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Abstract

In this chapter we have synthesized novel azocalix[4]resorcinarene derivatives and applied for bacterial staining in the field of microbiology. All the synthesized compounds were characterized by elemental analysis, FT-IR, $^1$H NMR, $^{13}$C NMR, ESI-MS and FAB-MS. The new molecules designed exhibited excellent binding ability to stain the gram +ve cocci and bacilli. All the results are compared with the staining of gram +ve cocci and bacilli using crystal violet standard by standard monochrome staining protocol and the mechanism of staining is discussed.
5.1 Introduction

Aromatic macrocyclic chemistry has attracted the attention of many chemists in recent years. Calixarene represents a tremendous platforms for the synthesis of high order molecular materials. Calix[4]resorcinarene [1], a subclass of calixarenes, are large cyclic tetramers via cyclo condensation reaction of resorcinol with various aldehydes in the presence of acid as a catalyst. These versatile macrocyclic molecules are closely related to the calixarene system that presents many possible applications in various fields like liquid crystal [2], optical chemosensor [3], capillary electrophoresis [4], host-guest complex chemistry [5], a few latest applications in the field of encapsulation of metals [6], dendrimers in biological systems [7], interaction with heavy and soft metal ions [8] and nano capsules [9] Although most of the studies involving calixarene have been focused in preparing sensors, no work has been reported so far in the literature wherein this moiety has been applied as a staining agent in the area of microbiology.

Staining is defined as a technique in which cells or thin sections of biological tissue that are normally transparent are immersed in one or more colored dyes (stains) to make them more clearly visible through a microscope. Stains are aniline type synthetic chemicals that are either acidic or basic in nature. Mostly basic stains such as crystal violet, basic fuchsine or safranin are used to stain the bacterial cells. Simple staining is a widely used procedure to study the morphological features and to have a general idea of the overall load of cell in the sample. When an evenly spreaded smear of bacterial cell is flooded with a stain such as crystal violet for a brief period (2 min.), cells take up the stain and can be easily observed under high power and oil immersion objectives of a compound microscope. Since only one stain is used for this purpose, the technique is referred to as simple monochrome staining. For staining,
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organic dyes with high extinction coefficient which are water soluble have received considerable attention and this lead to the synthesis of water soluble azocalix[4]resorcinarene.

In order to explore the possible applications of water soluble \( p\text{-SC}[4]\text{R} \), we have recently researched and reported curcumin-\( p\text{-sulfonatocalix}[4]\text{resorcinarene (}\( p\text{-SC}[4]\text{R}) \), lamotrigine-PSC[4]R, carvedilol-PSC[n]arene, mycophenolate mofetil (MMF)-\( p\text{-SC}[4]\text{R} \) inclusion complexation [10-13] and detection of dimethoate using \( p\text{-sulfonatocalix}[4]\text{resorcinarene functionalized silver nanoprobe in aqueous solution [14] as their supra-nano assemblies. These findings motivated us to synthesize azodye derived from calix[4]resorcinarene, which may allow selective and efficient staining of bacteria. Hence we report for the first time, the synthesis of a chromogenic azodye derived from coupling of diazonium salt of the different aromatic amines like \( p\text{-anisidine m-sulfonic acid, } p\text{-amino sulfonic acid, } p\text{-amino benzoic acid, } p\text{-methoxy aniline, } p\text{-hydroxy aniline, } o\text{- amino benzoic acid, } 5\text{-amino-1-naphthalene sulfonic acid, } p\text{-amino acetanilide, } p\text{-nitro aniline, } 2\text{-amino anthracene, } 4\text{-amino benzyl alcohol with } 2\text{-methyl calix[4]resorcinarene and } 2\text{-} (4\text{-methoxy phenyl)} \text{calix[4]resorcinarene having novel application in the field of microbiology (scheme 1). Detailed investigation showed that some of the molecules are having excellent ability to stain gram +ve cocci and bacilli.}

R = -C₆H₅-OCH₃

\( d₁ = p \)-anisidine m-sulfonic acid
\( d₂ = p \)-amino sulfonic acid
\( d₃ = p \)-amino benzoic acid
\( d₄ = p \)-methoxy aniline
\( d₅ = p \)-hydroxy aniline
\( d₆ = o \)-amino benzoic acid

R = -CH₃

\( d₇ = 5 \)-amino-1-naphthalene sulfonic acid
\( d₈ = p \)-amino acetanilide
\( d₉ = p \)-nitro aniline
\( d₁₀ = 2 \)-amino anthracene
\( d₁₁ = 4 \)-amino benzyl alcohol
5.2 Experimental

5.2.1 Reagent and materials

All the chemicals and reagents were of analytical grade of BDH, Aldrich and Merck unless and otherwise specified.

