cancer.[3] The antioxidant properties of calixarenes have gained considerable attention for their ability to neutralize ROS and mitigate their harmful effects.[4] Additionally, modification of calixarenes with various functional groups enhances their potential as enzyme inhibitors. Inhibition studies on enzymes such as acetylcholinesterase (AChE), α-glucosidase, and tyrosinase underscore the therapeutic promise of calixarenes.[5–6]

Functionalization of calixarenes with phenolic, sulfonate, and phosphate groups has been reported to improve their antioxidant properties. These modifications not only increase their water solubility but also enhance their biological activity.[7] For instance, the antioxidant activities of azocalixarene derivatives, synthesized by reacting calixarenes with diazonium salts, have demonstrated high free radical scavenging abilities.[8] Furthermore, functionalizing calix-

arenes with structures like sulfonamides and dihydropyrimidines has amplified both their antioxidant and enzyme inhibition capabilities.[9–10] The multivalent architecture of calix[n]arenes allows the simultaneous presentation of multiple functional groups, which can lead to synergistic effects in binding and biological activity. This multivalency is a key factor in their potential as effective enzyme inhibitors and free radical scavengers.[11–12] The biological activity of calixarene derivatives is strongly influenced by the nature and position of the substituents on their upper and lower rims. Subtle changes in functional groups can significantly alter their redox behavior, binding affinity, and selectivity toward enzyme active sites, highlighting the importance of structure-activity relationship (SAR) studies.[13–15]

Enzyme inhibition, particularly targeting acetylcholinesterase (AChE), α-glucosidase, and tyrosinase, has become a fundamental strategy in the treatment of chronic diseases such as Alzheimer's disease, type 2 diabetes, and hyperpigmentation disorders. AChE inhibitors play a crucial role in managing neurodegenerative diseases, while α-glucosidase inhibitors are widely used in diabetes therapy. Additionally, tyrosinase inhibitors are important for treating dermatological conditions related to abnormal skin pigmentation. In recent years, calixarene derivatives have emerged as promising supramolecular architectures with significant inhibitory effects on these enzymes. Their unique ability to interact with enzyme active sites enables calixarenes to serve as effective potential therapeutic agents. Various studies have demonstrated that functionalized calix[n]arenes exhibit potent inhibition against AChE, α-glucosidase, and tyrosinase, highlighting their potential for optimizing biological activities through structural modifications.[15] These findings underscore the value of calixarene-based compounds as versatile candidates in the development of novel treatments for neurodegenerative, metabolic, and dermatological disorders.[16–17]

In addition to their established antioxidant and enzyme inhibitory roles, calix[n]arenes have recently emerged as multifunctional platforms in nanomedicine. They self-assemble into nanostructures such as micelles, capsules, and cage like systems facilitating drug encapsulation and controlled release, which enhances solubility, stability, and membrane permeability.[18] For example, Fan and Guo[18] highlighted their potential to carry chemotherapeutics in tumor models, while Xu *et al.*[19] demonstrated hypoxia-responsive, tumor targeted delivery systems. Recently, Muley *et al.*[20] reviewed how calixarene guest architectures improve the bioavailability and targeting capacity of biologics including proteins and peptides.

In light of these considerations, the biological activities of calixarenes underscore their potential for diverse applications in the pharmaceutical field. The functional group modifications of calixarenes not only enhance their antioxidant and enzyme inhibition activities but also provide opportunities for the development of more efficient therapeutic approaches. Thus, research into the biological properties and enzyme inhibition of calixarenes paves the way for their use in drug development.

In this study, we report the design, synthesis, and biological evaluation of *N*-propionylcysteamine-functionalized calix[n]arenes. These compounds were synthesized through the functionalization of calixarene derivatives with *N*-propionylcysteamine and characterized using various techniques, including NMR and FT-IR spectroscopy. The synthesized compounds were tested for their antioxidant properties and inhibitory activities against AChE, α-glucosidase, and tyrosinase, providing valuable insights into the therapeutic potential of these functionalized calix[n]arenes for managing oxidative stress related diseases and enzyme modulation.

In light of these considerations, the biological activities of calixarenes underscore their potential for diverse applications in the pharmaceutical field. However, current research on *N*-propionylcysteamine-functionalized calix[n]arenes remains limited, especially in terms of structure-activity comparisons between macrocyclic sizes and the impact of sulfonation. Therefore, in this study, we aimed to synthesize and characterize novel **C4** and **C8** derivatives bearing *N*-propionylcysteamine moieties and evaluate their antioxidant and enzyme inhibition profiles through both experimental and spectroscopic methods. The results contribute to a deeper understanding of how calixarene frameworks and functional modifications influence biological performance and therapeutic potential.

2. Materials and methods

2. 1. Materials

2. 1. 1. Instrumental techniques

Melting point determinations were performed using a Barnsted/Electrothermal device. The ^{1}H NMR and ^{13}C NMR spectra were recorded using a JEOL ECX-400 MHz spectrometer, with $CDCl_3$ as the solvent. Tetramethylsilane (TMS) was used as the internal standard in the ^{1}H NMR spectra, and chemical shifts (δ) are reported in ppm. FT-IR spectra were obtained using a SHIMADZU IRAffinity–1S spectrophotometer, with KBr disks. In the enzyme inhibition assays, optical densities were measured with a ROMER BioTek ELx800 automatic microplate reader at specific wavelengths (nm). All aqueous solutions were prepared using water purified by a Millipore Milli-Q Plus water purification system.

