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Design, synthesis and anticoagulant activity of new flexible calix[8]arene sulfonic acids

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ABSTRACT

Four new calix[8]arene sulfonic acids (**2a-b**, **3a-b**) were synthesized. The anticoagulant activity and POM virtual screening of these flexible calixarenes have been established.

RÉSUMÉ

Quatre acides calix[8]arene sulfoniques (**2a-b**, **3a-b**) inédits ont été synthétisés. L'activité anticoagulante et le screening virtuel par la méthode de calcul POM de ces calixarenes flexibles ont été établis.

1. Introduction

Calix[*n*]arenes are macrocyclic molecules, consisting of several phenol units (four to eight) connected via methylene bridges into *ortho* position with respect to the hydroxy group. They are generally known to possess the properties-ability to complex both metal ions and organic molecules. Functionnalized calixarenes are of particular interest because of their potential uses in complexation electrochemistry, catalysis and in selective enrichment of rare earth metal ions [1]. Very recently, calixarene nano-baskets enabled the encapsulation of guest drugs and show different biological activities [2]. For the synthesis of calix[*n*]arenes, we can use the one-pot synthesis via the base-catalyzed condensation of *para*-substituted phenol or the stepwise procedure from acyclic phenol–formaldehyde precursors [3]. The purpose of our research is to investigate the synthesis of flexible calix[8]arenes, bearing a propanosulfonic or a butanosulfonic group at the lower ring. In this study, we will examine the effect of these water-soluble calix[8]arene sulfonic acids by sub-chronic administration on some hematological parameters using blood samples obtained from rat. We will investigate whether a change in blood coagulation parameters after natural calixarenes administration in Wistar rats at single dose inhibits platelet aggregation induced by vitamin K or after *in vitro* incubation of these compounds with rat fresh blood. The investigation of the anticoagulant activity of the synthesized water-soluble calix[8]arene sulfonic acids is justified by the structure–activity relationship, which is presented here by the use of POM calculations.

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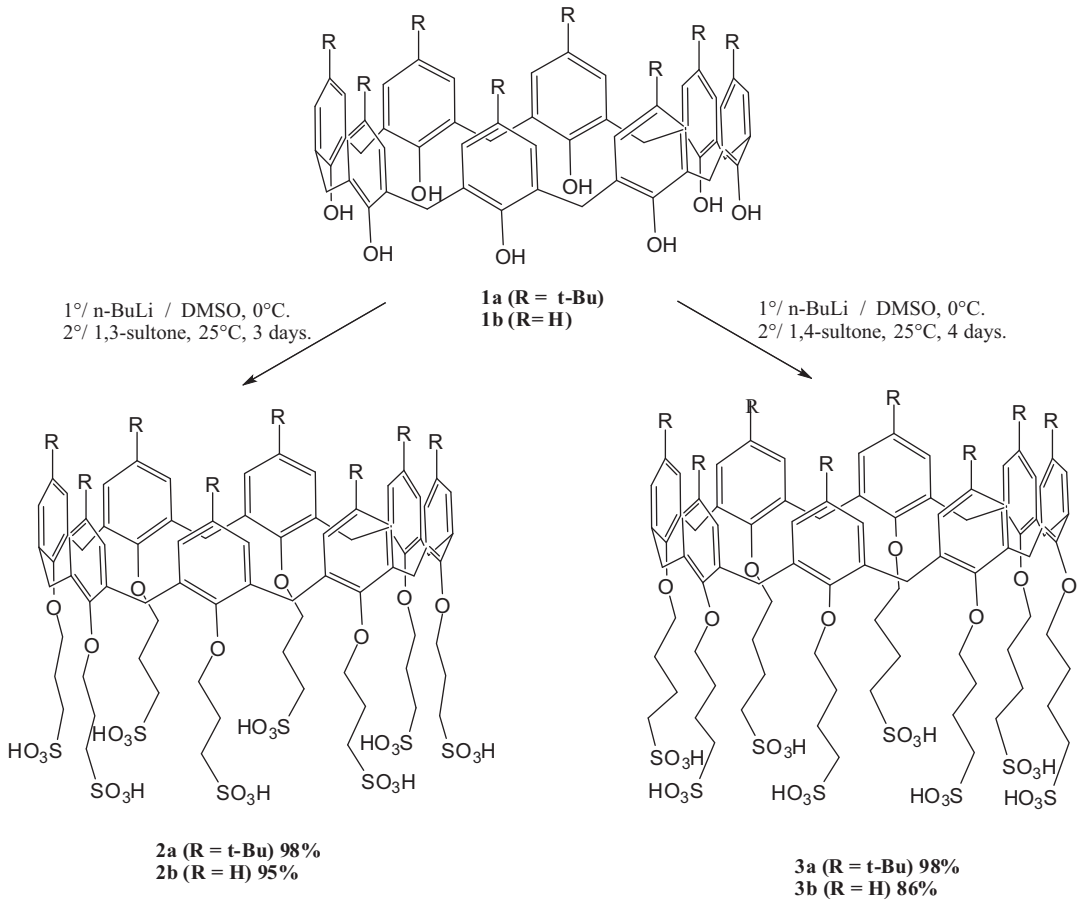


