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Research Paper

Novel Azocalix [4] resorcinarene based monochrome staining agent for gram +ve cocci and bacilli

Chemistry

KEYWORDS : Azocalix[4]resorcinarene, Gram +ve cocci and bacilli, Biological Staining

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ABSTRACT

In the present research work demonstrates the application of a novel azocalix[4] resorcinarene (C4R) derivative synthesized and characterized in the field of microbiology. The new molecule designed showed excellent binding ability to stain the gram +ve cocci and bacilli. The staining has been carried out by standard method of grams staining. The results are compared with the staining of gram +ve cocci and bacilli using crystal violet standard and the mechanism of staining is discussed.

INTRODUCTION:

Calixarene represents an excellent platform for the synthesis of high order molecular materials. Calix[4] resorcinarene [1], analogs of calixarene, is synthesized by the acid catalyzed cyclocondensation of resorcinol with various aliphatic and aromatic aldehydes. These versatile macrocyclic molecules are closely related to the calixarene system that presents many possible applications in various fields like liquid crystal properties [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

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, organic dyes with high extinction coefficient which are water soluble have received considerable attraction and this lead to the synthesis of water soluble azocalix[4]resorcinarene.

In order to explore the possible applications of water soluble *p*-SC[4]R, we have recently reported curcumin-p-sulfonatocalix[4] resorcinarene(*p*-SC[4]R)inclusion complexation[10] and detection of dimethoate using p-sulfonatocalix[4] resorcinarene functionalized silver nanoprobe in aqueous solution[11] as their supra-nano assemblies.These findings motivated us to synthesize an 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 2-amino benzoic acid with 2-methyl calix[4] resorcinarene and 2-(4-methoxy phenyl) calix[4] resorcinarene having novel application in the field of microbiology. Detailed investigation showed that this molecule is having excellent ability to stain gram +ve cocci and bacilli.

EXPERIMENTAL

Materials and Method

Reagent and materials

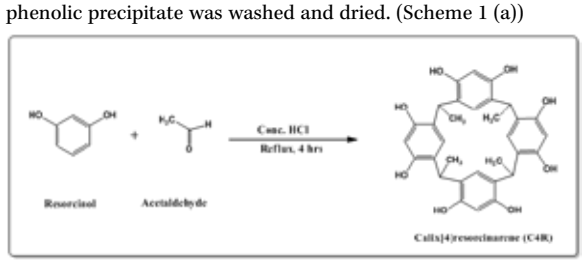
All the chemicals and reagents were of analytical grade of BDH, Aldrich and Merck unless and otherwise specified.

Apparatus

Trinocular compound microscope of KRUSS, A. KRUSS OPTRONIC GERMANY

Synthesis of calix[4]resorcinarene

The calix[4] resorcinarene was synthesized according to the literature procedure[12]. 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 to 10 ml of conc. HCl. The precipitate obtained were stirred at 75 ^{0}C for 4 h, cooled in ice bath and filtered. The phenolic precipitate was washed and dried. (Scheme 1 (a))



Scheme 1 (a): synthesis of calix[4]resorcinarene

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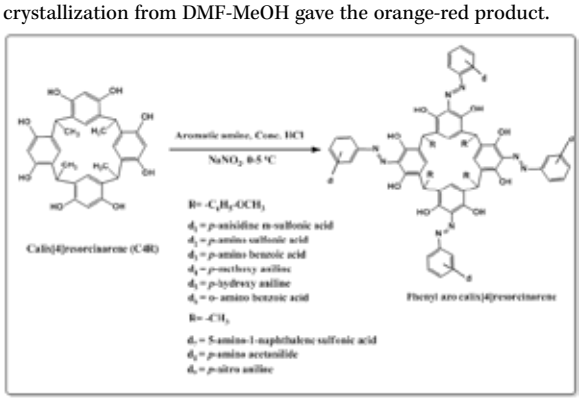
Synthesis of Azo calix[4]resorcinarene

The novel azo calix[4]resorcinarene dyes **(d_1-d_9)** 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 and *p*-amino acetanilide. The selected amines are diazotized and coupled with calix[4]resorcinarene to get the azo calix[4]resorcinarene **d_1-d_9.** The procedure followed for the synthesis is essentially the same for all the azo calix[4]resorcinarene **(d_1-d_9)** dyes. (Scheme 1(b))