Analytical thin-layer chromatography (TLC) was performed on pre-coated aluminum plates with silica gel (SiO_2, Merck 60 F_{254}). NaH (80% paraffin) was washed twice with *n*-hexane before use. Tetrahydrofuran (THF) and toluene were refluxed over sodium/benzophenone and subsequently distilled fractionally to obtain fresh solvents for use. Acetone was distilled over anhydrous $MgSO_4$ and used fresh.

2. 2. Synthesis

The compounds C4–1, C4–2, and C8–1, C8–2 were synthesised according to the literature.[2,21–22] Compound C4–3, C4–4 and C8–3, C8–4 used in this work as illustrated in Figure 1, and Figure 2 have been synthesised according to the method given next.

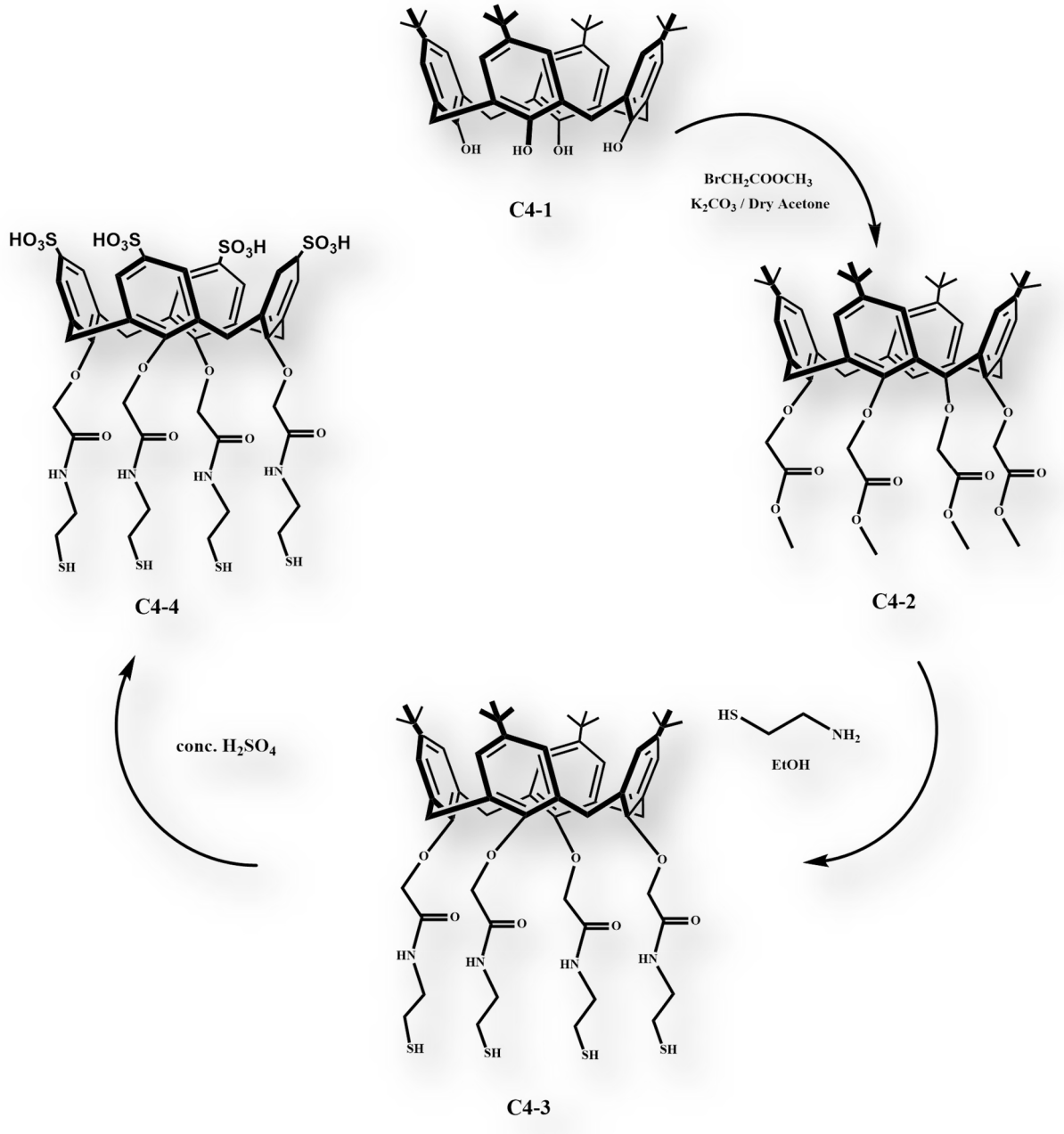

Figure 1. Synthesis scheme of *para-tert*-butylcalix[4]arene and its derivatives

2. 2. 1. Synthesis of *para-tert*-Butylcalix[4]aren-tetrakis(3-mercaptopropanamid) (C4–3)