Scheme 1. Synthesis of new water-soluble sulfonated calix[8]arenes **2a-b** and **3a-b**.

2. Results and discussion

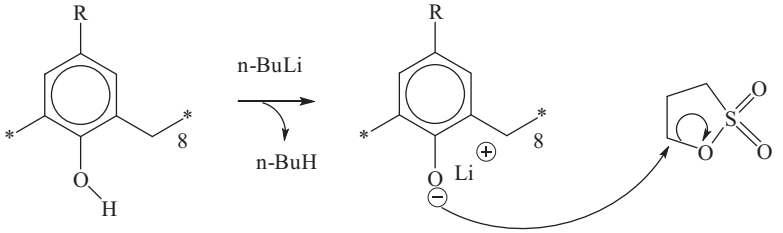
2.1. Chemistry

The treatment of the calix[8]arenes (**1a-b**) with 1,3-propane sultone, during three days at room temperature, led to the new corresponding water-soluble calix[8]arenes (**2a-b**), bearing a propane sulfonic group with excellent yields (98 and 95%, respectively). The reaction of (**1a-b**) with a pyrane sultone afforded, after four days, two new calix[8]arenes bearing a butane sulfonic group (**3a**) in 86 and 98% yields, respectively (Scheme 1).

The *in situ* produced phenolate from n-BuLi in DMSO reacts with the sultone through a ring opening reaction (Scheme 2). The reaction was controlled by ^{1}NMR spectroscopy and the products were purified by recrystallization in ethanol.

Compared with the starting calixarene, the ^{1}HNMR spectra of (**2a**) showed the appearance of three new signals at 3.73, 2.73 and 2.06 ppm, due to the resonance of the methylene groups, CH_2–O–, CH_2–O–SO_3 and C–CH_2–C, respectively. The IR spectra exhibited bands at 3423 cm^{-1} (OH), 2958 cm^{-1} (*t*-Bu), 2872 cm^{-1} (Ar–H), 1421 cm^{-1} (Ar–CH_2), 1193 cm^{-1} (CH_2–O), 1057 cm^{-1} (S=O) and 617 cm^{-1} (S=O). The ES/MS showed a molecular ion at *m*/*z*: 2273.87 corresponding to the $C_{112}H_{160}O_{32}S_8$ formula.

The introduction of the butylsulfonate group on the calixarene squeleton was confirmed by the presence of the



Scheme 2. Possible pathway of the reaction with the sultone.

Table 1
Concentration-dependent effect of calixarenes **(2b, 3a, 3b)** on *in vitro* blood coagulation. One milliliter of rat blood is incubated with 0.1 mL of tested anticoagulant compound.

Final concentration	10^{-2} M	10^{-3} M	10^{-4} M	10^{-5} M
Sodium citrate	NC	NC	C	C
EDTA 2.8%	NC	NC	C	C
2b	NC	PC	C	C
3a	NC	NC	NC	C
3b	NC	NC	C	C

NC: no coagulation; P: partial coagulation; C: coagulation.

correct molecular peak in the mass spectra ES/MS of (**3a**), *m*/*z*: 2385.99 corresponding to the $C_{120}H_{176}O_{32}S_8$ formula and by multiplets at 3.61, 2.54, 1.75 ppm due to the resonance of CH_2–O–, –CH_2–SO_3, C–CH_2–CH_2–C, respectively.