For the synthesis of azo calix[4]resorcinarene derivatives (**d_1-d_9**), a solution of phenyl diazonium chloride, which was prepared from different amine (20 mmol), sodium nitrite (1.30 g, 11 mmol) and conc. HCl (7ml) in water (25 ml), was added slowly to a cold (0-5^{0}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. After 1 h the solution was removed from ice bath and stirred for further 1 h at room temperature. 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^{0}C for 30-35 min to give dark orange solids. This was filtered and washed with water and MeOH. All the obtained compounds(**d_1-d_9**) was dissolved in 50 ml of hot solution of $NaHCO_3$ (3.0 g), 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 (room temperature, 30^{0}C) and acidified with concentrated HCl (1-2 ml). The solution was heated to 60^{o}C for 30-35 min. and then cooled. The resulting solid was filtered and wash with water and dried. Recrystallization from DMF-MeOH gave the orange-red product.



Scheme 1 (b): synthesis of azocalix[4]resorcinarene (d_1 – d_9)

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 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 [13, 14]. The prepared smear was stained with the crystal violet as a control sample for comparison and with newly synthesized azocalix[4]resorcinarene dye.

CHARACTERIZATION:

Characterization of *p*-(4-methoxy-m-sulfophenylazo) calix[4]resorcinarene (d1)

Elemental analysis calculated for $C_{84}H_{76}N_8O_{28}S_4$, %C 56.88, %H 4.28 %N 6.22 **Found** %C 56.75 %H 4.12 %N 6.28. **FT-IR** (KBr) υ: 3250 (-OH), 2830 (Ar-CH), 1457 (-N=N-) cm^{-1} **^{1}H 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), **^{13}C 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), 106.7, 73.3, 70.5, 26.8, 13.5 (-CH2) **ESI-MS** observed m/z 1773 (M+).

Characterization of *p*-(4-sulfo phenylazo) calix[4]resorcinarene (d2)

Elemental analysis calculated for $C_{80}H_{68}N_8O_{24}S_4$, %C 58.11, %H 4.11 %N 6.77 **Found** %C 58.27 %H 4.0 %N 6.52. **FT-IR** (KBr) υ: 3336 (-OH), 2855 (Ar-CH), 1452 (-N=N-) cm^{-1} **^{1}H 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), **^{13}C 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). **FAB-MS** observed m/z 1653 (M+).

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

Elemental analysis calculated for $C_{84}H_{68}N_8O_{20}$ %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}. **^{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), **^{13}C 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). **FAB-MS** observed m/z 1510 (M+2).

Chracterization of *p*-(4- methoxy phenylazo) calix[4]resorcinarene (d4)

Elemental analysis calculated for $C_{84}H_{76}N_8O_{16}$ %C 69.42, %H 5.23 %N 7.71 **Found** %C 69.61 %H 5.15 %N 7.83. **FT-IR** (KBr) υ: 3378 (-OH), 2999 (Ar-CH), 1547 (-N=N-) cm^{-1},**^{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), **^{13}C 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).

Chracterization of *p*-(4-hydroxy phenylazo) calix[4]resorcinarene (d5)

Elemental analysis calculated for $C_{80}H_{68}N_8O_{16}$ %C 66.84, %H 4.50 %N 7.42 **Found** %C 66.68 %H 4.25 %N 7.58. **FT-IR** (KBr) υ: 3299 (-OH), 2849 (Ar-CH), 1545 (-N=N) cm^{-1},**^{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), **^{13}C 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 (-CH3), 13.5(-CH2). **ESI-MS** observed m/z 1397 (M+).

Chracterization 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) υ: 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), 123.66, 126.0, 140, 151, 152.2, 163,166 (Ar-C). **ESI-MS** observed m/z 1509 (M+).

RESULTS AND DISCUSSION

Our present investigation shows that the azocalix [4] resorcinarene dye is having novel application in the field of micro biology for the monochrome staining of gram +ve cocci and bacilli.

