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Antimicrobial activity of calixarenes

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Abstract – The pursuit of antimicrobially active compounds against a variety of microorganisms is an area of intense and important research. In the present study, we examined the relative antimicrobial activity of calixarenes having different side chains, moieties and/or substitution groups against a diverse set of bacteria and fungi. Antimicrobial activity against the various species was evaluated by growth rate and inhibition rate comparisons to distinguish between the compounds for this desired property. Preliminary screening of 57 calixarenes was conducted to assay their potential as antimicrobially active compounds against *Corynebacterium*. Of these compounds, seven calixarenes numbered 25, 26, 27, 28, 30, 34 and 50 were found to exhibit suitable antimicrobial activity. These seven samples were then further tested to elucidate any antimicrobial activity they might have versus additional species. After examining the growth and inhibition values of these selected compounds, calixarenes sample numbers 25, 26, 27, 28 and 50 were shown to also display antimicrobial activity against *Fusarium solani f. sp. mori* [F.s.-26] with an inhibition range of approximately 60–70%. Additionally, sample numbers 25, 26, 27, and 28 exhibited excellent and selective antimicrobial activity against the fungal strains, *Rosellinia necatrix* [R-8], and *Colletotrichum dematium* [C.d. 8901]. ***To cite this article: R. Lamartine et al., C. R. Chimie 5 (2002) 163–169*** © 2002 Académie des sciences / Éditions scientifiques et médicales Elsevier SAS

antimicrobial activity / macrocycles / calixarenes / pesticide

Résumé – La recherche de composés à activité antimicrobienne vis-à-vis de divers microorganismes demeure un défi important. Dans cette étude, nous déterminons et présentons l'activité antimicrobienne de calixarènes de chaînes latérales et/ou de groupements substitués différents vis-à-vis de différentes bactéries et champignons, par comparaison des vitesses de croissance et d'inhibition. Un criblage préliminaire des 57 calixarènes vis-à-vis de *Corynebacterium* a été effectué pour déterminer les composés présentant une activité antimicrobienne. Sept d'entre eux présentent une activité antimicrobienne satisfaisante. On a ensuite étudié l'activité antimicrobienne de ces derniers vis-à-vis d'autres espèces. La détermination des vitesses d'inhibition et de croissance montre que les calixarènes **25**, **26**, **27**, **28** et **50** présentent une activité antimicrobienne vis-à-vis de *fusarium solani f. sp. mori* [F.s.-26] avec une vitesse d'inhibition de 60–70%. Par ailleurs, les calixarènes **25**, **26**, **27** et **28** possèdent une activité antimicrobienne très forte et sélective vis-à-vis de souches de champignons *Rosellinia necatrix* [R-8] et *Colletotrichum dematium* [C.d. 8901]. ***Pour citer cet article : R. Lamartine et al., C. R. Chimie 5 (2002) 163–169*** © 2002 Académie des sciences / Éditions scientifiques et médicales Elsevier SAS

activité antimicrobienne / macrocycles / calixarènes / pesticides

1. Introduction

Calixarenes are a very important class of compounds in supramolecular chemistry [1–5]. As shown in Fig. 1, calixarenes are cavity-shaped cyclic molecules made up of phenol units linked via the ortho positions by methylene bridges. Calixarenes are synthetic macrocycles produced by condensation of p-substituted phenols with formaldehyde. They have an original molecular architecture and are considered

