

SYNTHESIS OF WATER SOLUBLE L-ALANIN-CALIX[N]ARENES AND THEIR COMPLEX FORMATION STUDY WITH WARFARIN AND ACENOCOUMAROL

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Abstract

Calixarenes are cyclic oligomers which can be successfully used as selectors in the molecular recognition of a large variety of compounds with different sizes and shapes. When using water soluble calixarenes, these derivatives can be used as target carriers for different types of drugs. For the present study, we have synthesized three water soluble calix[n]arenes of different cavity sizes ($n = 4, 6, 8$), derivatized with L-alanine in the endo-position. The obtained calixarene derivatives were characterized by spectroscopic techniques, such as UV and Attenuated Total reflection - Fourier - Transform - Infrared (ATR-FT-IR). The formation and the stability of the inclusion complexes with acenocoumarol and warfarin were studied by capillary electrophoretic techniques, where the most stable complexes were with the calix[4]arene derivative and with the calix[6]arene derivative. In the same time, the results proved the key role of hydrogen bonds in the complex formation.

Rezumat

Calixarenele sunt oligomeri ciclici care pot fi utilizați cu succes ca selectori în recunoașterea moleculară a unei largi varietăți de compuși cu diferite forme și dimensiuni. Prin utilizarea calixarenelor solubile în apă, acești compuși pot deveni molecule transportoare pentru diferite tipuri de substanțe medicamentoase. Pentru prezentul studiu, am sintetizat trei calix[n]arene solubile în apă cu diferite dimensiuni ale cavităților ($n = 4, 6, 8$), derivatizate cu L-alanină în poziția endo. Derivații de calixarene obținuți au fost caracterizați prin metode spectroscopice, cum ar fi UV și ATR-FT-IR. Formarea și stabilitatea complexelor de incluziune cu acenocoumarol și warfarină au fost studiate prin tehnici de electroforeză capilară, rezultatele demonstrând că cei mai stabili complecși sunt cei formați cu derivații de calix[4]arenă, respectiv de calix[6]arenă. În același timp, rezultatele au demonstrat rolul cheie al legăturilor de hidrogen în formarea complexelor.

Keywords: water soluble calixarenes; oral anticoagulant; capillary electrophoresis; inclusion complex

Introduction

Calixarenes are cyclo-oligomers forming a cavity which can trap different ions [6] and small molecules [9] and can be even used in chiral recognition [3]. Their high disadvantage is their high hydrophobicity, which makes them soluble only in highly non-polar solvents such as benzene, toluene or chloroform [3, 13]. But, through introduction of polar substituents in certain positions of the molecule, the calixarene derivatives can conserve the hydrophobicity of the cavity and can get a higher polarity of the entire molecule, leading to a higher solubility in polar solvents, even in water [4, 12]. Warfarin and acenocoumarol are two oral anticoagulants widely used in thrombotic diseases, post-myocardial infarction or in stroke prevention. However, they present a low therapeutic index and a high patient-to-patient variability, which makes difficult to control the dose [10]. The use of water

soluble calixarenes as target transport carriers of these anticoagulants would be beneficial, considering also the low toxicity of these compounds [7, 8].

Capillary electrophoresis (CE) is a widely used separation technique, which comes with several advantages, such as high efficiency, reduced sample volume, relative short analysis times. CE has been used not only for the separation of molecules, but also as a chiral technique [1] for establishing the reaction mechanisms [2] or for characterizing different compounds [14].

The aim of the present work was to synthesize new water soluble amino-acyl calix[n]arene derivatives which could work as selectors for the recognition of the above mentioned oral anticoagulants. In the same time, we studied the interactions between the cavity shaped selectors and the two analytes taken in study - warfarin and acenocoumarol.