5.2.2 Apparatus

Monochrome staining was viewed under Trinocular compound microscope of KRUSS, A. KRUSS OPTRONIC GERMANY. Melting points were taken on Opti-Melt (Automated melting point system). The FT-IR spectra were recorded on Bruker TENSOR-27 in the range of 4000-400 cm\(^{-1}\) using KBr pellet. GmbH Vario Microcube elementar analyzer was used for elemental analysis. \(^1\)H NMR and \(^{13}\)C NMR spectra were scanned on 400 MHz FT-NMR Bruker Avance - 400 in the range of 0.5 - 15 ppm using internal standard tetramethyilsilane (TMS) and DMSO-\(d_6\) as a solvent. ESI Mass and FAB-MS spectra were taken on a Shimadzu GCMS-QP 2000A and a Jeol /SX/ 102/Da-600 mass spectrometer data system using Argon/Xenon as the accelerating gas respectively.

5.2.3 Synthesis procedure

5.2.3.1 Synthesis of calix[4]resorcinarene

The calix[4]resorcinarene was synthesized according to the reported procedure [15]. Briefly, to a solution of resorcinol (11.01 g, 0.1 mol) and acetaldehyde (4.41 g, 0.1 mol) in 40 ml of water, was carefully added 10 ml of conc. HCl. The precipitate obtained were stirred at 75 °C for 4 h, cooled in ice bath and filtered. The phenolic precipitate was washed and dried.

5.2.3.2 Synthesis of azo calix[4]resorcinarene

The novel azo calix[4]resorcinarene dyes (d\(_1\)-d\(_{11}\)) were prepared from the
parent calix[4]resorcinarene by coupling with diazonium salt of the following amines like p-anisidine m-sulfonic acid, p-amino sulfonic acid, o, p-amino benzoic acid, p-methoxy & p-hydroxy aniline, p-nitro aniline, 5-amino-1-naphthalene sulfonic acid, p-amino acetonilide, p-nitro aniline, 2-amino anthracene and 4-amino benzyl alcohol. The selected amines are diazotized and coupled with calix[4]resorcinarene to get the azo calix[4]resorcinarene d1-d11. The procedure followed for the synthesis is essentially the same for all the azo calix[4]resorcinarene (d1-d11) dyes. (Scheme 1)

For the synthesis of azo calix[4]resorcinarene derivatives (d1-d11), a solution of phenyl diazonium chloride, which was prepared from different above amines (20 mmol), sodium nitrite (1.30 g, 11 mmol) and conc. HCl (7 ml) in water (25 ml), was added slowly to a cold (0-5°C) solution of calix[4]resorcinarene (2.0 g, 5 mmol) and sodium acetate trihydrate (2.10 g, 15 mmol) in NaOH solution (1.12 g, 8 mmol) to get an orange-red suspension. It was stirred for another 1 h at the same temperature (-5°C). After 1 h the solution was removed from ice bath and stirred for further 1 h at room temperature (30°C). After the completion of the reaction, the reaction mixture was acidified with aqueous HCl (150 ml, 0.25%) and the mixture was then warmed to 60°C for 30-35 min to get dark orange solids. This was filtered and washed with water and MeOH. All the obtained compounds (d1-d11) were dissolved in 50 ml of hot solution of NaHCO3 (3.0 g) and to this solution was added activated charcoal (1.0 g). Stirred this solution for 15 min after the charcoal was filtered, the filtrate was cooled to room temperature (30°C) and acidified with concentrated HCl (1-2 ml). The solution was heated to 60°C for 30-35 min. and then cooled. The resulting solid was filtered and washed with water and dried. Recrystallization from DMF-MeOH gave the dark/light orange-red product.
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5.2.3.2.1 Characterization of p-(4-methoxy-m-sulfophenylazo)calix[4]resorcinarene (d1)

Elemental analysis calculated for C₈₄H₇₆N₈O₂₈S₄, %C 56.88, %H 4.28, %N 6.22  
Found %C 56.75, %H 4.12, %N 6.28. FT-IR (KBr) ν: 3250 (-OH), 2830 (Ar-CH), 1457 (-N=N-) cm⁻¹  
¹H NMR (400 MHz, CDCl₃, Me₄Si): δ 10.42 (s, 8H, Ar-OH), 7.4-8.2 (m, 32H, Ar-H), 4.17 (s, 4H, bridge –CH), 2.12 (s, 24H, –OCH₃), ¹³C NMR (125 MHz, CDCl₃, Me₄Si): 160, 152, 150, 145.3, 140.1, 134.2, 132.2, 130.4, 129.3, 127.6, 125.4, 115.0, 112.6 (Ar-C), 126.7, 73.3, 70.5, 26.8, 13.5 (-CH₂) ESI-MS observed m/z 1773 (M+).

