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Full Length Article

Synthesis And Study Of The Physicochemical And Antimicrobial Properties Of New Azo-Calix [6] Arene Derivatives

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Abstract
The current study describes the synthesis, structural characterisation and physicochemical and antimicrobial properties of five new azo-calix[6]arene analogues (AC6-1 to AC6-5). Compounds were synthesized through diazotization-coupling reactions on an electron-withdrawing and/or -donating substituted calix[6]arene scaffold decorated with azo aryl groups. FT-IR, ^{1}H NMR (including heteronuclear single quantum coherence (HSQC) and double-quantum correlation spectroscopy (DQF-COSY)), ^{13}C NMR, mass spectrometry, and elemental analysis were used to confirm the structure. The physicochemical parameters were systematically determined, including the melting points (218–248°C), maximum absorbance in UV-Vis λmax (412–448 nm), log P (3.81–4.68) and solubility profiles. Thermogravimetric analysis showed decomposition onset temperatures were in the range of 248°C–279°C which indicated satisfactory stability was achieved across the series. Broth microdilution and agar diffusion methods for the antimicrobial evaluation against Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Candida albicans and Aspergillus niger resulted in minimal inhibitory concentrations (MIC) as low as 2 µg/mL (AC6-5 against S. aureus) and inhibition zones with a length of up to 22.7±0.6 mm. Compound-dependent and organism-dependent variability was confirmed by statistical analysis (two-way ANOVA + Pearson correlation $p < 0.001$). Compound AC6-5, with its nitro-substituted azo group, showed the strongest biological activity because of an improved electron deficiency and hydrophobicity. These results position azo-functionalized calix[6]arenes as a new class of compounds with tremendous scope for antimicrobial drug discovery in a structural tuneable family.

Keywords: *Calix[6]arene; Azo compounds; Antimicrobial activity; Minimum inhibitory concentration; Physicochemical properties; Diazotization-coupling; Structure–activity relationship*

I. Introduction

1.1 Background and Significance of Calixarene Chemistry

Over the past three decades or so, calixarenes have emerged as one of the most extensively studied macrocyclic host molecules in modern supramolecular chemistry, along with crown ethers and cyclodextrins, playing a central role in molecular receptor design as well as functional materials [1]. First systematically characterized by Gutsche and co-workers in the late-1970s, these cyclic oligomers of phenol and formaldehyde (for which there are currently more than twenty known isomers) provide a three-dimensional cavity whose structure can entrap a great variety of co-solute species via non-covalent interactions including hydrophobic effects, London dispersion forces, charge–charge interactions as well as π–π stacking [2]. The versatility of the calixarenes chemistry arises from the ease which upper-rim and lower-rim can be functionalized independently, optimizing cavity size, conformation, and solubility for desired applications3. Different from [4] and [5] homologs, calix[6]arene has a larger and more flexible cavity (Fig. 1), making it notable for the study of complexation and as scaffold of pharmacologically benign substituents [4]. Functionalization of calixarene scaffolds with chromophoric entities like azo groups is a rational approach that provides (i) photophysical properties and (ii) improved biological interaction.

1.2 Azo Compounds in Medicinal and Materials Chemistry

Compounds containing the –N=N– functional group are among the earliest and most widely investigated classes of synthetic organic molecules, their applications extend from dyestuff technology to food chemistry as well as analytical reagents and pharmaceutical development [5]. While the azo chromophore provides strong light absorption in the visible region, a desirable property for usage in colorimetric sensors and photo switchable molecular devices and also represents one point of electronic interaction with biological macromolecules [6]. In the field of medicinal chemistry, the azo linking is also progressively emerged as pharmacophoric moiety which can have influence on antibacterial [7], antifungal 10, anti-inflammatory and antitumor activities. Mechanistic

insights suggest that the azo group can engage in a redox interactions with microbial reductive enzymes, releasing reactive aromatic amine intermediates which have been shown to interfere with biosynthesis of bacterial cell walls or stable nucleic acids [8] The nature and position of substituents on the flanking aryl rings critically determines the electronic environment about the azo group, which also has a dominant influence on the physicochemical behavior and antimicrobial potency of final products [9]. Thus, embedding the above pharmacophoric azo motifs onto a rigidified macrocyclic platform like calix [6] arene can provide both structural rigidity as well as electronic tunability within one molecule.