The ^{1}HNMR spectra of (**2b**) exhibited three multiplets at 3.17, 1.96, 1.37 ppm corresponding to the resonance of CH_2O, CH_2SO_3 and C–CH_2–CH groups, respectively. The IR spectra showed the appearance of the new bands at 3207 cm^{-1} (OH), 2935 cm^{-1} (–CH_2), 2877 cm^{-1} (Ar–H), 1448 cm^{-1} (Ar–CH_2), 1186 cm^{-1} (CH_2O) and 1051 cm^{-1} (S=O). The mass spectra showed a molecular ion at *m*/*z*: 1824.37 corresponding the $C_{80}H_{96}O_{32}S_8$ formula. The ^{1}HNMR spectra of (**3b**) was characterized by the presence of three multiplets at 3.71, 2.35, 1.73 ppm attributed to CH_2O, CH_2SO_3, C–CH_2–CH_2–CH groups, respectively. ES/MS showed a molecular ion at *m*/*z*: 1937.49 corresponding to the $C_{88}H_{112}O_{32}S_8$ formula.

2.2. Anticoagulant activity

We assessed routine blood haematology including platelet, red blood cells, white blood cells counts and routine coagulation parameters as prothrombin time (PT), cephalin kaolin time (CKT) and fibrinogen. A slight effect on thromboplastin time, fibrinogen levels or CKT was observed after a single intravenous administration of calixarenes **2b** and **3b** (10 mg/kg) after pretreatment with vitamin K 10 mg/kg, while treatment with **3b** for the same dose and period resulted in significant changes in the parameters (Table 1).

In the specific field of *in vitro* diagnostics, anticoagulants are commonly added to collection tubes either to maintain blood in the fluid state for hematological testing or to obtain suitable plasma for coagulation and clinical chemistry analyses. Preincubation of blood with calixarene **3a** decreased the rate of coagulation in a concentration-dependent manner, a total inhibiting effect being obtained at 100 μM. Identical results were observed with control (EDTA or sodium citrate) and with calixarenes **2b** and **3b** but at 1000 μM.

Compared to EDTA (used as control in the study), the calixarenes (**2a**, **3a**, **3b**) may act by the chelation of calcium necessary for a range of enzyme reactions of the coagulation cascade and its removal irreversibly prevents blood clotting.

2.2.1. Blood cells counts

Results showed a remarkable decrease in platelets count after vitamin K treatment (245×10^3/ul vs 850×10^3/ul for control) and a slight decrease in red blood cells in the group of animals treated with calixarenes **2b** and **3b**. It seems that calixarenes (**2b**, **3a** and **3b**) prevent blood clotting by inhibiting the action of vitamin K at the first stage of clotting (adhesion of blood platelets at the site of damage) followed by aggregation of the platelets (Tables 2–4).

No change in other blood cells counts is observed.

Table 2
Effect of intravenous administration of calixarenes **(2b**, **3a** and **3b)** on platelets parameters during a vitamin K (10 mg/kg) treatment in Wistar rats.

Experience	PTL (10^3/ul)	MPV (fl)
Control	850	5.6
Vitamin K[a]	245	5.8
Vitamin K[a] + **Heparin**[b]	394	5.6
Vitamin K[a] + **2b**[a]	440	5.7
Vitamin K[a] + **3a**[a]	886	5.4
Vitamin K[a] + **3b**[a]	401	5.6

[a] 10 mg/kg.
[b] 3000 U.

2.2.2. Anticoagulant plasma parameters

The PT is a basic coagulation screening test, useful in the assessment of deficiencies of the extrinsic coagulation pathway (factors II, V, VII and X). The PT is commonly used for monitoring anticoagulant therapy because of its sensitivity to variations in the concentration or effects of the vitamin K-dependent factors II, VII and X.

Prolonged PT has been observed during treatments with calixarene **3a** and partially with calixarene **3b**. These compounds inhibit the conversion of vitamin K to its different forms as it participates as a cofactor in the synthesis of the vitamin K-dependent factors (Table 5).