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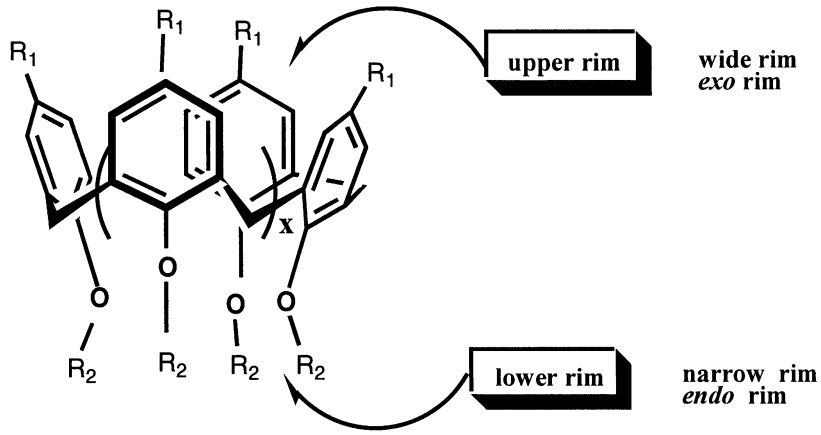

Fig. 1. Representation of the calixarenes and designation of the faces.

to be important starting materials in the design of novel host-molecules for molecular recognition.

In the past ten years, parent calixarenes have been widely used as molecular platforms for the synthesis of selective molecular receptors for cations, anions and neutral molecules, in order to develop applications [6] in the fields of separation [7] purification [8], recovery of metals [9,10], pollution control [11] membranes for electronic devices [12,13], and phase transfer agents [14].

Although some calixarenes have been quoted for their antitumor and antiviral properties, very few publications appeared in the literature on their biological activities. Only recently, in August 2000, a communication [15] on the cellular toxicity of calixarenes stated that calixarene sulfonates, the calix[6] and [8] derivatives display the same level of toxicity as glucose, while slight toxicity associated with the calix[4]arene sulfonate does exist.

In the present study 57 kinds of calixarenes were screened to find antimicrobially active compounds toward a variety of fungal and bacterial microorganisms. These novel compounds are not only important as new antimicrobial substances but are also attractive from a materials-based perspective in that calixarenes have metal binding properties.

2. Experimental

Materials

The calixarenes synthesized for these experiments are listed in Table 1, along with the different side chain moieties and/or substitution groups they contain.

All even numbered parent calixarene samples **1, 4, 6, 8, 13, 15, 16** have been synthesized according to the Gutsche procedures [16–18]. Odd numbered parent calixarene samples **3**, **5**, **7** were produced according to modified synthesis procedures [19,20]. Dihomooxacalixarenes **2, 14** were synthesized in basic conditions by optimised synthetic procedures [21], while C-alkylcalix[4]resorcinarene **33, 34, 35** were obtained by acid-catalysed procedures [1]. The upper rim *tert*-butyl groups were removed [22] by retro Friedel–Crafts reactions to form H-calixarenes **9**–**12**.

Additionally, the alkylated upper rim calixarene samples **17–23, 29, 32** were obtained by Friedel–Crafts alkylation of H-calixarenes with alkyl chlorides in the presence of $AlCl_3$ in $CHCl_3$ solution [3]. Sulfonato-calixarene samples **25**–**27** were produced by ipso-sulfonation [23], and phenylazocalixarene samples **28**–**30, 37, 53** were formed via the diazo coupling scheme [24,25]. Functional groups were introduced into the lower rim of isopropylcalixarenes by means of Williamson-type OH-modifications. Literature procedures were used to introduce on the *p*-isopropylcalix[4]arene lower rim, ether, ester, acyl, ketone and amine groups for samples **36, 38, 40, 41, 43, 46, 49, 50**. The exhaustive functionalisation of *p*-isopropylcalix[6] and [8]arene samples **39, 42, 44, 45, 47, 48**, utilised literature procedures that were subsequently modified and optimised for these specific reactions [14].

3. Measurement

3.1. Antimicrobial activity towards bacteria

The antimicrobial activity of calixarenes was assessed against a variety of microorganisms, including several kinds of bacteria [26] and fungi, listed in Table 2. Phytoanthorogenic microorganisms were mainly used to evaluate antimicrobial activity of these compounds. Factors for these choices included ease of experiment, non-toxic quality and prevalent existence in nature.

Table 1. Synthesized calixarenes having different side chain moieties and/or substitution groups (Ph: phenyl; Ad: adamantyl; Py: pyridyl; bPy: bipyridyl).