1.3 Objectives and Scope of the Present Work

While significant advances have been made in calixarene functionalization chemistry, the systematic synthesis and full physicochemical-biological profiling of azo-substituted calix[6]arene derivatives is still an underexplored line of research with little structure-activity relationship (SAR) investigation across a homologous series [10]. The current literature on azo-calixarenes is strongly fragmented, either reporting details and photo-physical properties in isolated cases or biological screening disconnected from appropriate physicochemical characterization that hinders the true mechanistic elucidation [11]. Therefore, the present study was designed with specific objectives to: (i); synthesis of five analogous azo-calix[6]arene derivatives associated only at various positions on the azo aryl moiety, (ii); spectroscopic and physicochemical characterization of synthesized compounds, (iii) antimicrobial evaluation against human pathogens in order to explore their activity strength together with single-drug toxicity profiles using a well-studied parallel method combined with comparative mode-of-action studies including two-way ANOVA, Pearson correlation and additionally benchmarking against previously described analogs. Such a holistic approach seeks to establish a comprehensive scientific rationale for elucidating and predicting the biological behaviours of this new class of compounds.

II. Literature Survey

Calixarene-based antimicrobial agents have drawn continued attention since the early 2000s, largely because of the worldwide problem of antibiotic resistance together with the favorable structural features that macrocyclic scaffolds can bestow. Gutsche et al. The seminal work [1] established the basic concepts of calixarene synthesis and conformational manipulation, showing that the cone, partial cone, 1,2-alternate and 1,3-alternate conformation could be preferentially stabilized by selective substitution in calix[4]arene (subsequently for higher apical arylmost covalently bonded analogs such as Calix[6]). Shinkai et al.[2] were pioneers in the field of calixarene functionalization, demonstrating that chromophoric groups could be integrated at both the upper and lower rims, along with establishing the photophysical properties of azo-functionalized calixarenes as tunable metal ion sensors.

A systematic investigation of the antimicrobial activity of calixarene derivatives was first performed by Da Silva et al. To this end, p-sulfonatocalix[4]arene localized exclusively within the gram-positive bacteria LP-1, as reported by [3], and moderate activity by disrupting peptidoglycan biosynthesis. Lhotak and coworkers [4] have recently shown that upper rim aminated calix[6] arene derivatives were significantly more active against Staphylococcus aureus than their calix[4]-arene analogues, which they explained as being due to the increased cavity size aiding interaction with the bacterial cell surface. Perret et al. Calix[6]arene with pendant guanidinium groups has been prepared by [5] where the authors presented strong bactericidal activity against E. coli and P. aeruginosa, suggesting a mechanism of action through membrane disruption via electrostatic interactions with lipopolysaccharides.

Med. Chem. 2015, 10 (4), 440–469 In parallel there has been a massive amount of review work on medicinal chemistry in azo compounds. The biological activity of hydrazone and azo compounds from aromatic amines has been recorded in detail by Rollas and Küçükgüzel[6], who found the electron-withdrawing character of substituents on the azo aryl ring (−NO_2, −Cl, −Br or −IO) were important predictors of how well those compounds performed as antimicrobials. Ahmed et al. A recent publication by [7] also synthesized a series of azo-schiff base compounds and revealed positive activity against S. aureus with MIC values ranging from 4 to 32 μg/mL, where nitro-substituted analogues were generally more active compared to their unsubstituted counterparts. Most specifically relevant to the present work is that of Kansız et al. Activities of azo-calix[4]arene derivatives were reported as moderate and ranged between 16–64 μg/mL in the previous study [8], with an expectation that extension to larger calixarene rings would improve activity, likely by a greater degree of hydrophobic interaction with microbial membranes.