The synthesized compound **C4–2**, (0.54 g, 0.57 mmol) and cysteamine (3 g, 38.76 mmol) were dissolved in 22.5 mL of ethanol, and the reaction mixture was heated under reflux for 36 hours. After cooling to room temperature, the resulting white solid was filtered and washed with ethanol. The crystalline pure compound **C4–3** was obtained. Yield: 0.509 g (80%). FT-IR (KBr): 3200, 2600, 1652, 1530 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.27 (s, 36H, But), 1.41 (s, 4H, SH), 2.47 (t, 8H, SCH_2), 3.45 (d, J = 13.2 Hz, 4H, Ar–CH_2–Ar), 3.66 (t, J = 7.03 Hz, 8H, $NHCH_2$), 4.3 (m, 12H, OCH_2, Ar–CH_2–Ar), 6.85 (s, 8H, ArH), 7.73 (br, 4H, NH). ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm): 26.7, 31.3, 33.0, 34.8, 42.3, 67.6, 119.3, 126.0, 143.2, 151.4, 168.6. FAB MS *m/z* 1116.52 $[M+1]^+$. Anal.

calcd for $C_{60}H_{84}N_4O_8S_4$: C, 64.48; H, 7.58; N, 5.01; S, 11.47. Found: C, 64.10; H, 7.25; N, 4.91; S, 11.04.

2. 2. 2. Ipso sulfonation of *para-tert*-Butylcalix[4]aren-tetrakis(3-mercaptopropanamid) (C4–4)

A reaction mixture consisting of 0.5 g (0.5 mmol) of compound **C4–3**, and 5 mL of 98% H_2SO_4 was heated under reflux at 50°C in an oil bath for 5 hours, maintaining the temperature constant. The completion of the reaction was confirmed by solubility testing with water, using a pasteur pipette to collect a sample. The mixture was then cooled to room temperature. Approximately 50 mL of diethyl ether was added to a beaker, and the mixture was transferred dropwise onto the diethyl ether using a Pasteur pipette. The precipitated compound was filtered and dried, yielding compound. Yield: 0.49 g (82%) (**C4–4**). FT-IR (KBr): 1665, 1520, 1200, 1141 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.41 (s, 4H, SH), 3.37 (s, 8H, SCH_2), 3.80 (s, 12H, Ar–CH_2–Ar, N–CH_2), 4.27 (m, 4H, Ar–CH_2–Ar), 5.18 (s, 8H, OCH_2), 7.42 (s, 8H, ArH), 7.72 (s, 4H, NH). 9.31(s, 4H, SO_3H), ^{13}C NMR (400 MHz, $CDCl_3$) δ (ppm): 26.5, 42.1, 67.3, 116.2, 129.2, 130.1, 142.6, 161.8, 168.5. FAB MS *m/z* 1212.09 $[M+1]^+$. Anal. calcd for $C_{44}H_{52}N_4O_{20}S_8$: C, 43.55; H, 4.32; N, 4.62; S, 21.14. Found: C, 43.26; H, 4.23; N, 4.35; S, 20.91.

2. 2. 3. Synthesis of *para-tert*-Butylcalix[8]aren-oktakis(3-mercaptopropanamid) (C8–3)

The synthesized compound **C8–2**, (0.74 g, 0.4 mmol) and 2 g (27.2 mmol) of cysteamine were mixed and 22.5 mL of ethanol was added to the reaction mixture. The reaction was heated under a reflux condenser for 36 hours. After cooling to room temperature, the white solid precipitate was filtered and washed with ethanol. The resulting crystalline pure compound **C8–3** was obtained. Yield: 0.69 g (78%). FT-IR (KBr): 3320, 2600, 1654, 1530 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$) δ (ppm): 1.24 (s, 72H, But), 1.4 (s, 8H, SH), 2.38 (t, J = 4.9 Hz, 16H, SCH_2), 3.32 (br, 16H, $NHCH_2$), 4.02 (br, 32H, Ar–CH_2–Ar, OCH_2), 6.89 (br, 16H, ArH), 7.72 (br, 8H, NH). ^{13}C NMR (400 MHz $CDCl_3$) δ (ppm): 26.3, 31.5, 34.6, 42.9, 67.3, 111.8,