Materials and Methods

Materials

The three calixarene derivatives (compound I), p-tert-butyl-calix[4]arene tetra-ethylacetic ester, p-tert-butyl-calix[6]arene hexa-acetic-ester, p-tert-butyl-calix[8]arene octa-acetic-ester, from which was started the synthesis, were kindly donated by the research team from the Inorganic Chemistry Department from the Institute of Chemical Researches "Raluca Ripan", Cluj-Napoca, Romania. All other reagents and solvents: tetramethyl-ammonium hydroxide ($\text{Me}_4\text{N}(\text{OH})$), tert-butyl-L-alanine hydrochloride (t-bu-Ala), diisopropylethylamine (DIPEA), 1- [Bis-(dimethylamino)methylene]- 1H- 1,2,3- triazolo [4,5-b] pyridinium 3-oxid hexa-fluorophosphate (HATU), trifluoroacetic acid (TFA), dimethyl-formamide (DMF), dichloromethane (DCM), tetrahydrofuran (THF), phosphoric acid, boric acid, tris(hydroxymethyl)aminomethane (Tris), sodium hydroxide, hydrochloric acid, warfarin (W), acenocoumarol (A), acetone were purchased from Sigma Aldrich® (Germany) and were of analytical grade. Ultrapure bidistilled water for capillary electrophoretic analysis was produced *in situ* using a Barnstead EASYpure RoDi ultrapure water purification system.

Apparatus and methods

Synthesis. The synthesis procedure was performed according to Hamilton [5], slightly modified and adapted for the three cavity dimensions of calixarene derivatives. Briefly, the p-tert-butyl-calix[n]arene ethylacetic esters and corresponding amount of aqueous 10% $\text{Me}_4\text{N}(\text{OH})$ were dissolved in 50 mL THF and stirred for 24 hours at room temperature. The reaction was stopped by adding 1 mL 37% HCl and the mixture was stirred other 16 hours at room temperature. The solvent was completely evaporated using a rotary evaporator (Rotavapor RE, Büchi Laboratoriums Technik AG CH-9230), washed with bidistilled water and dried in a vacuum desiccator. In the next step, the crude product, p-tert-butyl-calix[n]arene carboxylic acid, was esterified with t-bu-Ala using HATU. 0.5 mmol of the hydrolysis product, 4 mmol t-bu-Ala and 8.8 mmol DIPEA was added to 25 mL anhydrous DMF. Next, 4.4 mmol HATU were added to the reaction mixture and this was stirred at 0°C for one hour and then at room temperature for the next 4 hours. Water was added in order to stop the reaction. The white precipitate was filtered and dried in a *vacuum* desiccator during the night. The resulted compound was dissolved in 5 mL DCM and cooled in a dry ice bath, then 10 mL TFA was added drop by drop. This mixture was left at room temperature for 30 minutes and after that the solvent was evaporated.

Characterization of calixarene derivatives. The characterization of the obtained compounds was performed by UV and ATR-FT-IR spectroscopy. UV analysis was realized using a SPECORD 200 spectrophotometer, in the range of 190 - 400 nm, with a speed of 1nm/s. The ATR-FT-IR analysis was realized using a Jasco spectrometer, with ZnSe crystal, in the range of 4000 - 500 cm^{-1} , having an average of 60 spectra *per* analysis. For each IR analysis, a blank analysis was performed before, in order to eliminate the air signals.

Capillary electrophoresis. The obtained calixarene compounds were added to the running buffer in a capillary electrophoresis system Agilent CE 3D. All buffers were prepared by dissolving the necessary amount of the weak acid or weak base in pure water, the pH was adjusted with 0.1 N NaOH or 0.1 N HCl, as applicable, using a Consort C931 potentiometer, then water was added as needed, into a volumetric flask.

Every beginning of an analysis day was started with a capillary washing protocol: 15 min 1N NaOH, 15 min 0.1N NaOH, 10 min bidistilled water, 10 min butyl glycol ether (BGE).