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 ($\mathbf{d_1}$-$\mathbf{d_9}$) for 1 min. Usually 8 to 9 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 [15].

After completion of all the procedure of the staining, the slides were observed under the low power objective of a binocular compound microscope. 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).**

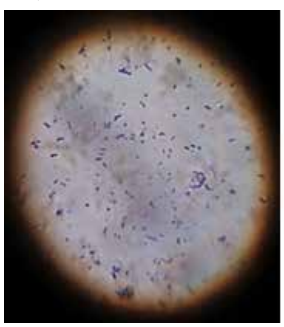


Figure 1

Figure 2

Figure 3

Figure 4

Figure 1,2 : Staining of +ve cocci and bacilli using crystal violet

Figure 3, 4: Staining of +ve cocci and bacilli using our synthesized azocalix[4] resorcinarene dye

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 peptidoglycen 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.

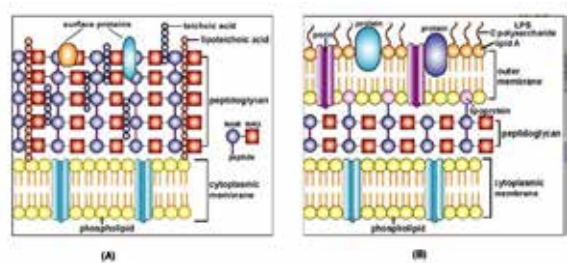


Figure 5:Schematic representation of the cell wall of gram-positive and gran-negative cocci and bacilli.

(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.

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]resorcinare was equally bright as standard crystal violet used for staining.

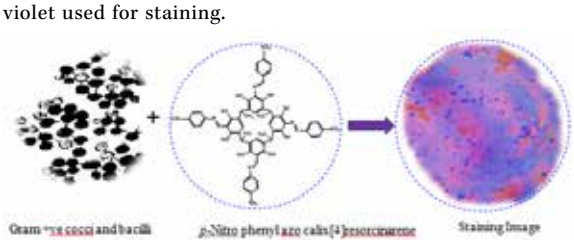


Figure 6: proposed mechanism of staining of gram +ve cocci and bacilli.

CONCLUSION

In summary, we have designed a very simple molecule derived from a calix[4]resorcinarene(C4R) which showed excellent ability to stain the bacterial cell wall. Results 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. 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.

Acknowledgements

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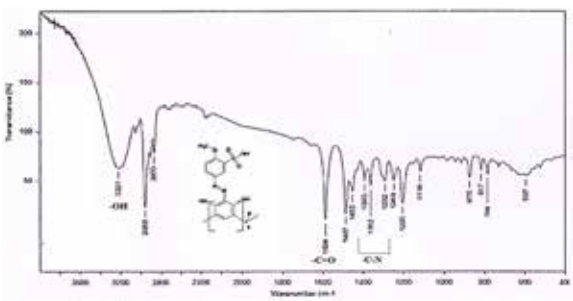
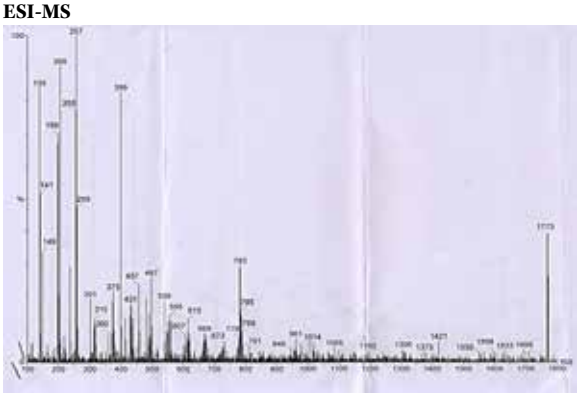
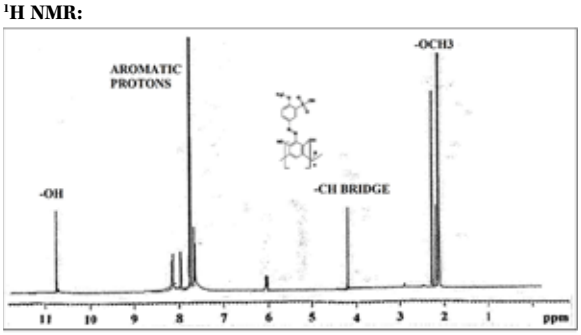
Compound d_1:
FT-IR:



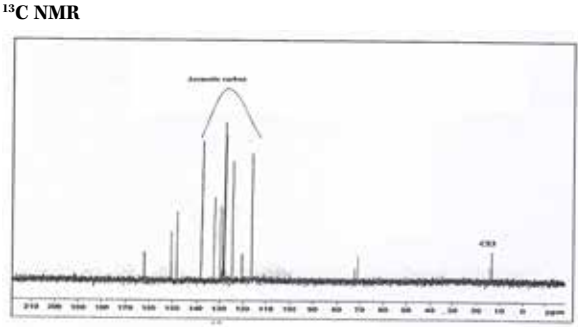
ESI-MS



^{1}H NMR:



^{13}C NMR



Compound d_2:
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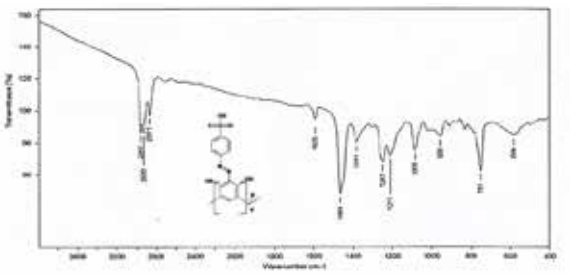

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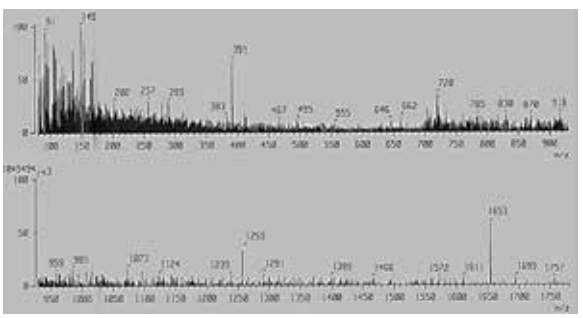
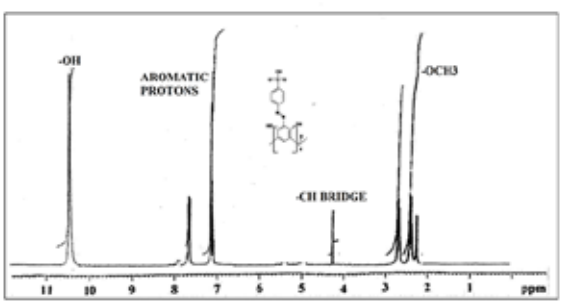
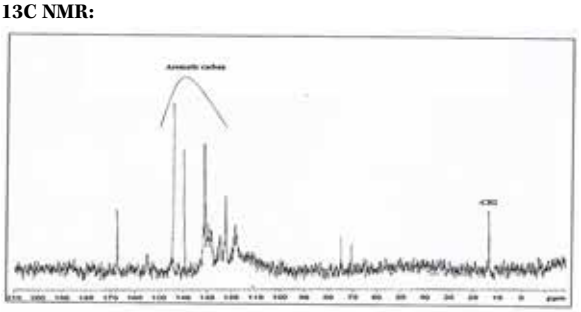


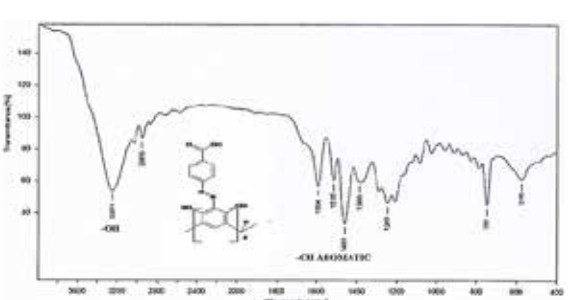
^{1}H NMR



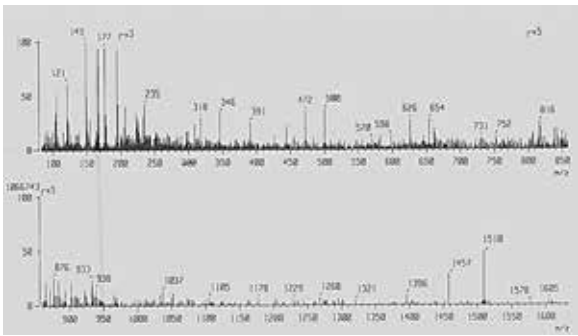
13C NMR:



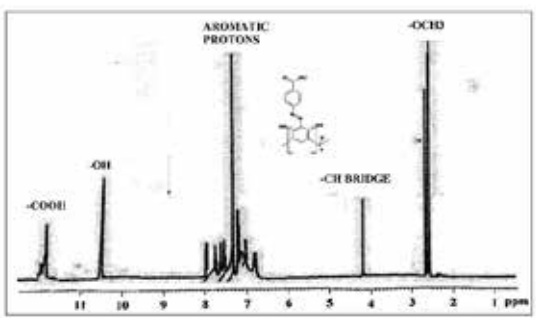
Compound d_3:
FT-IR:



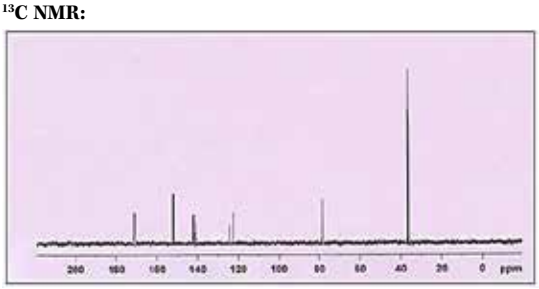
FAB-MS:



^{1}H NMR:



^{13}C NMR:



Compound d_4:

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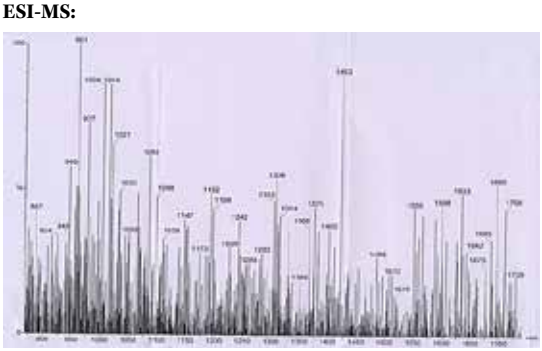
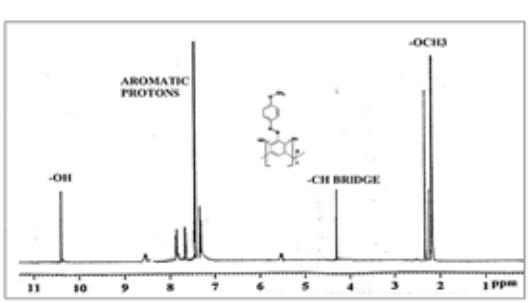
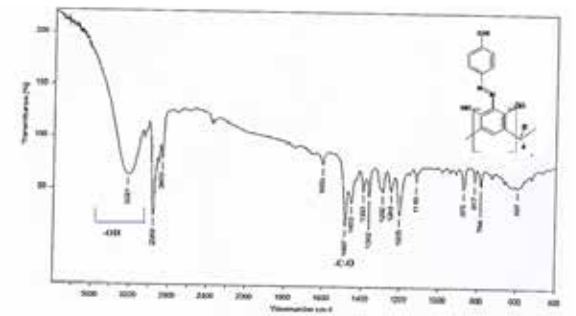


^{1}H NMR:



Compound d_5:

FT-IR:



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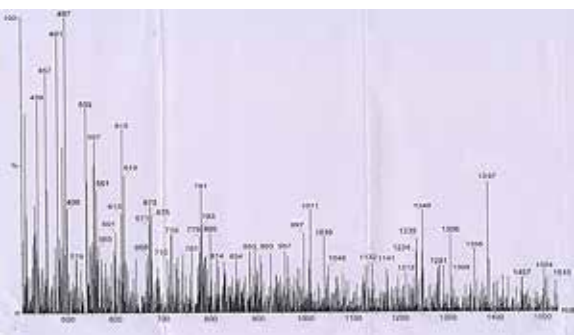
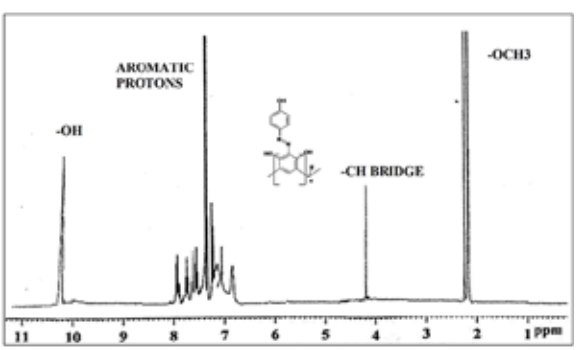
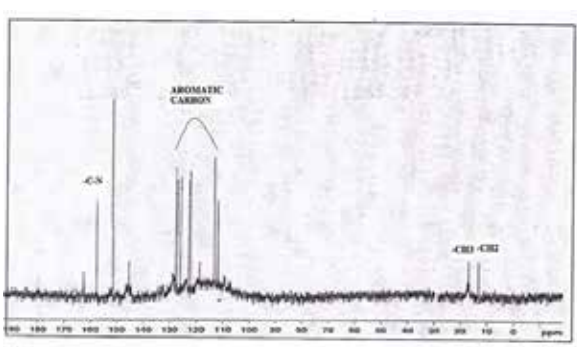


^{1}H NMR:



^{13}C NMR:



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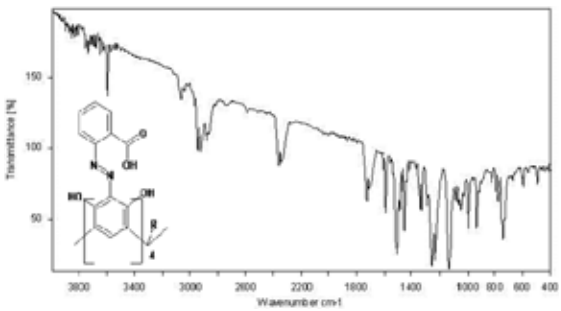
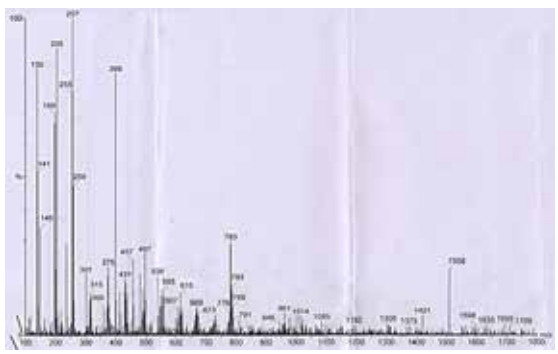
Compound d_6:

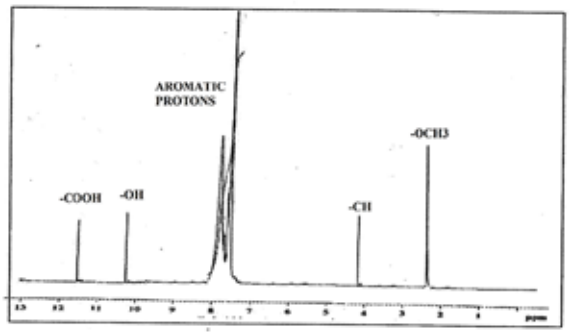
FT-IR:



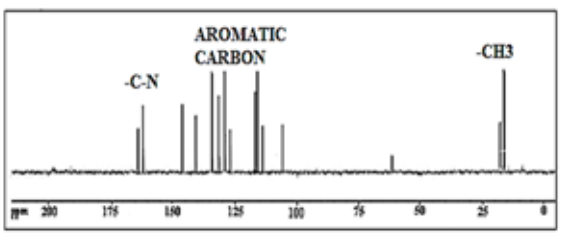
ESI-MS:



^{1}H NMR:



^{13}C NMR:



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