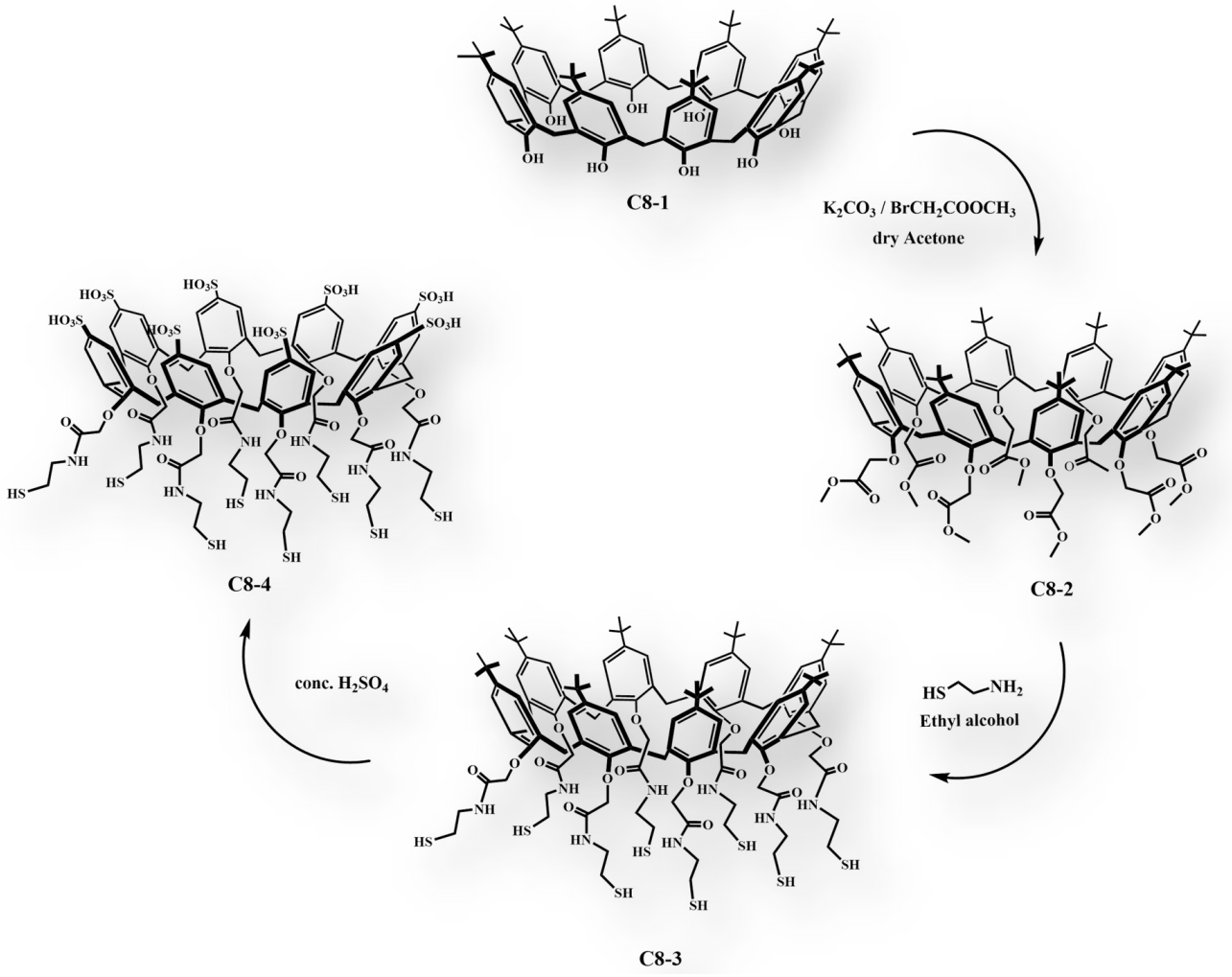

Figure 2. Synthesis scheme of *para-tert*-butylcalix[8]arene and its derivatives

123.2, 127.5, 143.4, 154.7, 168.6. FAB MS *m/z* 2234.04 $[M+1]^+$. Anal. calcd for $C_{120}H_{168}N_8O_{16}S_8$: C, 64.48; H, 7.58; N, 5.01; S, 11.47. Found: C, 64.35; H, 7.50; N, 4.94; S, 11.04.

2. 2. 4. Ipsosulfonation of *para-tert*-Butylcalix[8] aren-oktakis(3-mercaptopropanamid) (C8–4)

The reaction mixture consisting of 0.5 g (0.25 mmol) of compound **C8–3**, and 5 mL of 98% H_2SO_4 was heated under a reflux condenser in an oil bath set at 50 °C for 5 hours, maintaining a constant temperature. Upon completion of the reaction, the mixture was tested for solubility in water using a Pasteur pipette. The mixture was then cooled to room temperature. Approximately 50 mL of diethyl ether was added to the reaction mixture, and it was dropped onto the diethyl ether using a pasteur pipette. The precipitate formed was filtered and dried, resulting in the compound. Yield: 0.48 g (79%). (**C8–4**). FT-IR (KBr): 1660, 1530, 1210, 1142 cm^{-1}. ^1H NMR (400 MHz $CDCl_3$) δ (ppm): 1.4 (br s, 8H, SH), 3.14 (br, 16H, SCH_2), 3.53 (br, 16H, $NHCH_2$), 4.19 (s, 16H, Ar–CH_2–Ar), 4.88 (br, 16H, OCH_2), 7.37 (s, 16H, ArH), 7.72 (br, 8H, NH). 9.34(s, 8H, SO_3H), ^{13}C NMR (400 MHz $CDCl_3$) δ (ppm): 26.2, 41.9, 66.8, 115.5, 128.1, 129.9, 143.3, 160.1, 168.4 FAB MS *m/z* 2424.19 $[M+1]^+$. Anal. Calcd for $C_{88}H_{104}N_8O_{40}S_{16}$; C, 43.55; H, 4.32; N, 4.62; S, 21.14. Found: C, 43.43, H, 4.25, N, 4.55; S, 21.03.