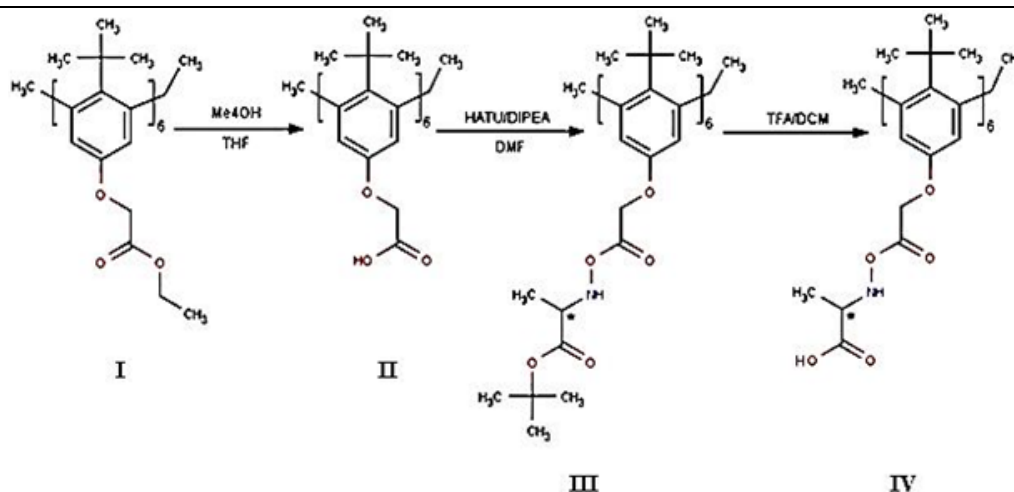
The concentration of calixarene compound was set at 1 mM for all analyses. The initial analysis was performed using "standard" electrophoretic parameters: phosphate buffer 50 mM, +20 kV, hydrodynamic injection 250 mbar/sec. The DAD detector was set to three wavelengths: 310 nm, 200 nm and 210 nm. Acetone was used as electroosmotic flow (EOF) marker.

Results and Discussion

Synthesis and characterization of p-tert-butyl-calix[n]arene L-alanine derivatives

The synthesis of the p-tert-butyl-calix[n]arene L-alanine derivatives was performed in a three step process (Figure 1).

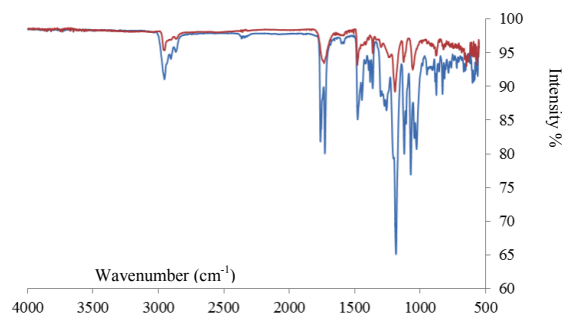
In the first step, it was performed a hydrolysis of the ethyl-ester group on the endo-rim of the calixarene compound, leading to a calixarene derivative with a free carboxylic group (compound II), which is also soluble in water. Further, the carboxylic group reacts with the tert-butyl-L-alanine, to form a calixarene chiral amide, non-soluble in water (compound III). The reaction takes place in the presence of a base (DIPEA), using HATU as peptidizing agent, under the nucleophilic attack of the carboxyl group. The tert-butyl group is then eliminated in acidic media, forming the final hydrosoluble compound (compound IV). The yields for the synthesis, total yield and partial yield, were calculated to be between 50% and 99%.

**Figure 1.**

The synthesis of p-tert-butyl-calix[6]arene L-alanine derivative, starting from p-tert-butyl-calix[6]arene hexa-acetyl-ester

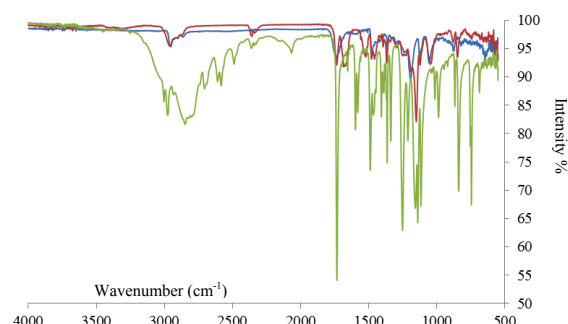
All the compounds have been characterized by ATR-FT-IR spectroscopy and also, the UV spectra of the two water soluble compounds have been recorded, because the water non-soluble intermediates are soluble in solvents which are not appropriate for UV analysis.