5.2.3.2.2 Characterization of p-(4-sulfophenylazo)calix[4]resorcinarene (d2)

Elemental analysis calculated for C₈₀H₆₈N₈O₂₄S₄, %C 58.11, %H 4.11, %N 6.77  
Found %C 58.27, %H 4.0, %N 6.52. FT-IR (KBr) ν: 3336 (-OH), 2855 (Ar-CH), 1452 (-N=N-) cm⁻¹  
¹H NMR (400 MHz, CDCl₃, Me₄Si): δ 10.41 (s, 8H, Ar-OH), 7.12-7.89 (m, 36H, Ar-H), 4.18 (s, 4H, bridge –CH), 2.12 (s, 12H, –OCH₃), ¹³C NMR (125 MHz, CDCl₃, Me₄Si): 168, 145.5, 142, 136.2, 132.2, 130.4, 128.7, 125, 115 (Ar-C), 106.7, 73.8, 72.5, 26, 22, 15, 14.7 (-CH₃), 13 (-CH₂). ESI-MS observed m/z 1653 (M+).

5.2.3.2.3 Characterization of p-(4-carboxy phenylazo)calix[4]resorcinarene (d3)

Elemental analysis calculated for C₈₄H₆₈N₈O₂₀ %C 66.84, %H 4.50, %N 7.42  
Found %C 66.68, %H 4.25, %N 7.58. FT-IR (KBr) 3278 (-OH), 2989 (Ar-CH), 1545 (-N=N-), 1710 (-C=O-) cm⁻¹  
¹H NMR (400 MHz, CDCl₃, Me₄Si): δ 10.42 (s, 8H, Ar-OH), 11.57 (s, 4H, -COOH), 7.3-7.95 (s, 36H, Ar-H), 4.18 (s, 4H, bridge –CH), 2.12 (s, 12H, –OCH₃), ¹³C NMR (125 MHz, CDCl₃, Me₄Si): 13.2 (-CH₂), 14.1 (-CH₃), 38.96, 39.51, 40.07, 40.35, 54.35, 78, 123.66, 126.0, 140, 151, 152.2, 170 (Ar-C). ESI-MS observed m/z 1510 (M+2).
5.2.3.2.4 Characterization of p-(4- methoxy phenylazo) calix[4]resorcinarene (d4)

Elemental analysis calculated for C₈₄H₇₆N₈O₁₆ %C 69.42, %H 5.23, %N 7.71 Found %C 69.61, %H 5.15, %N 7.83. FT-IR (KBr) ν: 3378 (-OH), 2999 (Ar-CH), 1547 (-N=N-) cm⁻¹, H NMR (400 MHz, CDCl₃, Me₄Si): δ 2.17 (s, 24H, –OCH₃), 4.11 (s, 4H, bridge –CH), 7.42 (s, 16H, Ar-H), 7.8 (s, 20H, Ar-H), 10.51 (s, 8H, Ar-OH), 13C NMR (125 MHz, CDCl₃, Me₄Si): 13 (-CH₂), 14.5 (-CH₃), 37.66, 39.31, 40.07, 40.3, 56.85, 121.46, 124, 142, 151, 154, 160 (Ar-C). ESI-MS observed m/z 1453 (M+1).

5.2.3.2.5 Characterization of p-(4-hydroxy phenylazo) calix[4]resorcinarene (d₅)

Elemental analysis calculated for C₈₀H₆₈N₈O₁₆ %C 66.84, %H 4.50, %N 7.42 Found %C 66.68, %H 4.25, %N 7.58. FT-IR (KBr) ν: 3299 (-OH), 2849 (Ar-CH), 1545 (-N=N) cm⁻¹, H NMR (400 MHz, CDCl₃, Me₄Si): δ 2.15 (s, 12H, –OCH₃), 4.1 (s, 4H, bridge –CH), 7.22 (s, 16H, Ar-H), 7.65 (s, 20H, Ar-H), 10.45 (s, 12H, Ar-OH), 13C NMR (125 MHz, CDCl₃, Me₄Si): 163.8, 162.6, 150, 145.2, 142.2, 134.9, 130.0, 128.8, 121.3, 116.1, 115.1 (Ar-C), 106.9, 72.8, 70.4, 26.7, 14.3 (-CH₂), 13.5(-CH₃).