2. 3. Antioxidant activity studies and acetylcholinesterase, α-glucosidase, and tyrosinase enzyme inhibition methods

The antioxidant properties of the functional calix[n] arenes obtained after synthesis and whose structures have been elucidated have been determined using the methods outlined below. The results are expressed graphically using appropriate standards for the methods. For ease of coding during analysis, the ipso-sulfonated calix[4]arene compound is referred to as **C4–4**, and the ipso-sulfonated calix[8]arene synthesis as **C8–4**.

2. 3. 1. Antioxidant activity assays

The antioxidant potential of the synthesized **C4–4** and **C8–4** calix[n]arene derivatives was evaluated through three complementary in vitro assays: DPPH radical scavenging, iron(II) ion chelation, and ABTS• radical cation inhibition.

For the DPPH assay, sample solutions at concentrations ranging from 0.05 to 1 mM were prepared, and 40 µL aliquots were combined with 160 µL of DPPH solution. Following 30 minutes of incubation at ambient temperature in the dark, absorbance was recorded at 517 nm. Ultrapure water served as the blank. The radical scavenging activity was quantified by comparing the decrease in absorbance relative to the control, and results were expressed as percent inhibition. Butylated hydroxyanisole (BHA) was employed as a reference antioxidant.[23]

Iron(II) chelation capacity was determined based on the competition between the calix[n]arene compounds and ferrozin for Fe^{2+} ions. Sample mixtures containing 100 µL of test solution, $FeCl_2$ and ferrozin were incubated sequentially, with final absorbance measured at 562 nm after a 40 minute reaction period. The degree of chelation was calculated by comparing absorbance values of the control and samples, using EDTA as a standard chelating agent.[4]

The ABTS• radical cation assay involved generating the radical by mixing equal volumes of 7.4 mM ABTS and 2.6 mM potassium persulfate, followed by 16 hour dark incubation. The resultant solution was diluted to an absorbance of approximately 1.1 at 734 nm. Samples (150 µL) were then reacted with the ABTS• solution (2850 µL) for 6 minutes, and absorbance reduction was measured at 734 nm. Trolox was used as a standard for comparison. Radical inhibition percentages were calculated based on absorbance changes relative to controls and blanks.[24]

2. 3. 2. Determination of enzyme inhibitory activities

The inhibitory effects of the synthesized compounds on acetylcholinesterase (AChE), butyrylcholinesterase (BChE), tyrosinase, and α-glucosidase enzymes were assessed using spectrophotometric microplate assays.

Cholinesterase inhibition was measured by the Ellman *et al.*[25] method, which quantifies the production of 5-thio-2-nitrobenzoic acid (TNB) from the enzymatic hydrolysis of acetylthiocholine or butyrylthiocholine substrates. Sample solutions (50 µL) were combined with DTNB reagent and AChE or BChE enzyme, incubated at 25 °C for 15 minutes, followed by substrate addition. Absorbance was read at 405 nm after 10 minutes. Galantamine served as a positive control, and inhibitory activity was expressed as mg galantamine equivalents per gram of extract.

Tyrosinase inhibition was evaluated via the dopachrome assay, based on L-DOPA oxidation catalyzed by tyrosinase. Samples were preincubated with enzyme and phosphate buffer, then reacted with L-DOPA. After 10 minutes at 25 °C, absorbance was recorded at 492 nm. Kojic acid was used as the standard inhibitor, and results were presented as mg kojic acid equivalents per gram of extract.[26]

For α-glucosidase inhibition, samples were incubated with enzyme prior to adding *para*-nitrophenyl-β-D-glucopyranoside substrate. After 15 minutes at 37 °C, the reaction was stopped with sodium carbonate, enhancing the yellow *para*-nitrophenol product. Absorbance was measured at 400 nm, with inhibition percentages calculated relative to control samples without inhibitors.[27]

In all assays, enzyme-free blanks were included, and inhibition percentages were calculated as follows:

$$\text{Inhibition (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

3. Results and discussion

3. 1. Synthesis and characterization of *para-tert*-Butylcalix[4]arene derivatives

The compound **C4–3** was obtained by refluxing compound **C4–2** with cysteamine in ethanol yield 80% following the method of Pandya *et al.*[2] Reaction progress was monitored by TLC. The FT-IR spectrum showed the disappearance of the ester carbonyl band at 1754 cm^{-1} and the appearance of a new amide band at 1652 cm^{-1}. In the ^1H NMR spectrum, the absence of the 3.82 ppm methyl ester signal and the emergence of a new signal at 7.73 ppm for four amide –NH protons confirmed the conversion. Additional signals at 1.41 ppm (four –SH protons) and 2.47 ppm (eight –S–CH_2 protons) indicated successful cysteamine incorporation.

Finally, sulfonation of **C4–3** was performed by heating with H_2SO_4 at 50 °C under reflux for 5 hours, yielding ipso-sulfonated derivative (**C4–4**).[2] Reaction completion was confirmed by a water solubility test. The FT-IR spectrum showed a new absorption band at 1141 cm^{-1} corresponding to the sulfonic acid group (O=S=O). The ^1H NMR spectrum lacked the 1.11 ppm signal for *tert*-butyl protons, indicating their removal during sulfonation. The synthesis scheme is presented in Figure 1.