For the first compound, the ATR-FT-IR spectrum shows characteristic vibrational bands for the calixarene groups in the 3000 cm^{-1} region, more specific one main vibrational band at 2955 cm^{-1} and two bands at 2905 cm^{-1} and 2862 cm^{-1} , respectively. This group of IR bands is present in all four compounds encountered in the synthesis process, being characteristic to the calixarene ring. In the same time, the C=O bonds of the ester groups in compound I have been identified close to the fingerprint region, at 1761 cm^{-1} and 1728 cm^{-1} . When analysing the IR spectrum of the compound, it can be seen a modification in the spectrum, where the vibrational intensity of the C=O vibrational band is higher, due to the influence of hydrogen on the -C=O group (Figure 2).

**Figure 2.**

Overlaid ATR-FT-IR spectra of p-tert-butyl-calix[6]arene hexa-acetyl-ester (I) (blue) and p-tert-butyl-calix[6]arene hexa-carboxyl (II) (red)

In the same time, when overlaying the spectrum of the second, the third compound and tert-butyl-L-alanine, the specific vibrational bands can be identified (Figure 3): the C=O bond can be identified for all the three compounds at $1733 - 1736\text{ cm}^{-1}$. The amidic C=O in compound III gave another close vibrational band at 1676 cm^{-1} . Two very small vibrational bands of the N-H amidic group appeared in the spectrum of the compound III at 3413 cm^{-1} and 3314 cm^{-1} . The free amino group in tert-butyl-L-alanine was observed at $2582 - 3005\text{ cm}^{-1}$.

**Figure 3.**

Overlaid ATR-FT-IR spectra of p-tert-butyl-calix[6]arene hexa-carboxyl (II) (blue), p-tert-butyl-calix[6]arene p-tert-butyl-L-alanine-ester (III) (red) and p-tert-butyl-L-alanine hydrochloride (green)

Finally, the specific vibrational bands of the compound of interest, compound IV, can be seen in Figure 4. The group of amidic vibrational bands are still initially present at 3413 cm^{-1} and 3314 cm^{-1} in compound III spectrum are shifted and form one single vibrational band for the amidic group at 3314 cm^{-1} . The calixarene vibrational bands are still present in the region of $2955 - 2862\text{ cm}^{-1}$, while the

carboxylic group that appeared could be identified in the fingerprint region.

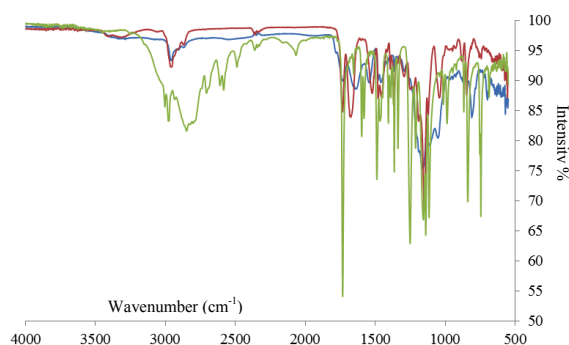


Figure 4.

Overlaid ATR-FT-IR spectra of p-tert-butyl-calix[6]arene p-tert-butyl-L-alanine-ester (III) (red), p-tert-butyl-calix[6]arene L-alanine-ester (IV) (blue) and p-tert-butyl-L-alanine hydrochloride (green)

The UV analysis was performed for the two compounds which were soluble in water: compound II and compound IV. The results showed that the two compounds presented totally different spectra, indicating that there are two different compounds and the reaction took place (spectra not shown). The spectrum of compound II showed two maximum absorption peaks, at 209 nm and 233.4 nm, while the spectrum of compound IV showed one maximum absorption peak at 196.2 nm.

Capillary electrophoresis

By capillary electrophoresis we studied the formation of complexes between the synthesized calix[n]arene derivatives and two oral anticoagulants: warfarin and acenocoumarol.

The type of buffer, the running temperature, the length of capillary, the buffer organic additive were taken in study as parameters which could influence the formation of inclusion complexes between the synthesized calixarenes and the two oral anticoagulants. We have also studied the influence of the size of the cavity of the three synthesized calix[4,6,8]-arenes.