Calixarenes	***x***	**R_1**	**R_2**	**Calixarenes**	***x***	**R_1**	**R_2**
1	1	$C(CH_3)_3$	H	**30**	1	N=N–Ph	H
2	1	$C(CH_3)_3$	H	**31**	1	NO_2	H
Homooxa	ether bridge						
3	2	$C(CH_3)_3$	H	**32**	1	CH_2–CH=CH_2	H
4	3	$C(CH_3)_3$	H	**33**	1	C-ethyl bridges	
				Resorcin.			
5	4	$C(CH_3)_3$	H	**34**	1	C-nonyl bridges	
				Resorcin.			
6	5	$C(CH_3)_3$	H	**35**	1	C-benzylbridges	
				Resorcin.			
7	6	$C(CH_3)_3$	H	**36**	1	$CH(CH_3)_2$	C(=O)–CH_3
8	7	$C(CH_3)_3$	H	**37**	3	N=N–Ph	H
9	1	H	H	**38**	1	$CH(CH_3)_2$	CH_3
10	2	H	H	**39**	3	$CH(CH_3)_2$	CH_3
11	3	H	H	**40**	1	$CH(CH_3)_2$	CH_2–CH_3
12	5	H	H	**41**	1	$CH(CH_3)_2$	CH_2–CH=CH_2
13	1	$CH(CH_3)_2$	H	**42**	3	$CH(CH_3)_2$	C(=O)–Ph
14	1	$CH(CH_3)_2$	H	**43**	1	$CH(CH_3)_2$	CH_2–C(=O)–O–C_2H_5
Homooxa	ether bridge						
15	3	$CH(CH_3)_2$	H	**44**	3	$CH(CH_3)_2$	CH_2–C(=O)–O–C_2H_5
16	5	$CH(CH_3)_2$	H	**45**	5	$CH(CH_3)_2$	CH_2–C(=O)–O–C_2H_5
17	4	CH_2–CH_3	H	**46**	1	$CH(CH_3)_2$	CH_2–C(=O)–O–C_2H_5
18	3	CH_2–CH_2CH_3	H	**47**	5	$CH(CH_3)_2$	CH_2–C(=O)–CH_3
19	5	$CH_2(–CH_2)_6$–CH_3	H	**48**	3	$CH(CH_3)_2$	CH_2–C(=O)–Ph
20	2	$C(CH_3)_2$–CH_2–$C(CH_3)_3$	H	**49**	1	$CH(CH_3)_2$	CH_2–C(=O)–Ad
21	3	$C(CH_3)_2$–CH_2–$C(CH_3)_3$	H	**50**	1	$CH(CH_3)_2$	CH_2–C(=O)–N(C_2H_5)$_2$
22	5	$C(CH_3)_2$–CH_2–$C(CH_3)_3$	H	**51**	1	$C(CH_3)_3$	CH_2–C(=O)–O–C_2H_5
23	5	CH_2–$(CH_2)_{12}$–CH_3	H	**52**	1	$CH(CH_3)_2$	3 CH_3, 1H
24	3	CH_2–$(CH_2)_{16}$–CH_3	H	**53**	1	N=N-Ph-NO_2	H
25	1	SO_3H	H	**54**	1	$C(CH_3)_3$	2 Py + 2 bPy
26	3	SO_3H	H	**55**	1	$C(CH_3)_3$	2 Py + 2bPy,Cu(I)
27	5	SO_3H	H	**56**	1	2 NO_2,2 $C(CH_3)_3$	H
28	3	N=N–Ph–SO_3H	H	**57**	1	3 $C(CH_3)_3$,1 H	3 C(=O)–Ph, 1H
29	1	N=N–Ph–SO_3H	H				

Table 2. Microorganisms examined in the present study.