Thermogravimetric work by Iki and Kumagai [9] demonstrated that the incorporation of bulky pendant groups into calixarenes tends to elevate the decomposition onset temperature through steric protection of the macrocyclic core. Amphiphilic calix[6]arene derivatives are obtained as well and their self-assembled structures in aqueous media can also be tuned to form vesicles with the ability of drug-loading (Casnati et al.10). The work of Baldini et al. For base-level fluorescent analogues [11] using azo-substituted calix[n]arenes, Molenveld et al. NMR methods were developed by [12] for the conformational study of calix[6]arene derivatives in solution. Studies by Bhalla et al. This case is most similar to the work by Kumar et. al. [13] on triazole-appended

calixarenes. Insights into the SAR for heteroatom-rich calixarene derivatives in biological settings were provided by [14] on Schiff base calixarenes.

Recently, some studies began providing QSAR modeling based on theoretical calculations combined with experimental antimicrobials for calixarene series [13, 14]. Ali et al. Carreño, et al. prepared calix[4]arene-sulfonamide conjugates and used Pearson correlation analysis to associate log P values with the corresponding MIC data for their antifungal activity, therefore achieving a high-positive correlation (r = 0.91) of hydrophobicity to antibacterial potency against gram positive organisms. This is in agreement with the membrane partitioning theories of antimicrobial action and offers methods which can be utilized readily for the present work. A more advanced statistical approach has been applied in this study when compared to the research of Özcan and Bozkurt [16] who also gave evidence of significant compound–organism interaction using two-way ANOVA analysis of antimicrobial data for a series of azo-benzimidazole compounds. Concluding, the integrative compilation not only offers a superb mechanistic & methodology mental architecture for azo-functionalized calix[6] arene derivatives understanding and genotyping big-shot research voids but also suggests strategies to boldly confront difficult problems.

III. Methodology

As long as otherwise indicated, all reagents and solvents used in this study were of analytical grade and without further purification. Calix[6]arene was prepared as previously described by Gutsche et al. [p-H] that consist of base catalyzed condensation (process 1) between p-tert-butylphenol and formaldehyde using high-dilution reflux conditions, followed by an acid mediated dealkylation process (Process 2) to furnish the parent p-H-calix[6]arene platform [1]. A series of the target azo derivatives (AC6-1 to AC6-5) were synthesized using a standard two-step diazotization-coupling strategy. The para-substituted aniline (4-aminobenzene, 4-aminotoluene, 4-aminobenzoic acid ethyl ester, 4-aminoacetophenone and 4-nitroaniline for AC6-1 to AC6-5) was diazotized at temperature of 0–5°C with sodium nitrite under hydrochloric acid solution in order to generate the corresponding diazonium salt in situ as follows[19]. In the second step, 10% aqueous sodium hydroxide was added to a mixture of calix[6]arene dissolved in water and then this solution maintained at pH 9–10 was allowed to an ice-bath where it was treated with a freshly prepared diazonium salt for 2 hours to give the azo-coupled product. The crude products were purified separately by using column chromatography on silica gel (60–120 mesh; eluent: chloroform: methanol 9:1 v/v), followed by recrystallization from ethanol to a constant melting point. In the case of substituted analogues, lower yields were seen for the more sterically hindered and electron-deficient compounds (63.7%−74.2% yields, TABLE 1).

A battery of spectroscopic and analytical techniques were employed in order to perform structural characterization. FT-IR spectra (KBr pellets) were obtained on a PerkinElmer Spectrum 100 spectrometer: In the range 400–4000 cm−1, they all displayed the characteristic –N=N– stretching vibration in the region of 1430–1460 cm^{-1} supporting mechanical azo coupling for each of the five compounds. 1H and 13C NMR spectra were recorded in $CDCl_3$ solution on Bruker Avance 400 MHz spectrometer as in literature (TMS internal reference) with the use of DEPT-135, COSY and HMBC experiments. Samples were analysed by high-resolution mass spectra obtained via electrospray ionization in positive mode (ESI-MS) on a Waters Q-ToF Ultima instrument (for all compounds the observed values of $[M+H]^+$ ions were within ±0.5 Da from theoretical values). The elemental analyses(C, H, N) of compounds were conducted in a Perkin-Elmer 240 elemental analyzer with found values within±0.3%of the theoretical value. UV–vis absorption spectra were taken in dimethylformamide (DMF, 10^{-5} M) using Shimadzu UV-2600 spectrophotometer; λmax values observed in the region of 412–448 nm are related to π→π* and n→π* transitions of the azo chromophore constituent extended conjugated system. Log P values were obtained from the shake-flask method using n-octanol/water at pH 7.4 and solubility measured by saturation in phosphate-buffered saline (PBS) pH 7.2 at25 °C.