After several test analysis, the borate buffer was chosen as election buffer among phosphate buffer, borate buffer and Tris buffer (tris(hydroxymethyl)-aminomethane), in terms of obtained signal-to noise ratio, conductivity of the buffer, influence over solubility of calixarene derivatives and analysis reproducibility. The higher temperature (40°C) used for the running analysis seemed to give a higher reproducibility. A higher total length of the capillary, 80 cm over 40 cm, gave a better total overview of the migration times of the complexes.

In positive polarity, the cathode was set at the capillary outlet end. At pH = 8 which was taken in study, both warfarin (pKa = 5.45) and acenocoumarol

(pKa = 5.18) [11] presented a low negative charge. In the same time, the calixarene derivatives (compounds IV) will be under their carboxylate forms, presenting a negative charge. Despite this fact, some types of non-covalent bonds can be formed (van der Waals, hydrogen bonds) and can eliminate or compensate the electrostatic repulsions, leading to the formation of inclusion complexes. It must be mentioned that the pH = 8 was chosen because the synthesized calixarene derivatives are soluble in water only at alkaline pH, when the carboxylates are formed.

In the complex formation study between the synthesized calix[n]arenes and the two anticoagulants, we have followed the electrophoretic retention times of the single molecules and the retention times of the supposed formed complexes. As it can be seen in Table I, the single molecule guests presented much higher retention times than their calixarene complexes. This can be explained by the fact that warfarin and acenocoumarol, respectively, present the negative charge which is sufficient to “fight” the electro-osmotic flow (EOF) and to obtain a higher retention time. When in the presence of a ligand, there can appear different types of non-covalent interactions which can even cancel the anode attraction, thus the complex being (almost) only by the EOF. For example, in our study, complexes of acenocoumarol with the calix[4 and 6]arene derivative had the same electrophoretic mobility as the EOF, when analysed in presence of methanol as buffer additive.

We have studied the migration of complexes using two buffer organic additives: methanol (MeOH) and acetonitrile (ACN). In all cases, there is a significant difference between the mobility of single guest molecules and the electrophoretic mobility of calixarene anticoagulant mixtures, suggesting the complex formation. The higher the difference in electrophoretic mobility is, the strongest the complex stability is. Thus, as it can be observed in Table I, the most stable complexes formed were be those analysed in presence of ACN, their mobility becoming even greater than the one of the EOF, even if the mixture contained initially two negative charged molecules.

In the case of complexes analysed in the presence of MeOH, as it can be seen in Table I, the obtained electrophoretic mobility have values similar to the EOF mobility. Therefore, we can state that the non-covalent interactions which appear in the complex formation can lead to at least the cancelation of the effect of the ionic charge of the two molecules forming the inclusion complex, having a greater impact in the electrophoretic migration than the ionic charge taken as it is.

Table I

Migration times and electrophoretic mobility obtained in case of single runs for anticoagulants and for complexes with calix[n]arenes, using different buffer additives

Media	Retention times (min)								
	acetone	W	A	W/C4	A/C4	W/C6	A/C6	W/C8	A/C8
MeOH	8.153	14.617	14.338	8.388	8.139	8.323	8.158	7.869	7.882
ACN	6.282	18.934	18.350	5.406	5.282	5.437	5.287	5.216	5.197
Media	Electrophoretic mobilities (10^{-4}) ($\text{cm}^2/(\text{s}\cdot\text{V})$)								
	EOF	W	A	W/C4	A/C4	W/C6	A/C6	W/C8	A/C8
MeOH	350.791	195.669	199.477	340.963	351.395	343.626	350.576	363.452	362.852
ACN	455.269	151.054	155.861	529.042	541.462	526.025	540.949	548.313	550.317

Running conditions: borate buffer 50 mM pH = 8, voltage +20kV, injection 400 mbar/sec, temperature 40°C, capillary: 50 μm ID diameter, total length 80 cm (effective length 71.5 cm). W = warfarin, A = acenocumarol, MeOH = methanol, ACN = acetonitrile.

The two analysed buffer additives can be used in different situations: ACN gave better stability of the complexes, but no difference between cavity sizes, while with MeOH there can be observed a difference in electrophoretic mobility, which is **benefic** for the separation of the two guest molecules.