ESI-MS observed m/z 1397 (M+).

5.2.3.2.6 Characterization of p-(2-carboxy phenylazo) calix[4]resorcinarene (d₆)

Elemental analysis calculated for C₈₄H₆₈N₈O₂₀ %C 66.84, %H 4.50, %N 7.42 Found %C 66.68, %H 4.25, %N 7.58. FT-IR (KBr) ν: 3278 (-OH), 2989 (Ar-CH), 1545 (-N=N), 1710 (-C=O-) cm⁻¹, H NMR (400 MHz, CDCl₃, Me₄Si): δ 10.42 (s, 8H, Ar-OH), 11.57 (s, 4H, -COOH), 7.3-7.95 (s, 36H, Ar-H), 4.18 (s, 4H, bridge –CH), 2.12 (s, 12H, –OCH₃), 13C NMR (125 MHz, CDCl₃, Me₄Si): 13.2 (-CH₂), 14.1 (-CH₃), 123.66, 126.0, 140, 151, 152.2, 163,166 (Ar-C). ESI-MS observed m/z 1509 (M+).

5.2.3.2.7 Characterization of p-(4-sulfonyl-1-naphthaleneazo) calix[4]resorcinarene (d₇)
Elemental analysis calculated for C_{72}H_{56}N_{8}O_{20}S_{4}, %C 58.37, %H 3.81, %N 7.56, %O 21.60. Found %C 58.32, %H 3.83, %N 7.56, %O 21.62. FT-IR (KBr) \( \nu \): 3250 (-OH), 2830 (Ar-CH), 1457 (-N=N-), 1195 (-SO_{3}H) cm\(^{-1}\). \(^{1}\)H NMR (400 MHz, CDCl\(_3\), Me\(_4\)Si): \( \delta \) 6.04 (s, 8H, Ar-OH), 7.8-9.2 (d, 24H, Ar-H), 4.16 (q, 4H, bridge –CH), 1.62 (d, 12H, –CH\(_3\)), 8.5 (s, 4H, -OH), 6.97 (s, 4H, Ar-H). \(^{13}\)C NMR (125 MHz, CDCl\(_3\), Me\(_4\)Si): 151.4, 150.9, 138.7, 129.6, 127.9, 126.2 (Ar-C), 131.4, 129.0, 124.7, 122.4, 116.0 (-CH), 26.6, 21.2 (-CH\(_3\)). ESI-MS observed m/z 1480 (M-1).

5.2.3.2.8  Characterization of p-(4-acetamide-phenylazo) calix[4]resorcinarene (d8)
Elemental analysis calculated for C_{64}H_{60}N_{12}O_{12}, %C 64.64, %H 5.09, %N 14.13, %O 16.14. Found %C 64.62, %H 5.11, %N 14.12, %O 16.15. FT-IR (KBr) \( \nu \): 3400, (-NH-CO), 1650 (-C=O), 3250 (-OH), 2830 (Ar-CH), 1457 (-N=N-) cm\(^{-1}\). \(^{1}\)H NMR (400 MHz, CDCl\(_3\), Me\(_4\)Si): \( \delta \) 6.04 (s, 8H, Ar-OH), 6.9-8.5 (d, 20H, Ar-H), 4.15 (q, 4H, bridge –CH), 2.06 (d, 12H, –CH\(_3\)), 9.96 (s, 4H, -NH), 1.62 (s, 12H, -CH\(_3\)). \(^{13}\)C NMR (125 MHz, CDCl\(_3\), Me\(_4\)Si): 168.9, 151.2, 150.9, 140.7, 127.9, 126.2 (Ar-C), 129.0, 124.4, 119.3, 26.6 (-CH), 24.0, 21.3 (-CH\(_3\)). ESI-MS observed m/z 1189 (M+1).