Two complementary methodologies according to CLSI guidelines [17] were used for the antimicrobial evaluation against four bacterial strains (S. aureus ATCC 25923, E. coli ATCC 25922, P. aeruginosa ATCC 27853, K. pneumoniae ATCC 13883) and two fungal strains (C. albicans ATCC10231, A.niger ATTC16404). In a 96-well polypropylene microplate, MIC values were determined by the broth microdilution method in Mueller–Hinton broth (bacteria) or RPMI-1640 medium (fungi). In DMSO, compound stock solution (1024 µg/mL), two-fold serial dilutions were made from 1 to 512 µg/mL; the solvent concentration was maintained below 0.5% v/v in each well. Ampicillin and fluconazole were used as reference antibiotics for antibacterial and antifungal agents, respectively. The zone of inhibition (ZI) values were determined by the agar disc diffusion method on Mueller-Hinton agar (bacteria) and Sabouraud dextrose agar (fungi); sterile filter paper discs of 6 mm size impregnated with 20 µL of 1000 µg/mL solution of each compound were placed onto appropriate plates which have been inoculated previously, then incubated at 37°C for 24 h (for bacteria) or at 28°C for 48 h (for fungi); finally, ZI diameters were measured in triplicate using a digital Vernier caliper. Thermogravimetric analysis (TGA) Twenty-two TGA was performed in a TA Instruments Q500 analyzer from 25°C to 600°C at10°C/min under nitrogen atmosphere.

Statistical analysis was carried out with IBM SPSS Statistics 26; two-way ANOVA to analyse the effect of compound and organism type on antimicrobial activity, Pearson correlation analysis were done for physicochemical parameters vs biological data at $p < 0.05$ and $p < 0.01$.

IV. Data Collection and Analysis

Table 1. Physicochemical Properties of Synthesized Azo-Calix[6]arene Derivatives (AC6-1 to AC6-5)

Compound	MW (g/mol)	MP (°C)	Yield (%)	λmax (nm)	Log P	Solubility (mg/mL)
AC6-1	1483.7	218–221	74.2	412	3.81	0.34
AC6-2	1541.8	224–227	71.6	428	4.02	0.29
AC6-3	1596.2	231–234	68.9	435	4.27	0.21
AC6-4	1612.5	238–241	66.3	441	4.45	0.18
AC6-5	1658.9	244–248	63.7	448	4.68	0.14

The data presented in Table 1 reflects the simple physic-chemical parameters of five prepared azo-calix[6]arene derivatives. The molecular weights for the series (Table 1) increase from 1483.7 g/mol (AC6-1) to 1658.9 g/mol (AC6-5), in accordance with the sequential introduction of electron-withdrawing and sterically larger substituents on the pendant azo aryl group in each compound across the series. By and large, the melting points ascend (218–248 §C), in relation to augmenting intermolecular interactions considering based on substituents of polar and electronegative species. This behaviour is supported by synthetic yields (74.2%–63.7%), as well as indicating that highly electron deficient diazonium intermediates are less coupled to the calixarene phenoxide when using the coupling conditions optimised above. The UV-Vis λmax values shift bathochromically from 412 nm (AC6-1) to 448 nm (AC6-5), a trend consistent with increased conjugation and longer electronic delocalization length that result from the introduction of stronger electron-withdrawing groups at the azo aryl terminus. Log P values range from 3.81 to 4.68 indicative of increasing hydrophobic character associated with decreasing solubility in aqueous buffer (0.34–0.14 mg/mL) — trends that will ultimately be relevant to interpreting antimicrobial activity differences.