3. 2. Synthesis and characterization of *para-tert*-Butylcalix[8]arene derivatives

The compound **C8–3** was obtained by refluxing compound **C8–2** with cysteamine in ethanol yield 78% following the method of Pandya *et al.*.[2] Reaction progress was monitored by TLC. The FT-IR spectrum showed the disappearance of the ester carbonyl band at 1753 cm^{-1} and the appearance of a new amide band at 1654 cm^{-1}. In the ^1H NMR spectrum, the absence of the 3.51 ppm methyl ester signal and the emergence of a new signal at 7.72 ppm for eight amide –NH protons confirmed the conversion. Additional signals at 1.4 ppm (eight –SH protons) and 2.38 ppm (sixteen –S–CH_2 protons) indicated successful cysteamine incorporation.

Finally, sulfonation of **C8–3** was performed by heating with H_2SO_4 at 50 °C under reflux for 5 hours, yielding ipso-sulfonated derivative (**C8–4**).[2] Reaction completion was confirmed by a water solubility test. The FT-IR spectrum showed a new absorption band at 1141 cm^{-1} corresponding to the sulfonic acid group (O=S=O). The ^1H NMR spectrum lacked the 1.24 ppm signal for *tert*-butyl protons, indicating their removal during sulfonation. The synthesis scheme is presented in Figure 2.

3. 3. *In vitro* assessment of antioxidant properties and Inhibitory effects on acetylcholinesterase, α-glucosidase, and tyrosinase enzymes

The antioxidant properties of the synthesized calix[n]arenes were systematically evaluated employing a range of free radical scavenging assays, including DPPH, ABTS, and iron(II) chelation methods. The obtained results were analyzed and compared with the established standards in the literature, providing a comprehensive assessment of their efficacy in neutralizing reactive oxygen species.

According to the results of the DPPH method, both **C4–4** and **C8–4** compounds exhibited free radical scavenging properties. The % inhibition values of the tested samples are shown in Figure 3. Specifically, at a concentration of 1 mM, the **C4–4** and **C8–4** compounds demonstrated inhibition effects of 36.45% and 36.02%, respectively, highlighting the radical scavenging potential of these compounds. These values are consistent with previously reported sulfonato-calix[4]arene derivatives, which typically exhibit moderate scavenging activity (40–50%) in the DPPH assay. Moreover, calixtyrosol a tyrosol-functionalized calix[4]arene has been reported to show a remarkable DPPH stoichiometric factor of n = 4.83, highlighting the effectiveness of multivalent antioxidant scaffolds.[28]

However, these values are considerably lower compared to the synthetic antioxidant BHA (89.47% inhibition) (Figure 3). This suggests that while BHA remains superior in potency, calixarene-based systems still offer a meaningful contribution to radical scavenging especially when appropriately derivatized.

The chelating capacity of calixarenes for iron(II) ions is dependent on their structural features and binding sites. At a concentration of 1 mM, **C4–4** exhibited a chelation activity of 33.33%, while **C8–4** showed 32.65%. It is evident that EDTA, with a chelation activity of 56.46%, is more effective than these compounds (Figure 3). This result aligns with prior studies reporting that the steric bulk and spatial arrangement of calixarene frameworks often limit their metal-chelating ability, particularly when compared to classical chelators like EDTA.

In the tests conducted with the $ABTS^{\bullet}$ radical, the compound **C8–4** exhibited a higher inhibitory effect compared to **C4–4** (Figure 3). At a concentration of 1 mM, **C8–4** demonstrated an inhibition of 39.54%, indicating a stronger $ABTS^{\bullet}$ radical scavenging activity than that of **C4–4** (33.41%). Although these results are notably lower than Trolox (83.47%), they fall within the activity range reported for phenol-containing calixarene derivatives.[29] This highlights that calixarenes, especially when modified with electron-donating groups, maintain consistent but improvable antioxidant capabilities.

The inhibitory effects of the compounds **C4–4** and **C8–4** against acetylcholinesterase, tyrosinase, and glucosi-