Conclusions

In this study we presented the detailed three step synthesis of three calix[4-, 6- and -8]arene L-alanine derivatives which are soluble in water. The obtained compounds were characterized by UV and ATR-FT-IR spectroscopy. They were used as additives to the background electrolyte in capillary electrophoresis in order to evaluate of stability of inclusion complexes formed with two oral anticoagulants - warfarin and acenocumarol. As the study revealed, the complexes with higher stability were those formed in acetonitrile media, but the presence of methanol could lead to their separation.

Acknowledgement

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References

1. Ali I., Sanagi M.M., Aboul-Enein H.Y., Advances in chiral separations by nonaqueous capillary electrophoresis in pharmaceutical and biomedical analysis. *Electrophoresis*, 2014; 35(7): 926-936.
2. Bodoki E., Chira R., Zaharia V., Săndulescu R., Mechanistic Study Of Colchicine's Electrochemical Oxidation. *Electrochim. Acta*, 2015; 178: 624-630.
3. Chiriță C., Cioroianu D.M., Chiriță C.I., Negreș S., Marian B., Zbârcea C.E., Synthesis and pharmacological activity of new acyloximines derivatives. *Farmacia*, 2016; 64(1): 61-66.
4. Chen H., Zou Z.L., Tan S.L., Bi J.H., Tian D.M., Li H.B., Efficient synthesis of water-soluble calix[4]-arenes via thiol-ene “click” chemistry. *Chinese Chem. Lett.*, 2013; 24(5): 367-369.
5. Hamilton K., Synthesis, Characterization, And Application Of Water-Soluble Chiral Calix[4]Arene Derivatives In Spectroscopy And Capillary Electrokinetic Chromatography. 2003, Ph. Thesis.
6. Ma J., Song M., Boussouar I., Tian D., Li H., Recent progress of calixarene-based fluorescent chemosensors towards mercury ions. *Supramol. Chem.*, 2015; 27(5-6): 444-452.
7. Mokhtari B., Pourabdollah K., Applications of calixarene nano-baskets in pharmacology. *J. Incl. Phenom. Macrocycl. Chem.*, 2012; 73(1-4): 1-15.
8. Mokhtari B., Pourabdollah K., Applications of nano-baskets in drug development: high solubility and low toxicity. *Drug Chem. Toxicol.*, 2013; 36(1): 119-132.
9. Oniga S., Duma M., Oniga O., Tipericiu B., Pirnau A., Araniciu C., Palage M., Synthesis of some new 4-methyl-2-(4-pyridyl)-thiazole-5-yl-azoles as potential antimicrobial agents. *Farmacia*, 2015; 63(2): 171-178.
10. Rathore S.S., Agarwal S.K., Pande S., Singh S.K., Mittal T., Mittal B., Therapeutic Dosing of Acenocoumarol: Proposal of a Population Specific Pharmacogenetic Dosing Algorithm and Its Validation in North Indians. *PLoS One*, 2012; 7(5): e37844.
11. Remko M., Broer R., Remkova A., A comparative study of the molecular structure, lipophilicity, solubility, acidity, absorption and polar surface area of coumarinic anticoagulants and direct thrombin inhibitors. *RSC Adv.*, 2014; 4(16): 8072-8084.
12. Rodik R.V., Anthony A.S., Kalchenko V.I., Mely Y., Klymchenko A.S., Cationic amphiphilic calixarenes to compact DNA into small nanoparticles for gene delivery. *New J. Chem.*, 2015; 39(3): 1654-1664.
13. Tiuca I., Saponar A., Bodoki A., Ziémons E., Bodoki E., Hubert P., Oprean R., ATR-IR and Raman spectroscopic study of interaction between major calixarene derivatives and oral anticoagulants. *Farmacia*, 2013; 61(5): 874-882.
14. Vuignier K., Veuthey J.L., Carrupt P.A., Schappler J., Characterization of drug-protein interactions by capillary electrophoresis hyphenated to mass spectrometry. *Electrophoresis*, 2012; 33(22): 3306-3315.