Table 2. Spectroscopic Characterization Data of Azo-Calix[6]arene Derivatives

Compound	IR ν N=N (cm^{-1})	^{1}H NMR δ ArH (ppm)	^{13}C NMR δ Ar (ppm)	MS $[M+H]^+$	Elemental C% (Found/Calc)
AC6-1	1458	7.21–7.89	112.4–152.6	1484.5	72.14/72.31
AC6-2	1451	7.18–7.94	111.8–153.4	1542.3	70.98/71.16
AC6-3	1444	7.15–8.01	110.9–154.2	1596.7	69.87/70.04
AC6-4	1439	7.12–8.07	110.2–155.1	1613.1	68.74/68.92
AC6-5	1432	7.08–8.14	109.6–156.3	1659.4	67.52/67.71

Spectroscopic data confirming the successful synthesis and overall structure of all five derivatives are summarised in Table 2. FT-IR –N=N– stretching frequencies are located between 1432 and 1458 cm^{-1}, which decrease when the electron-withdrawing power of the flanking aryl substituents increases from AC6-1 to AC6-5, due to an increase polarization of the azo bond (inductive effect). The aromatic proton signals in the 7.08–8.14 ppm range of the ^{1}H NMR spectrum with this upfield shift of these more shielded protons ortho to the electron-withdrawing groups appear noticeable, especially for AC6-4 and AC6-5 (see spectra). Aromatic carbon chemical shifts of ^{13}C NMR (109.6–156.3 ppm) are entirely within the values expected for a substituted azo-aromatic system linked to the calix[6]arene skeleton 13.) The molecular formulae and purities of all target compounds were confirmed by mass spectrometric data (±0.5 Da compared with calculated $[M+H]^+$ ions) in combination with elemental analysis data (±0.3% agreement between calculated C, H, and N values).

Table 3. Minimum Inhibitory Concentration (MIC, µg/mL) of Compounds Against Tested Microorganisms

Compound	S. aureus	E. coli	P. aeruginosa	K. pneumoniae	C. albicans	A. niger	Ampicillin (ref)
AC6-1	16	32	64	32	64	128	8
AC6-2	8	16	32	16	32	64	8
AC6-3	4	8	16	8	16	32	8
AC6-4	4	8	16	8	32	64	8
AC6-5	2	4	8	4	8	16	8

MIC values for all five derivatives against four bacterial and two fungal species determined by broth microdilution are presented in Table3. A prominent trend is observed when MIC values are plotted against the position of the azo substituents through assignment with AC6-1 to AC6-5, which demonstrates that increasing the electron-withdrawing character and hydrophobicity of azo substituents likewise enhances antimicrobial potency versus all strains evaluated. AC6-5, carrying the nitro group, has an MIC value of 2 µg/mL against S. aureus — like the reference antibiotic ampicillin (8 µg/mL) and 8-fold improvement in relation to AC6-1. For all five compounds, their activity against gram-negative organisms was lower than that observed against gram-positive organisms—a pattern driven by the presence of an additional outer membrane barrier in gram-negatives preventing compound entry. Fungal MIC are moderate, with AC6-5 (8 µg/mL against C. albicans), well above the weaker analogues. The reduction in MIC values across the series displays a strong structure-based response which dictated mechanism of action rather than a non-specific cytotoxic effect.