Microorganisms	Strain number of MAFF
Bacteria from Plant pathogens	
Agrobacterium tumefaciens	302307
Clavibacter michiganensis subsp. *michganensis*	301368
Pseudomonas cichorii	118079
Fungi from Plant pathogens	
Colletotrichum dematium	840066
Fusarium solani f. sp .mori	840046
Rosellinia nocatrix	840051

3.2. Antimicrobial activity against bacteria

Initial antimicrobial studies screened 57 types of calixarenes against the specific microbe, *Corynebacterium*, chosen for its high sensitivity to a wide variety of antimicrobial substances, such as Ag ions, and phytoalexin. After screening, the antimicrobial activity of the calixarenes was examined against many strains of bacteria and fungi.

From a Corynebacterium cell culture with a cell density of 100 cells/ml, 2 ml were taken and mixed with 25 ml agarose containing King B growth medium (Wako Pure Chemical Industries, Ltd.) at 55 °C, poured into a glass Petri dish and allowed to solidify at 25 °C. The calixarenes (approximately 0.5 mg) in powder form were placed onto the surface of the solid gel and incubated at 25 °C for two days. The antimicrobial activity was evaluated by measuring the radius of growth inhibition around the sample.

3.3. Antimicrobial activity against fungi

The antimicrobial activity of various calixarenes against several fungi, including *Rosellinia sclerotiorum* (R-8), *Colletotrichum dematium* (C.d.8901) and *Fusarium solani f. sp. mori* (F.s.26) was evaluated according to the following procedures. Solidified potato sucrose agar medium (A medium) was prepared by pouring liquid potato sucrose agar into a glass Petri dish at 25 °C. One of the fungi selected was inoculated at 25 °C on the surface of the fresh A medium. All fungal culture strains were thus inoculated on the A medium and allowed to grow at room temperature until a mycelia growth radius of approximately 3 cm had been reached (usually four days after inoculation). Another batch of solidified potato sucrose agar (B medium) was prepared according to the same methods as those described above. From the initial mycelia growths, a small portion from the mycelial periphery (size: 0.5 mm × 0.5 mm × 0.5 mm) was removed with a scalpel and transferred to a different place on the surface of the solidified B medium. Approximately 0.5 mg of calixarenes in powder form was then placed on these mycelia to assess antifungal activity. Growth rate (GR) and inhibition rate (IR) were both monitored for three days as a measure of the antifungal activity according to the equation below. The results presented here are the average of four mean values.

Growth Rate (GR)

$$GR = B/A \times 100\ (\%) \tag{1}$$

A: diameter size (mm) of control fungal colony without sample exposure.

B: diameter size of fungal colony with exposure to calixarene material.

Inhibition Rate (IR)

$$IR = (1 - B/A) \times 100\ (\%) \tag{2}$$

A: diameter size (mm) of control fungal colony without sample exposure.

B: diameter size of fungal colony with exposure to calixarene material.

3.4. Antimicrobial activity

A preliminary evaluation of the antimicrobial activity of calixarenes against *Corynebacterium* was performed and gauged according to the following three stage criteria:

++ high antimicrobial activity; size of inhibition zone is approximately 3.5 mm;

+ relatively high antimicrobial activity; size of the inhibition zone is approximately 2.5 mm;

± weak antimicrobial activity; size of inhibition zone is less than 1.5 mm;

– no antimicrobial activity.

4. Results and discussion

For the preliminary screening studies of the 57 calixarenes, the antimicrobial activities of all samples were determined against *Corynebacterium*. *Corynebacterium* is useful because of its high sensitivity toward a wide variety of microorganisms of which the results are listed in Table 3.

From Table 3, it was found that the calixarenes exhibited a range of antimicrobial activities from complete growth inhibition to little or no inhibition.

Table 3. Preliminary screening data for antimicrobial activity of calixarenes against *Corynebacterium*.

Sample No.	25	26	27	28	30	34	50
Activity	+	+	+	+	±	±	±

Table 4. Antimicrobial activity of selected calixarenes against *Corynebacterium* as a function of material concentration.