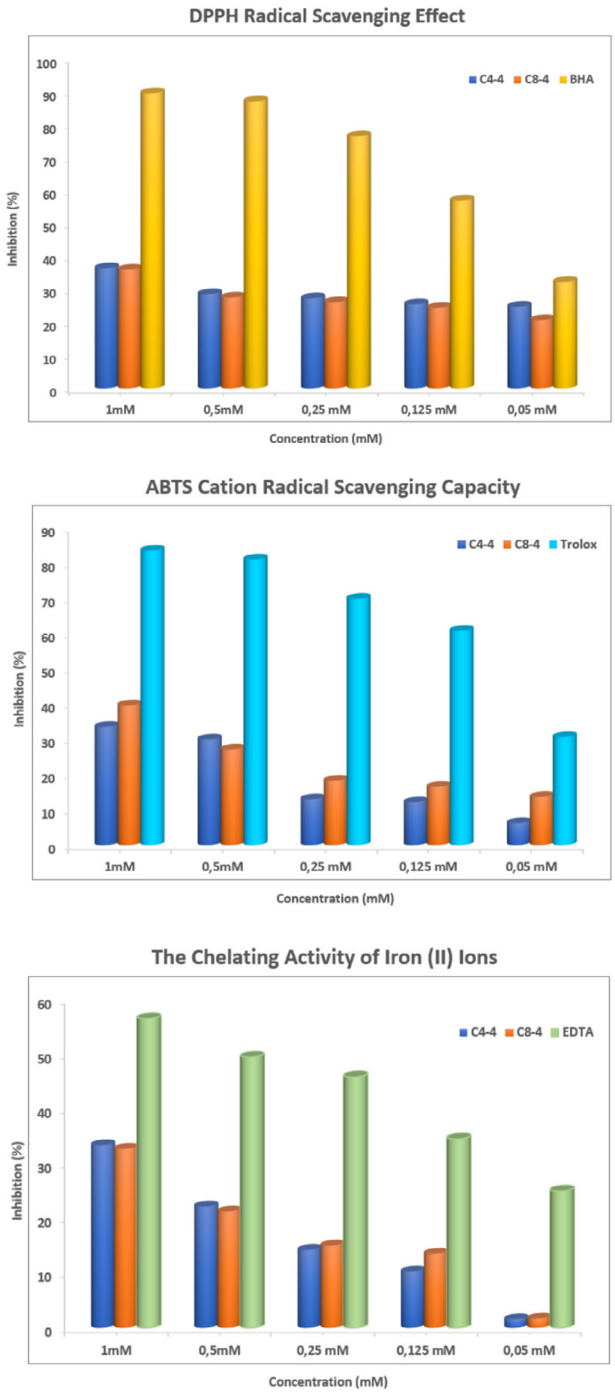

Figure 3. Antioxidant Activity Assays

dase enzyme activities were comparatively evaluated. For **C4–4**, the inhibition rate against acetylcholinesterase was found to be 41.09%, while inhibition against tyrosinase activity was observed as 9.14%. The glucosidase inhibition of **C4–4** was determined to be 56.27%. In the case of **C8–4**, the compound exhibited 37.81% inhibition against acetylcholinesterase, 17.71% inhibition against tyrosinase, and 39.43% inhibition against glucosidase. These results indicate that **C4–4** exhibits a higher inhibitory effect on glucosidase activity, whereas **C8–4** is more effective in inhibiting tyrosinase.

These data suggest that **C4–4** demonstrates a stronger glucosidase inhibition capacity, whereas **C8–4** is more effective in inhibiting tyrosinase activity. Regarding acetylcholinesterase, both derivatives show moderate inhibition levels consistent with earlier findings on calix[4]azacrown and calix[4]amide derivatives, which commonly inhibit AChE activity within the 35–45% range.[10] The glucosidase inhibition values, particularly for **C4–4**, are also comparable to those of plant-derived phenolic compounds such as those found in *Rhamnus formosana*, which demonstrated potent activity with IC_{50} values in the 2–10 μg/mL range.[30] Table 1 summarizes the enzyme inhibition results, confirming that the tested calixarene derivatives exhibit differential enzyme selectivity depending on structural features.

Table 1. Enzyme Inhibition Assays

Samples	Acetylcholinesterase Inhibition[a] *(% inhibition)*	Tyrosinase Inhibition[a] *(% inhibition)*	Glucosidase Inhibition[b] *(% inhibition)*
C4–4	41.09 ± 0.59	9.14 ± 0.53	56.27 ± 0.13
C8–4	37.81 ± 0.90	17.71 ± 0.21	39.43 ± 0.34
Galantamine[b]	81.99 ± 1.28	–	–
Kojic acid[b]	–	52.00 ± 0.82	–
Acarbose[b] (1 μmol/mL)	–	–	87.55 ± 0.23

[a] % Percentage inhibition values 0,5mM

[b] Standard compounds

In this project, various calixarene derivatives modified with different functional groups were successfully synthesized and characterized by spectroscopic methods such as FT-IR, ^{1}H NMR, and ^{13}C NMR. In this context, the following derivatives were synthesized: *para-tert*-butylcalix[4]arene (**C4–1**), tetraester (**C4–2**), tetraamide (**C4–3**) derivatives; ipso-sulfonated calix[4]arene (**C4–4**); *para-tert*-butylcalix[8]arene (**C8–1**), octaester (**C8–2**), octaamide (**C8–3**) derivatives, and ipso-sulfonated calix[8]arene (**C8–4**).

Particularly, the ipso sulfonated calix[n]arene derivatives with **C4–4** and **C8–4** codes exhibited free radical scavenging properties in antioxidant activity tests. According to the DPPH test results, **C4–4** showed 36.45% and **C8–4** showed 36.02% inhibition. These values are significantly lower than the standard antioxidant BHA (89.47%); however, considering the phenolic structure of calixarenes, they indicate that these compounds possess a certain antioxidant potential. These results suggest that the antioxidant capacity of calixarenes could be enhanced depending on their structural features.

Previous reports have indicated that calixarenes derivatized with azo, dihydropyrimidine, oxadiazole, and sulfonate groups exhibit enhanced radical scavenging ac-

tivity and enzyme inhibition.[9,28,31–32] In this study, the ipso-sulfonated derivatives **C4–4** and **C8–4** displayed moderate activity across all assays, suggesting that further derivatization (*e.g.*, addition of phenolic or azo-aromatic substituents) may significantly improve their biological performance.