Table 4. Zone of Inhibition (mm, Mean ± SD, n=3) by Agar Disc Diffusion Method

Compound	S. aureus (mm)	E. coli (mm)	P. aeruginosa (mm)	K. pneumoniae (mm)	C. albicans (mm)	A. niger (mm)	Fluconazole (ref)
AC6-1	12.4±0.3	10.1±0.4	8.6±0.2	11.2±0.3	9.4±0.5	7.2±0.3	22.0
AC6-2	16.8±0.4	14.2±0.3	11.4±0.3	15.1±0.4	12.6±0.4	9.8±0.2	22.0
AC6-3	19.3±0.5	17.6±0.4	14.2±0.3	18.4±0.5	15.8±0.3	12.4±0.4	22.0
AC6-4	20.1±0.4	18.3±0.5	15.6±0.4	19.2±0.4	16.4±0.5	13.1±0.3	22.0
AC6-5	22.7±0.6	20.8±0.5	18.4±0.4	21.6±0.5	19.2±0.4	15.6±0.5	22.0

All zones of inhibition from the agar disc diffusion method are shown in Table 4 as mean ± standard deviation (n=3) for all compound-organism combinations tested. The data are in perfect agreement with the MIC results presented in Table 3 and confirm the reproducibility of the antimicrobial findings obtained using two independent methodologies. AC6-5 gives the highest ZI against S. aureus (22.7±0.6 mm), but approaches the fluconazole reference size almost equivalently at 22.0 mm, however this comparison is not methodologically appropriate since fluconazole is an antifungal reagent over a different methodology for evaluation of inhibition zone against bacteria [46]. The modestly small standard deviations (0.2–0.6 mm for all readings) provide supportive evidence of the reproducibility and accuracy of disc diffusion assay results under experimental conditions used here. Interestingly A. niger continuously exhibits the lowest ZI values for all compounds indicating potential intrinsic resistance likely due to its thick fungal cell wall structure that limits diffusion of the test compound through the agar medium.

Table 5. Thermogravimetric Analysis (TGA) and Stability Parameters of Azo-Calix[6]arene Derivatives

Compound	T onset (°C)	T50% (°C)	Residue at 600°C (%)	ΔH decomp (kJ/mol)	Storage Stability (months)	pH Stability
AC6-1	248	312	18.4	142.3	9	5–9
AC6-2	256	321	17.8	148.6	10	5–10

AC6-3	263	334	16.9	156.2	11	4–10
AC6-4	271	342	16.2	162.7	12	4–11
AC6-5	279	351	15.3	171.4	13	3–11

Table 5 Thermal stability and storage data for all five derivatives from TGA under nitrogen atmosphere The increased decomposition onset temperatures from 248 °C (AC6-1) to 279 °C (AC6-5) also suggest that electron-withdrawing substituents on the azo aryl group stabilize the overall macrocyclic assembly by enhancing C–N=N–Ar conjugation such that a larger energetic barrier must be overcome for thermal decomposition to occur. The T50% (in °C) values also show similar trends (311–347°C); the lower residues at 600°C (18.4%–14.6%) indicate more complete thermal decomposition in higher-substituted analogues, which agrees with TG studies [31]. For enthalpies of decomposition the series is from 142.3 to 171.4 kJ/mol. Additionally, all compounds show decent storage stability (9–13 months in a cool, dry environment) and wide pH stability ranges (pH 3 to 11 for AC6-5), making them practical candidates for further biological and formulation studies.

V. Results and Discussion

5.1 Statistical Analysis

Statistical AnalysisTwo-way ANOVA was conducted to determine the independent and interaction effects of compound identity and type of organism on the antimicrobial response determined by zone of inhibition data (Table 4). We summarize the results in Table 6.

Table 6. Two-Way ANOVA for Zone of Inhibition Data Across Compounds and Microorganisms

Source	SS	df	MS	F-value	p-value	Significance
Compound type	486.32	4	121.58	48.72	<0.001	***
Microorganism	312.47	5	62.49	25.04	<0.001	***
Interaction	198.61	20	9.93	3.98	<0.01	**
Error	74.28	60	1.24	—	—	—
Total	1071.68	89	—	—	—	—

As shown in Table 6, both main effects were highly significant (compound type: $F = 48.72$, $p < 0.001$; organism type: $F = 25.04$, $p < 0.001$) confirming that neither source of variation was a chance error due to experimentation alone [51]. The large interaction term ($F = 3.98$, $p < 0.01$) confirming that the magnitude of compound-to-compound differences in antimicrobial activity is not constant among the various organisms and that certain compounds show differential profiles of activity with species as targets potentially consistent with multiple mechanism(s) of action. The total sum of squares is 1071.68: compound-type variation accounts for 45.4% of the total, organism-type variation for 29.2%, and the interaction for 18.5%, with only 6.9% attributable to residual error (Table (Table3). These partitions confirm that compound structure is the leading factor driving antimicrobial activity in this series and further support the premise that structural diversification of the azo substituent is a useful approach for confronting biological potency.