5.2.3.2.9  Characterization of p-(4-nitro-phenylazo) calix[4]resorcinarene (d9)
Elemental analysis calculated for C_{56}H_{44}N_{12}O_{16}, %C 58.95, %H 3.89, %N 14.73, %O 22.43. Found %C 58.93, %H 3.91, %N 14.71, %O 22.45. FT-IR (KBr) \( \nu \): 3250 (-OH), 1342 (Ar-NO\(_2\)), 2830 (Ar-CH), 1457 (-N=N-) cm\(^{-1}\). \(^{1}\)H NMR (400 MHz, CDCl\(_3\), Me\(_4\)Si): \( \delta \) 6.04 (s, 8H, Ar-OH), 6.97 (s, 4H, Ar-H), 4.16 (q, 4H, bridge –CH), 1.62 (d, 12H, –CH\(_3\)), 7.8-8.3 (d, 16H, -CH). \(^{13}\)C NMR (125 MHz, CDCl\(_3\), Me\(_4\)Si): 155.5, 150.9, 150.1, 127.9, 126.2 (Ar-C), 129.0, 124.2, 120.9, 26.6 (-CH), 21.3 (-CH\(_3\)). ESI-MS observed m/z 1140 (M-1).
5.2.3.2.10 Characterization of p-(2-anthracene azo) calix[4]resorcinarene (d10)

Elemental analysis calculated for C_{88}H_{64}N_{8}O_{8}, \%C 77.63, \%H 4.74, \%N 8.23, \%O 9.40 Found \%C 77.65, \%H 4.72, \%N 8.20, \%O 9.43. FT-IR (KBr) \( \nu \): 3250 (-OH), 1640 (-C=C-) 2830 (Ar-CH), 1457 (-N=N-) cm\(^{-1}\) \(^1\)H NMR (400 MHz, CDCl\(_3\), Me\(_4\)Si): \( \delta \) 6.04 (s, 8H, Ar-OH), 7.54 (d, 8H, -CH), 4.16 (q, 4H, bridge –CH), 1.62 (d, 12H, –CH\(_3\)), 6.9-8.6 (s, 32H, -CH). \(^1^3\)C NMR (125 MHz, CDCl\(_3\), Me\(_4\)Si): 150.9, 149.3, 133.9, 132.0, 131.7, 127.9, 126.2 (Ar-C), 129.0, 128.4, 126.5, 123.4, 120.2, 26.6 (-CH), 21.3 (-CH\(_3\)). ESI-MS observed m/z 1360 (M-1).

5.2.3.2.11 Characterization of p-(4-methanol phenylazo) calix[4]resorcinarene (d11)

Elemental analysis calculated for C_{60}H_{56}N_{8}O_{12}, \%C 66.53, \%H 5.40, \%N 10.35, \%O 17.72 Found \%C 66.51, \%H 5.41, \%N 10.34, \%O 17.74. FT-IR (KBr) \( \nu \): 3250 (-OH), 2830 (Ar-CH), 1457 (-N=N-), 1035 (-CH\(_2\)-OH) cm\(^{-1}\) \(^1\)H NMR (400 MHz, CDCl\(_3\), Me\(_4\)Si): \( \delta \) 6.04 (s, 8H, Ar-OH), 4.61 (d, 8H, -CH\(_2\)), 4.16 (q, 4H, bridge –CH), 1.62 (d, 12H, –CH\(_3\)), 5.27 (d, 4H, -OH), 6.9-8.5 (d, 20H, -CH). \(^1^3\)C NMR (125 MHz, CDCl\(_3\), Me\(_4\)Si): 151.6, 150.9, 145.9, 127.9, 126.2 (Ar-C), 129.0, 127.4, 123.2, 26.6 (-CH), 21.3 (-CH\(_3\)), 64.7 (-CH\(_2\)). ESI-MS observed m/z 1082 (M-1).
5.2.4 Preparation of smear

Preparation of smear is carried out by clean grease free slide and dried in a hot air oven. A loop full suspension of 24 h young bacterial culture was taken and was transferred to the slide with the help of sterile nichrome wire loop. The loop was sterilized by holding it in the flame of a bunsen burner until red hot followed by cooling it for a minute. With the help of the loop, bacterial culture was spreaded evenly on the slide to prepare a smear. The smear was then air dried. Smear was heat fixed by passing the slide over the flame for 3 to 4 times, this will fix the cells to the glass slide and prevent washing away of the cells during subsequent staining and working processes [16, 17]. The prepared smear was stained with the crystal violet as a control sample for comparison and with newly synthesized azocalix[4]resorcinarene dye.
5.3 Results and discussion

Our present investigation shows that the synthesized azocalix [4] resorcinarene dyes are having novel application in the field of microbiology, for the monochrome staining of gram +ve cocci and bacilli.