Concentration (%)	25	26	27	28
1	+	+	+	++
0.1	–	–	–	+
0.01	–	–	–	–
0.001	–	–	–	–

Sample numbers **25**–**28** all exhibit relatively high antimicrobial behaviour, and contain a common SO_3H group in the basic form, which might play a role in this behaviour. The other calixarenes do not show any antimicrobial activity against *Corynebacterium*. It is of interest to note that calixarenes are generally able to selectively combine with metals; this feature could possibly lead to additional significant increases in the antimicrobial activity already displayed.

The extent of antimicrobial activity as a function of material concentration was also examined using the various samples in aqueous solution (Table 4). The 0.1% calixarene solution concentration corresponds to 1000 ppm.

As it is clear from Table 4, calixarene sample numbers **25**–**27** have slight antimicrobial activity against *Corynebacterium* when the sample concentration is above 1%, while calixarene sample number **28** has a much stronger antimicrobial activity beyond concentrations of 0.1%. Therefore, calixarene **28** has the strongest antimicrobial potential toward *Corynebacterium* among the 57 calixarenes studied. From an application-based perspective, the desired concentration for an effective pesticide is considered to be less than 100 ppm. The experimental data indicate that these calixarenes do not display antimicrobial activity below 0.1%, 1000 ppm. Therefore, it seems that these compounds cannot be used as effective pesticides at the moment; however, we should bear in mind that most of the calixarenes listed in Table 1 are safe for the human body.

Growth rate and inhibition rate of fungi from plant

The comparison of the growth and inhibition rates is an important measure to distinguish the antimicrobial activity of the samples against specific fungi and bacteria. Figs. 2 and 3 demonstrate the growth and inhibition rates of various calixarenes against differing fungal strains, respectively.

Calixarene sample numbers **25**–**27** and **50** displayed a range of antimicrobial activity against *Fusarium solani f. sp. mori* (F.s.-26), having an inhibition rate