Table 3
Red blood cells parameters during calixarenes treatment.

Experience	RBC (10^6/ul)	Hgb (g/dl)	HCT (%)	MCV(fl)
Control	6.75	12.6	38.2	60.1
Vitamin K[a]	6.15	11.8	33.3	58.1
Vitamin K[a] + **Heparin**[b]	5.49	11.2	33.1	60.2
Vitamin K[a] + **2b**[a]	4.90	10.1	29.2	59.5
Vitamin K[a] + **3a**[a]	6.35	12.2	36.2	57.0
Vitamin K[a] + **3b**[a]	6.53	12.0	34.8	53.2

RBC: red blood cell; Hgb: haemoblobin; HCT: haematocrit; MCV: mean corpuscular volume.
[a] 10 mg/kg.
[b] 3000 U.

Table 4
White blood cells parameters during calixarenes treatment.

Experience	WBC (10^3/ul)	LY (%)	MO (%)	GR (%)
Control	7	41	12.3	39.7
Vitamin K[a]	4.5	85.5	4.5	10.0
Vitamin K[a] + **Heparin**[b]	4.5	68.9	13.3	17.8
Vitamin K[a] + **2b**[a]	3.6	55.5	12.2	32.3
Vitamin K[a] + **3a**[a]	6.9	47.2	11.3	42.5
Vitamin K[a] + **3b**[a]	2.6	53.8	10.4	35.8

WBC: white blood cells; LY: lymphocytes; MO: monocytes; GR: granulocytes.
[a] 10 mg/kg.
[b] 3000 U.

Table 5
Effect of calixarenes treatment on plasma parameters.

Experience	PT (s)	PR (%)	Fb (g/L)	CKT (s)
Control	12	98	2.10	20
Vitamin K[a]	11	100	2.23	19
Vitamin K[a] + **Heparin**[b]	16	60	1.68	25
Vitamin K[a] + **2b**[a]	16	60	1.97	23
Vitamin K[a] + **3a**[a]	40	20	0.98	45
Vitamin K[a] + **3b**[a]	20	48	1.24	30

PT: prothrombin time; PR: prothrombin rate; Fb: fibrinogen level; CKT: cephalin kaolin time.
[a] 10 mg/kg.
[b] 3000 U.

Table 6
Osiris calculations of calixarenes **2a-b** and **3a-b**.

Compound	Toxicity Risks[a]				Osiris calculation[a]				
	MUT	TUMO	IRRI	REP	MW	CLP	S	DL	D-S
2a					2272	11.5	−21.9	−11.9	0.06
2b					1824	−0.62	−12.7	−9.4	0.12
3a					2384	15.22	−24.2	−15.6	0.06
3b					1936	3.09	−14.8	−1301	0.12
Melagatran					429	−0.88	−1.91	−0.41	0.58
Argatroban					507	0.34	−3.48	2.36	0.64
Dabigatran					471	1.14	−2.00	6.28	0.75
Warfarin					308	3.32	−3.72	2.01	0.44
BHC					336	2.66	−3.34	1.33	0.44
Anisidione					252	3.19	−4.05	−1.51	0.46

MUT: mutagenic; TUMO: tumorogenic; IRRI: irritant; REP: reproductive effective; MW: molecular weight in g/mol; CLP: ClogP; S: solubility; DL: drug-likness; D-S: drug-score; BHC: bishydroxycoumarin.
[a] The OSIRIS Property Explorer shown in this page is an integral part of Actelion's in-house substance registration system. It lets you draw chemical structures and calculates on-the-fly various drug-relevant properties whenever a structure is valid. Prediction results are valued and color coded. Properties with high risks of undesired effects like mutagenicity or a poor intestinal absorption are shown in red, whereas a green color indicates drug-conform behavior. http://www.organicchemistry.org/prog/peo/. Red appears darker in the printed version.

Namely, it prevents the synthesis by the liver of γ-carboxyglutamate from glutamate (factors VII, IX, X, la prothrombin and protein C). It is important to emphasize that anticoagulants like warfarin have no effect on previously synthesized functional factors already circulating in the plasma patients treated with these drugs.