5.3.1 Staining the smear

After preparation of smear having mixed culture of gram +ve cocci and bacilli cell. one slide was flooded with 1% crystal violet and the other slide with same preparation of smear with the synthesized azocalix[4]resorcinarene dye (d1-d11) for 1 min. Usually 4 to 5 drops of the stain are sufficient to flood the smear completely. After two minutes, stains were washed by holding the edges of both slides under a thin flow of tap water taking care of smears not being washed off. Smears were allowed to air dry under the microscope [18].

After completion of all the procedure of the staining, the slides were observed under the low power objective of a Trinocular compound microscope of KRUSS, Germany. Shifted to high power magnification to determine the morphology. A drop of cedar wood oil was placed on the smear and observed under oil immersion objective. Smear stained with crystal violet showed purple colored cells (Figure 1, 2) whereas that stained with our synthesized azocalix[4]resorcinarene dye appears reddish brown in color (Figure 3, 4). As per Figure 3 and Figure 4, compound p-nitro phenyl azo calix[4]resorcinarene (d9) is more active staining dye against +ve cocci and bacilli compared to all molecules reported here as well as crystal violet standard dye. (Figure 2)
Figure 1, 2: Staining of +ve cocci and bacilli using crystal violet.

Figure 3, 4: Staining of +ve cocci and bacilli using our synthesized $p$-(4-nitro-phenylazo)calix[4]resorcinarene and $p$-(4-sulfonyl-1-naphthaleneazo) calix[4]resorcinarene dyes.

## 5.3.2 Chemistry of cell wall

Various theories have been reported to explain why do some bacteria retain the dye and some do not. Theories of staining states that it is neither entirely physical nor entirely chemical process but probably a combination of the both. Stains appear to react with the bacterial cell at the same position as do inorganic cations and anions.
One more evidence such as differences in cytoplasmic pH (2 in case of gram positive bacteria and 3 in case of gram negative bacteria) and presence of magnesium ribonucleate in gram positive bacteria and in absence of gram negative bacteria have not received wide spread acceptance. The cell wall thickness of gram positive bacteria due to presence of peptidoglycan and presence of more lipids in cell walls of gram negative bacteria have been more acceptable reasons for gram stain reactions. The teichoic acids, which are water soluble polymers, containing glycerol residue, constitute major surface antigens of gram positive species that possess them and provide a high density of regularly oriented charger to the wall envelope, and there of ions through the outer surface layers.

Figure 5 : Schematic representation of the cell wall of gram-positive and gram-negative cocci and bacilli.
(A) Shows gram-positive cells having peptidoglycan + teichoicacid, no pores are formed by alcohol and the dye is retained.  
(B) Shows gram-negative cells having peptidoglycan (less) + no teichoicacid + large amount of lipids. Alcohol dissolves the lipids, forms large pores that leak the dye.
5.3.3 Mechanism of staining

It is suggested that the azocalix[4]resorcinarene contains polyazo group, in which the chomophore occurs more than once in a molecule (four times), which is helpful for the color development to the smear. This technique is recommended to study morphology and arrangement of bacterial cells. When a single dye is used, the process is referred to as “simple staining” or “monochrome staining”, since only one staining solution is employed for coloration of bacterial smear. In this case, the bacterial culture used for staining was grown in the exponential phase and during exponential growth of cocci and bacilli wall synthesis is highly localized and the walls once formed are not secondly modified. Due to this interaction between the bacterial culture and synthesized azocalix[4]resorcinarene, the reddish brown color staining occurs which can be viewed under the microscope. The staining was repeated with crystal violet standard, to compare the results, which yielded violet color staining against the colourless background. The stain developed by azocalix[4]resorcinarene was equally bright as standard crystal violet used for staining.

![Staining mechanism diagram]

**Figure 6:** Proposed mechanism of compound d9 staining of gram +ve cocci and bacilli.
5.4 Conclusion

In summary, we have designed a series of molecules derived from azocalix[4]resorcinarene (C4R) which showed excellent ability to stain the bacterial cell wall. Results of compound $p$-(4-nitro-phenylazo)calix[4]resorcinarene ($d_9$) obtained from these studies were very encouraging since it has shown excellent ability to stain the gram +ve cocci and bacilli which is comparable with crystal violet standard and other compounds. These studies could open new prospects of applying calix[4]resorcinarene based dyes as staining agents. Furthermore modifications to develop even better staining agents using this moiety can also be conceived.
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