Calixtyrosol and other tyrosol-based calixarene conjugates are particularly promising due to their multivalent antioxidant scaffolding, providing an opportunity for future structural optimization. Furthermore, steric hindrance observed in these large molecules may explain the lower metal chelation activity, which could be improved by rim modification or use of heterocyclic linkers.

When evaluating the chelation capacity of iron(II) ions, **C4–4** showed 33.33% and **C8–4** showed 32.65% chelation activity. These values are lower than the chelation capacity of EDTA (56.46%). This suggests that calixarenes, due to steric hindrance and limited binding sites, exhibit lower chelation activity for metal ions. Similar findings have been reported in previous studies, indicating that calixarenes have lower chelation capacities compared to EDTA.[4,10]

In the ABTS$^{\bullet}$ radical scavenging test, **C8–4** showed 39.54% and **C4–4** showed 33.41% inhibition. These results indicate that both compounds exhibit significant radical scavenging effects, although they are lower than the standard antioxidant trolox (83.47%). The literature indicates that the activity of calixarenes in the ABTS$^{\bullet}$ test varies depending on the functional group structure and their positions.[6,33–34]

In enzyme inhibition assays: **C4–4** showed 41.09% inhibition on acetylcholinesterase, 9.14% on tyrosinase, and 56.27% on glucosidase. **C8–4** showed 37.81% inhibition on acetylcholinesterase, 17.71% on tyrosinase, and 39.43% on glucosidase.

These data suggest that **C4–4** is more effective against glucosidase, while **C8–4** shows better inhibition against tyrosinase. Regarding acetylcholinesterase inhibition, both compounds exhibited moderate activity. AChE inhibition is a key strategy in Alzheimer's disease treatment, and the potential of calixarenes in this regard has been highlighted in the literature.[35]

4. Conclusion

In this study, a series of novel *para-tert*-butylcalix[n]arene derivatives (n = 4, 8) were successfully synthesized and structurally characterized *via* spectroscopic methods, including FT-IR and NMR analyses. The stepwise functionalization comprising amide formation followed by ipso-sulfonation enabled the generation of water-soluble calixarene analogs (**C4–4** and **C8–4**) with promising bioactive potential. The disappearance of characteristic ester signals and the emergence of sulfonic acid bands confirmed the targeted modifications.

Biological evaluations revealed that both **C4–4** and **C8–4** exhibit moderate antioxidant activity, with DPPH and ABTS$^{\bullet}$ scavenging capacities aligning with trends reported for sulfonated calixarene frameworks. Although their radical neutralization efficiency remains lower than standard antioxidants such as BHA and Trolox, their activity is still significant and modifiable through strategic structural enhancement. Notably, **C4–4** and **C8–4** also demonstrated measurable metal-chelating capacity, albeit inferior to classical chelators like EDTA likely due to steric limitations inherent to their macrocyclic architecture.

Enzyme inhibition assays further supported the multifunctional nature of these calixarene derivatives. **C4–4** displayed a pronounced glucosidase inhibition profile (56.27%), positioning it as a potential antidiabetic scaffold. Meanwhile, **C8–4** showed higher tyrosinase inhibition (17.71%), suggesting relevance in anti-hyperpigmentation or neuroprotective contexts. Both compounds achieved moderate inhibition of acetylcholinesterase activity (41.09% and 37.81%, respectively), underscoring their relevance in anti-Alzheimer's drug discovery pipelines.

Taken together, the present findings highlight the structural versatility and therapeutic promise of ipso-sulfonated calix[n]arenes. Their bioactivity spectrum particularly in antioxidant defense and selective enzyme inhibition can be fine tuned via rim functionalization or incorporation of additional pharmacophores. Future work should explore structure activity relationships (SAR), cytotoxicity profiling, and molecular docking analyses to further define their pharmacological potential.

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Povzetek

V okviru te študije smo z namenom povečanja topnosti v vodi kaliks[n]arene funkcionalizirali z *N*-propionilcisteaminom s pomočjo fenolnega fragmenta, s sledečo ipso-substitucijo. Sulfonirana derivata kaliks[4]arenov (**C4**–**4**) in kaliks[8] arenov (**C8**–**4**) smo karakterizirali s FT-IR, ^{1}H NMR in ^{13}C NMR. Antioksidativne lastnosti smo določili z metodami DPPH, lovljenjem ABTS$^{\bullet}$ radikalov in s keliranjem z Fe(II). Pri koncentraciji 1 mM je bila aktivnost z DPPH 36.45 % (**C4**–**4**) oz. 36.02 % (**C8**–**4**); keliranje Fe(II) 33.33 % oz. 32.65%; ABTS$^{\bullet}$ lovljenje pa 33.41 % oz. 39.54 %. Obe spojini sta izkazali zmerno inhibicijo acetilholinesteraze in α-glukozidaze. Rezultati kažejo, da sulfonirani cisteamin-kaliksareni izkazujejo opazen antioksidativni potencial in zmerno inhibicijo encimov, kar kaže, da so to primerna ogrodja za razvoj novih multifunkcionalnih bioaktivnih molekul.