2.3. Structure–activity relationship

A perusal of anticoagulant screening data indicates that all the four compounds under investigation were moderately active to the test coagulation. Prediction results of compounds **2a-b** and **3a-b** molecular properties (TPSA, GPCR ligand and ICM) are valued (Table 6).

The structure of synthesized calix[8]arene sulfonic acids (**2a-b**, **3a-b**), for ease of analysis, can be divided into three parts, Viz., cyclic octa-phenyl-methyl skeleton, alkyl-sulphonic acid side chain at C–1 of the phenyl ring and substituted phenyl ring at C–4 of the principal skeleton. We have fixed the former one part and varied the latter ones by substituting the phenyl rings with t–Bu groups at C–4 and replacing the 1,3-propane-sulphonic acid side chain at C–1 by the 1,4-butane-sulphonic acid side chain at C–1 in compounds **3a-b**. These modifications in the

Table 7
Molinspiration calculations of calixarenes **2b** and **3a-b**.

Compound	Molinspiration calculations [4]						Drug-likeness			
	MW g/mol	cLogP	TPSA	OH–NH Interract.	N.V.	Vol.	GPCRL	ICM	KI	NRL
2a	2275	7.65	509	8	4	2028	−6.7	−6.98	−6.97	−7.06
2b	1826	−4.96	509	8	3	1498	−6.05	−6.39	−6.36	−6.43
3a	2387	9.054	509	8	4	2162	−6.83	−7.11	−7.14	−7.20
3b	1938	−3.46	509	8	3	1632	−6.23	−6.55	−6.57	−6.62
Melagatran	429	−0.93	149	6	1	400	0.09	−0.10	−0.58	−1.09
Argatroban	508	1.52	174	7	2	462	0.12	−0.02	−0.89	−0.97
Dabigatran	472	0.97	150	5	0	420	0.10	−0.49	−0.229	−1.22
Warfarin	308	3.03	68	1	0	277	−0.76	−0.41	−0.98	−0.50
BHC	336	3.33	101	2	0	277	−0.39	−0.26	−0.37	−0.14
Anisidione	252	22.9221	43	0	0	225	−0.13	−0.40	−0.44	−0.13

TPSA: topological polar surface area; N.V.: number of violation; Vol.: volume; GPCRL: G protein couplet receptor ligand; ICM: ion channel modulator; KI: kinase inhibitor; NRL: nuclear receptor ligand; BHC: bishydroxycoumarin.

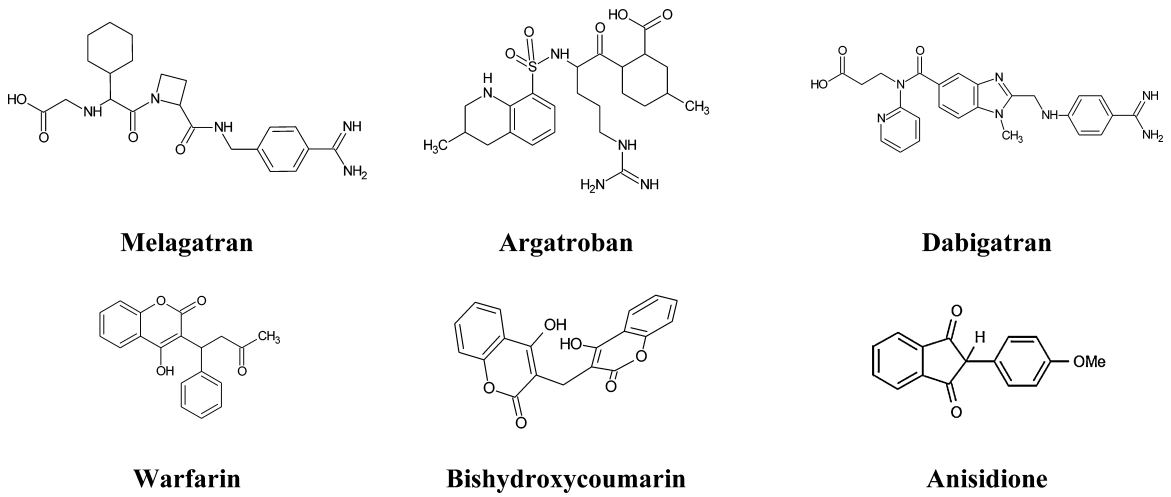


Fig. 1. a: structures of three direct thrombin inhibitors (DTIs); b: structures of three clinically useful coumarins and 1,3-indanediones.

calix[8]arene sulfonic acids skeleton followed by analysis of the resulting molecules' structure have resulted in the following findings.