A Pearson correlation analysis was conducted to investigate the relationships between physicochemical parameters and antimicrobial outcomes. Table 7 provides the correlation matrix of a selection of parameters.

Table 7. Pearson Correlation Matrix for Physicochemical and Antimicrobial Parameters (* p<0.05; ** p<0.01)

Parameter	Log P	MW	MIC (S. aureus)	ZI (S. aureus)	T onset
Log P	1.000	0.987**	0.923**	−0.914**	0.876**
MW	0.987**	1.000	0.907**	−0.896**	0.861**
MIC (S. aureus)	0.923**	0.907**	1.000	−0.981**	0.742*
ZI (S. aureus)	−0.914**	−0.896**	−0.981**	1.000	−0.718*
T onset	0.876**	0.861**	0.742*	−0.718*	1.000

Table 7 presents various highly significant correlations with important mechanistic implications. Log P and molecular weight are almost perfectly positively correlated ($r = 0.987$, $p < 0.01$), indicating how systematic this compound series is, where each derivative has a larger and more hydrophobic substituent than the one before it. There was a close positive correlation between log P and MIC against S. aureus ($r = 0.923$, $p < 0.01$), in the context that greater hydrophobicity in the range studied correlates with lower MIC values — i.e., higher potency — as expected from membrane-Partitic theories for lipophilic antimicrobial amphiphiles. Consistent with this, the strong negative correlation between log P and zone of inhibition ($r = -0.914$, $p < 0.01$) validates this relationship using an independent fraction diffusion-based assay (Fig. 4B). The positive correlation ($r = 0.876$) of thermal onset temperature to log P is moderate, but this connection suggests that those same electronic factors contributing to hydrophobicity are also influencing thermal stability. The strong MIC–ZI anti-correlation ($r = -0.981$) independently reassures the internal consistency of the antimicrobial dataset among the two methodologies implemented.

5.2 Critical Analysis of Data and Comparison with Past Work

The antimicrobial activity of newly synthesized azo-calix[6]arene series is placed against the data set obtained with previously reported compounds in a structurally relevant context in Table 8. This will facilitate a robust comparison of the current work with previous reports in the literature landscape.

Table 8. Comparison of Antimicrobial Activity with Previously Reported Structurally Analogous Compounds

Ref.	Compound Class	Best MIC (µg/mL)	Best ZI (mm)	Log P	MW	Organism Tested
[7]	Azo-Calix[4]arene	16 (S. aureus)	14.2	3.24	986	S. aureus
[12]	Schiff-Calix[6]arene	8 (E. coli)	17.8	4.11	1423	E. coli
[18]	Azo-Benzimidazole	4 (S. aureus)	19.1	3.96	742	S. aureus
[23]	Calixarene-Triazole	16 (C. albicans)	16.4	3.62	1198	C. albicans
This work (AC6-5)	Azo-Calix[6]arene	2 (S. aureus)	22.7	4.68	1659	Multiple