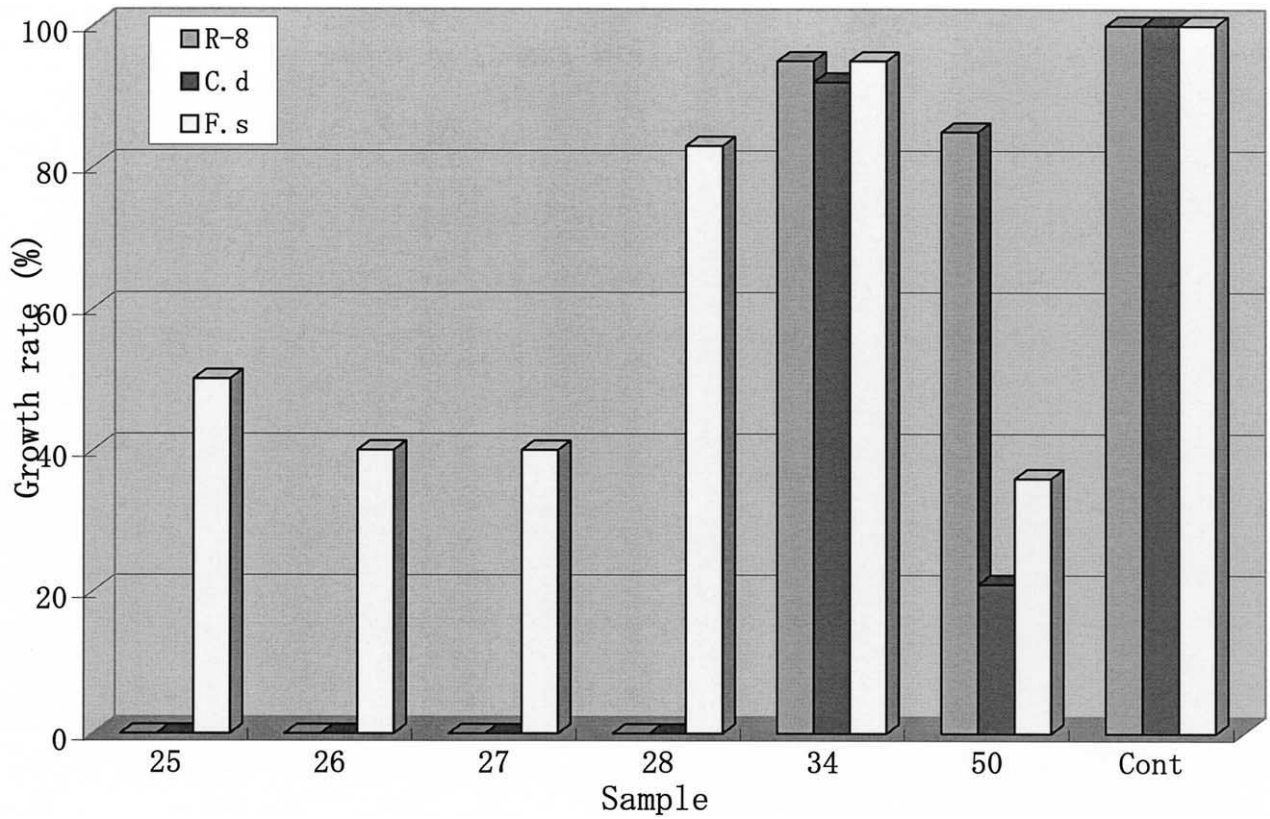

Fig. 2. Growth rates of various calixarenes against differing fungal strains.