The compound **2a** containing unsubstituted phenyl rings at C–4 is non-effective as a anticoagulant agent and **2b** is a moderately effective anticoagulant agent at 10^{-3} concentration against *in vitro* blood coagulation (Table 7).

The introduction of a butanosulfonic instead of a propanosulfonic group at the lower ring led to the more efficient analogue **3b**. In contrast to the alkanosulfonic group, the increase of the number of carbons of R group at position C–4 phenyl ring masked the potency in case of compounds **2a** and **3a** as compared with **2b** and **3b**. On the other hand, all compounds **2b** and **3a-b** had a lesser activity quite comparable to the commercial antibiotics (Fig. 1) tested under similar conditions.

This weak activity was probably due to the absence of a strong polar substituent –OH at any position in the phenyl ring on the calixarene moiety. In both cases, the oxygen can act as a hydrogen bond acceptor and the hydrogen can act as a hydrogen bond donor. One or all of these interactions may be important in binding the molecules to the binding site. Thus in both the cases, the hydroxyl group may be involved in some H-bonding, which increases the affinity of the molecule for the active site of the enzyme. So *n*-OH is necessary for high activity/solubility of the calixarenes.

A number of important points emerge concerning the electronic and steric factors, which have direct impact on bioactivity properties. The positive results we have recorded, while encouraging for purposes of new calixarene drug design, confirm that very likely most of these compounds could be used as potential anticoagulant agents after major modifications. Based on their structural properties, these compounds may be useful as selective chelating agents with higher potential activity.

3. Conclusion

The treatment of calix[8]arenes (**1a-b**) with 1,3-sultone, during three days at room temperature, led to the two new water-soluble calix[8]arenes (**2a-b**) bearing a propane sulfonic group with excellent yields (95–98%). Starting from 1,4-sultone, two new calixarenes (**3a-b**) with a butane sulfonic group were obtained with very good yields (86–98%).

Products of the cyclo-oxygenase pathway play important roles in platelet function and in the formation of platelet-plugs. So, blood exposed to the new water-soluble calix[8]arene sulfonic acids (**2b**, **3a**, **3b**) (*in vivo* and *in vitro*) is less capable of aggregating, and thus platelet-plug formation is hindered. Consequently, it is reasonable to assume that the pharmacological actions of these calixarenes could influence the flow properties of both blood and plasma. The anticoagulant activity of the calixarenes (**2b**, **3a**, **3b**) may also be explained by the inhibition of the liver synthesis of vitamin K-dependent coagulation factors but also by their role in preventing activation of thrombin and the activation of other factors.

Our results showed the possible interest of the new water-soluble calix[8]arene sulfonic acids (**2b**, **3a**, **3b**) in the domain of the diagnosis of the biologic phenomenon of blood coagulation and maybe of the therapeutic anticoagulant used.

4. Experimental

4.1. Chemistry

Melting points were determined on a Barnstead Electrothermal melting point apparatus and are uncorrected. ^{1}HNMR and ^{13}C–NMR spectra (δ, ppm) were recorded on a Bruker ALS 300 MHz spectrometer using tetramethylsilane as the internal reference. The IR spectra (ν, cm^{-1}) were obtained with a Shimadzu FTIR-8201 PC 1600 FTIR spectrometer in KBr pellets. The necessary chemicals were purchased from Merck and Fluka.

4.1.1. General procedure for synthesis of calix[8]arenes (*1a-b*)

Starting compounds (**1a-b**) were synthesized according to the literature [5].

4.1.2. General procedure for synthesis of new soluble sulfonated calix[8]arenes (*2a-b*)

0.772 mmol of calix[8]arene (**1a-b**) are dissolved in 40 ml of DMSO and stirred at 0 °C under nitrogen atmosphere. *n*-Butyllithium (7.8 ml, 12.4 mmol, 1.6 M in hexane) is added dropwise and the mixture is stirred during 24 hours at room temperature, then 1.508 g (1.236 mmol) of 1,3-sultone are dissolved in 10 ml of DMSO and added dropwise to the mixture at room temperature. After 3 days of stirring, the solvent is evaporated and the solid is washed with acetone then recrystallised in ethanol, affording **2a-b**.