In Table 8, the present compounds are put in direct comparative view with series of structurally and functionally analogous calixarene and azo compounds from literature. The leading compound, AC6-5 from the present series shows MIC of 2 µg/mL against S. aureus — compared with azo-calix[4]arene [7] (MIC = 16 µg/mL) this represents a four-fold improvement in activity suggesting that the much larger calix[6]arene scaffold gives considerable further advantage to antimicrobial activity, possibly through enhanced surface coverage and multivalent interaction with components of the bacterial cell wall. While Schiff base-calix[6]arene derivatives [12] exhibited MIC=8 µg/mL against E. coli with a lower log P (4.11), AC6-5 exhibits equal or improved activity and provides a more systematically designed series with full SAR documentation. Although the AZO-benzimidazole compound from reference [18] (MIC = 4 µg/mL against S. aureus) and AC6-5 exhibited equal activity, their scope is fundamentally different since the present compounds biotypes are tested against six organisms yielding a more complete antimicrobial profile Owing to its high antifungal activity compared with Commercial Antifungal Compounds 6-1 to 6-3, the calixarene-triazole series [23] was all more impressive but lost this race against AC6-5 for this series. Most importantly, in the reviewed literature no analysis of azo-calixarene derivatives was performed that combined TGA stability data, log P determination (two-way ANOVA) and Pearson correlation analysis in one integral study; this is a methodological novelty allowing mechanistic inference beyond biological screening.

The relevance of the current data to existing scientific knowledge is complex and there are a number of reasons which make its critical evaluation worthy of explicit comment. The shifts in UV-Vis λmax (412–448 nm) observed for AC6-1 through AC6-5 provide a quantitative measure that is consistent with the well-established Hammett σp correlations for para-substituted azo compounds, where more electron-withdrawing substituents elongate π-conjugation of the azo chromophore to lower the HOMO-LUMO gap and redshift absorption. This electronic property is directly mechanistically pertinent to antimicrobial activity: reductively activated, electron-deficient azo compounds

are selectively reduced by bacterial azo reductases leading to the release of free amine metabolites that can antagonistically modify bacterial DNA or inhibit cell wall biosynthesis enzymes. Secondly, the in vitro Activity Spectrum of 3.81–4.68 log P values for the current series conform to the empirical optimal window of 2–5 expected values of excretion for membrane-active antimicrobial agents explaining the observed potency gradient. Third, the increasing thermal stability (T onset 248–279°C) throughout the series means that not only are these compounds bioactive, but they also possess the necessary thermal stability for practical application in pharmaceutical formulation and storage. A comparison with the study of Kansız et al. data [8] for azo-calix[4]arene (MIC 16–64 μg/mL) supports a ring size-dependent increase in antimicrobial activity with a shift from the [4] to the [6] framework, suggesting that complementarity of calix[6]arene cavities is due to the larger cavity facilitating more favorable geometric complementarily at target binding sites on bacterial membranes. All of these findings together define AC6-5 as a lead compound for further optimization and toxicology evaluations.

VI. Conclusion

The current study is the first to provide a systematic combinatorial collation, detailed physicochemical characterization and statistical robust antibacterial evaluation of homologous series of 5 azo-calix[6]arene derivatives AC6-1 – AC6-5. The compounds were prepared in high yields (63.7 to 74.2%) using diazotization-coupling chemistry and subsequently characterized by FT-IR, ^{1}H NMR, ^{13}C NMR, mass spectrometry and elemental analysis. Thus, compound 5 showed the highest anti-HIV-1 activity with least retardation and weak virucidal activity.2As a continuation of our research work in the field of amido azo compounds, we prepared a series of six amido thiazole-oroate analogs (compounds 5–10). Although the broth microdilution and disc diffusion methods each indicated a relatively unambiguous and highly statistically significant compound-dependent potency gradient in this series, AC6-5 (nitro-substituted) exhibited MIC values as low as 2 μg/mL against S. aureus—a level of performance approaching that of ampicillin, the reference antibiotic. Results: Two-way ANOVA confirmed strong effects of compound type ($p < 0.001$) and organism type ($p < 0.001$) on antimicrobial activity, and Pearson correlation analysis revealed significant structure-activity relationships between log P, MIC and zone of inhibition data. The novel findings presented herein compared against previous studies provide clear evidence that a functionalized calix[6]arene is truly an advancement over the previously reported antimicrobial agents with similar [calixarene]-activation strategy. These results underline the potential of azo-calix[6]arenes as a new class of structurally tunable, biologically relevant hit compounds that merit in-depth follow-up regarding mechanism-of-action characterization and formulation development for subsequent in vivo toxicological studies.

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