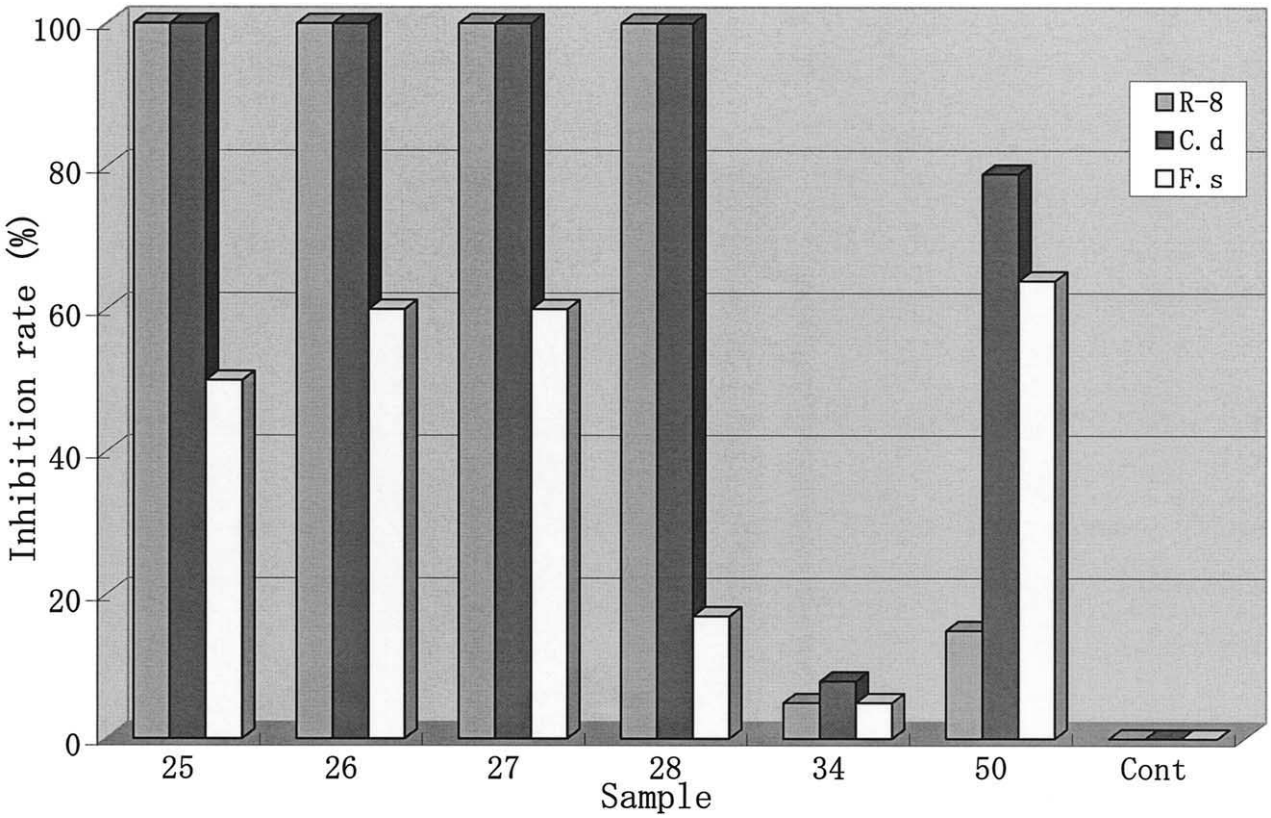

Fig. 3. Inhibition rates of various calixarenes against differing fungal strains.

of approximately 60–70%, while calixarene sample numbers **25**–**28** showed excellent and selective antimicrobial activity against fungal strains *Rosellinia necatrix* (R-8), and *Colletotrichum dematium* (C.d.8901).

5. Conclusion

Approximately 57 calixarenes were screened to determine compounds having antimicrobial activity against fungal and bacterial microorganisms. Sample numbers **25**–**28** all showed slight antimicrobial behaviour, and contain a common SO_3H group in the basic form, which might play a role in this aspect. The compounds in Table 5 were found to be the prominent calixarenes exhibiting antimicrobial activity.

Here we report the slight antimicrobial behaviour of calixarene sample numbers **25**–**28**, which all contain a common SO_3H group in the basic form. Additionally interesting is the fact that the antimicrobially active samples **28** and **30** contain a common –N=N–phenyl group.

This essential preliminary information should be further investigated as to why the above calixarenes are antimicrobially active towards microorganisms, as well as to elucidate the activity mechanisms leading to these observations.

These experimental results indicate that several kinds of calixarenes possess antimicrobial activity towards *Fusarium solani f. sp. mori* (F.s-26). Because

Table 5. Calixarenes exhibiting prominent character of antimicrobial activity.

Sample No.	Name	Character	*n*
25	*p*-sulfonatocalix[4]arene	OH, SO_3H	4
26	*p*-sulfonatocalix[6]arene	OH, SO_3H	6
27	*p*-sulfonatocalix[8]arene	OH, SO_3H	8
28	*p*-phenylazocalix[6]arene	OH, N=N–Ph–SO_3H	6
30	*p*-phenylazocalix[4]arene	OH, N=N–Ph	4
34	*C*-nonylresorcin[4]arene	OH, OH	4
50	Aminocarbonyl *p*-iPr-calix [4]arene	$CH_2CON(C_2H_5)_2$	4

F.s.-26 is a strong pathogen and produces harmful plant injuries, these calixarenes are very important substances to consider when developing new functional pesticides. At present, there are no pesticides against F.s-26, indicating that calixarenes comprise an exciting family of compounds that could be a leading materials substance for new pesticide developments.

Normally pesticides that are effective for plants contain a large quantity of harmful halogens such as sulphur and chloride. The advantage presented by these compounds for pesticide production is that calixarenes do not contain halogens in such high amounts. Therefore, it is hypothesized that calixarenes operate under a different antimicrobial mechanism as compared with that of commercialised pesticides. Since calixarenes combine specifically with metal ions, we can produce new antimicrobially active materials based from calixarenes combined with metals, such as Ag. Ag-combined calixarene could offer a safe alternative pesticide, which would be non-toxic to the human body, and a more durable antimicrobial material, which could be used widely in several applications. With this information in place, it will be possible to develop new techniques and materials utilizing antimicrobial calixarenes in the industrial and medical fields.

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