4.1.3. Spectral data of unknown compounds

[5,11,17,23,29,35,41,47-octa-tert-butyl-49,50,51,52,53,54,55,56-octakis-(3-propylsulphonic acid) calix[8]arene] (**2a**).

Yield (1 g, 98%); White solid. mp > 300 °C; IR: 3423 (OH), 2958 (t–Bu), 2872 (Ar–H), 1421 (Ar–CH_2), 1193 (CH_2–O), 1057 (S = O), 1057 (S = O) cm^{-1}; ^{1}HNMR (CD_3SOCD_3) δ (ppm): 6.81 (s, 16 H, H–Ar), 3.94 (s, 16 H, –CH_2–Ar), 3.73 (m, 16 H, –CH_2–O), 2.73 (m, 16 H, –CH_2-SO_3), 2.06 (m, 16 H, C–CH_2–C), 0.92 (s, 72 H, t–Bu); ES/MS for $C_{112}H_{160}O_{32}S_8$ $[M+H]^+$: Calcd, 2273,8658. Found, 2273.8648; Anal. Calcd: C, 59.13; H, 7.09; O, 22.50. Found. C, 59.01; H, 7.00; O, 22.33.

[49,50,51,52,53,54,55,56-octakis-(3-propylsulphonic acid) calix[8]arene] (**2b**).

Yield (1 g, 95%); White solid. mp > 300 °C; IR: 3207 (OH), 2935 (–CH_2–), 2877 (Ar–H), 1448 (Ar–CH_2), 1051 (S = O); 1186 (CH_2O) cm^{-1}; ^{1}HNMR (CD_3SOCD_3) δ (ppm): 6.08 (s, 24 H, H–Ar), 3.24 (s, 16 H, Ar–CH_2–Ar), 3.17 (m, 16 H, –CH_2–O), 1.96 (m, 16 H, –CH_2–SO_3), 1.37 (m, 16 H, C–CH_2–C); ES/MS for $C_{80}H_{96}O_{32}S_8$ $[M]^+$: Calcd, 1824.3650. Found, 1824.3625; Anal. Calcd: C, 52.62; H, 5.30; O, 28.04. Found. C, 52.40; H, 5.17; O, 27.89.

4.1.4. General procedure for synthesis of new soluble sulfonated calix[8]arenes (*3a-b*)

0.772 mmol of calix[8]arene (**1a-b**) are dissolved in 40 ml of DMSO and stirred at 0 °C under nitrogen atmosphere. *n*-Butyllithium (7.8 ml, 12.4 mmol, 1.6 M in hexane) is added dropwise and the mixture is stirred during 24 hours at room temperature then 1.74 g (1.27 mmol) of 1,4-sultone is dissolved in 10 ml of DMSO and added dropwise to the mixture at room temperature. After 3 days of stirring, the solvent is evaporated and the solid is washed with acetone then recrystallized in ethanol, affording **3a-b**.

4.1.5. Spectral data of compounds *3a-b*

[5,11,17,23,29,35,41,47-octa-*tert*-butyl-49,50,51,52,53,54,55,56-octakis-(4-butylsulphonic acid) calix[8]arene)] (**3a**).

Yield (1.61 g, 86%); Strong clear chestnut. mp > 300 °C; IR: 3247 (OH), 2869 (*t*-Bu), 1639 (Ar), 1458 (Ar–CH_2), 1184 (CH_2O), 1053 (S = O) cm^{-1}; ^{1}HNMR (CD_3SOCD_3) δ (ppm): 6.84 (s, 16 H, H–Ar), 3.93 (s, 16 H, Ar–CH_2–Ar), 3.61 (m, 16 H, –CH_2O–), 2.54 (m, 16 H, –CH_2–SO_3), 1.75 (m, 32 H, C-CH_2–CH_2–C), 0.93 (s, 72 H, t –Bu); ES/MS for C $C_{120}H_{176}O_{32}S_8$ $[M+H]^+$: Calcd, 2385,9910. Found, 2385,9920; Anal. Calcd: C, 60.38; H, 7.43; O, 21.45. Found. C, 60.27; H, 7.21; O, 21.36.

[49,50,51,52,53,54,55,56-octakis-(4-butylsulphonic acid) calix[8]arene)] (**3b**).

Yield (1.16 g, 98%); Yellow solid. mp > 300 °C; IR: 3224 (OH), 2942 (–CH_2–), 2873 (Ar–H), 1443 (Ar–CH_2), 1063 (S = O); 1183 (CH_2O) cm^{-1}; ^{1}HNMR (CD_3SOCD_3) δ (ppm): 6.48 (S, 24 H, H–Ar), 3.92 (s, 16 H, Ar–CH_2–Ar), 3.71 (m, 16 H, –CH_2O–), 2.35 (m, 16 H, -CH_2-SO_3), 1.73 (m, 32 H, C–CH_2–CH_2-C); ES/MS for $C_{88}H_{112}O_{32}S_8$ $[M+H]^+$: Calcd, 1937,4902. Found, 1937.4923; Anal. Calcd: C, 54.53; H, 5.82; O, 26.41. Found. C, 54.39; H, 5.71; O, 26.25.

4.2. Anticoagulant activity [6]

4.2.1. Treatment of animals (in vivo activity)

Female rats Wistar albinos, weighing 180–200 g, were randomly divided into six groups of six rats each, such that differences in average body weights were minimal. Each group was kept at uniform temperature with 12 h dark/light periodicity and fed with standard rat pellets and water ad libitum. Anticoagulants products were dissolved in distilled water. Group 1 (Control): rats received 0.1 ml of

distilled water intravenously. Group 2 (Vit K): rats received 0.1 ml of vitamin K (10 mg/kg) intravenously. Group 3 (Vit K + Heparin): rats received vitamin K (10 mg/kg) intravenously and two hours after, rats were administered intravenously 3000 U heparin (Fraxiparin®, Glaxo Smith and Kline, UK). Group 4 (**2b**): rats receiving 0.1 ml of vitamin K (10 mg/kg) intravenously and 2 hours after, rats received **2b** product (10 mg/kg) intravenously. Group 5 (**3a**): rats received 0.1 ml of vitamin K (10 mg/kg) intravenously and 2 hours after, rats received **3a** product (10 mg/kg) intravenously. Group 6 (**3b**): rats received 0.1 ml of vitamin K (10 mg/kg) intravenously and 2 hours after, rats received **3b** product (10 mg/kg) intravenously.

Treatment was stopped 24 hours after for all groups and venous blood was collected via retrorbital sinus puncture into EDTA sample and into sodium citrate for haematological and coagulation analyses.

In vitro study coagulation was carried on rat blood incubated with sodium citrate or with calixarenes (**2b**, **3a**, **3b**). One mL of rat blood was incubated with 0.1 mL of tested anticoagulant compound at concentrations ranging between 10^{-2} M and 10^{-5} M.

4.2.2. Haematological analyses

The blood samples were analyzed for white blood cells (WBC), red blood cells (RBC), and platelet count using the automated haematologic analyzer Coulter S (Beckman, USA). Haemoglobin (Hb), mean corpuscular volume (MCV), thromboplastin time (TPT), fibrinogen levels (Fb), prothrombin time (PT), prothrombin rate (PR and cephalin kaolin time [CKT]) were assessed.

4.3. Structure–activity relationship

4.3.1. Virtual screenings and molecular properties calculations

Osiris is already available online (http://www.organic-chemistry.org/prog/peo/).

4.3.2. Molinspiration calculations

CLogP (octanol/water partition coefficient) is calculated by the methodology developed by Molinspiration as a sum of fragment-based contributions and correction factors (Table 7). The method is very robust and is able to process practically all organic, and most organometallic molecules. Molecular Polar Surface Area (TPSA) is calculated based on the methodology published by Ertl et al. [7] as a sum of fragment contributions. O– and N– centered polar fragments are considered. PSA has been shown to be a very good descriptor characterizing drug absorption, including intestinal absorption, bioavailability, Caco–2 permeability and blood-brain barrier penetration.

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