NMR Studies of Ca^{2+} Complexes of Annonaceous Acetogenins

Jean-François Peyrat, Jacqueline Mahuteau, Bruno Figadère,* and André Cavé

Laboratoire de Pharmacognosie, associé au CNRS (BIOCIS), Université Paris-Sud, Faculté de Pharmacie, rue J. B. Clément, 92296 Châtenay-Malabry, France

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^{13}C NMR longitudinal relaxation times (T_1) have been determined for both annonacin (**1**) and squamocin (**2**) in the absence and presence of Ca^{2+} ions. These data are used to assess structural changes that accompany complexations. Even though acetogenins of the Annonaceae present no ionophoric effects in biological studies with living cells, we assume that they have a role in the bioavailability of the cations in the cell membranes, due to both their lipophilic and polar natures. These results also show differences in the stoichiometry of the complexes of a mono-tetrahydrofuranic acetogenin and a bis-tetrahydrofuranic acetogenin with Ca^{2+} ions.

Introduction

Acetogenins of the Annonaceae are potent bioactive natural products isolated from the tropical and subtropical plants belonging to the Annonaceae family.[1] To date, about 250 of these compounds have been isolated and characterized, and most of them have shown interesting biological properties, such as cytotoxic, antitumor, insecticidal, and antiparasitic activities.[1,2] These naturally occuring substances are structurally related and have in common a very long unbranched alkyl chain (32 or 34 carbon atoms), terminated by a γ-lactone (often α,β-unsaturated) and bearing other oxygen functions such as tetrahydrofuran(s), epoxide(s), and hydroxyl(s). Biogenetically, these compounds might be derived from the monoglyceride of lacceroic acid (for compounds with 35 carbon atoms) and from the monoglyceride of ghedoic acid (for compounds with 37 carbon atoms), through several enzymatic processes (e.g., hydroxylation and/or dehydrogenation, oxidation).[3] These molecules have recently been targets for several synthetic teams, and a few total syntheses, as well as hemisyntheses, have appeared in the literature.[3,4] Their mechanisms of action are still not completely understood, even though several hypotheses have been postulated, such as (i) related ionophoric abilities based on observed complexations with cations,[5] (ii) inhibition of cell proliferation through the deactivation of complex I in the respiratory chain in mitochondria,[6,7] and (iii) inhibition of NADH oxidase in the plasma membranes of tumor cells.[8] Although acetogenins of the Annonaceae present no ionophoric effects in biological studies with living cells,[9] in our laboratory we have shown that structurally related analogues of acetogenins present strong selective abilities to complex bivalent cations (such as Ca^{2+}, Ba^{2+}) that depend both on the nature of the studied cation and the stereochemical relationship of the stereogenic centers in the molecules.[10] Calcium has many roles in mammals; in addition to its important contribution to bone structure in vertebrates it serves as a messenger in signal transduction and neurotransmission. In this paper, we wish to present some results of NMR studies on the complexation of a mono-tetrahydrofuranic acetogenin, annonacin (**1**) (Figure 1), and a bis-tetrahydrofuranic acetogenin, squamocin (**2**) (Figure 1), with calcium ions in acetone-d_6.

Annonacin (**1**), the most cytotoxic type-A acetogenin extracted from several plant species of the Annonaceae,[1] was also found to exhibit immunosuppressive activity on mixed murine lymphocytes[2] with a $IC_{50} = 3$ nM for this model (compared to cyclosporin with 10 nM for the same model). Squamocin (**2**), also isolated from several plants,[1] is one of the most cytotoxic acetogenins belonging to type B. Both annonacin (**1**) and squamocin (**2**) were tested on particular cancer cell lines that are generally refractory to chemotherapy,[2] such as glioma (Fogarty), prostatic (PC3), ovarian (SKOV3), mammalian (MCF7), and colon (HT 29) carcinomas; see Table 1.

Annonacin (**1**) exhibited promising activity on PC3 and MCF7S cells, whereas squamocin (**2**) displayed similar activity against the five cells lines tested. The most intriguing aspect is that both compounds **1** and **2** are just as active on parental sensitive cell lines as on their corresponding resistant lines, particularly those homologues that expressed the multidrug resistance (MDR) phenotype.[2] It seems that acetogenins of the Annonaceae are not recognized by glycoprotein Gp170, overexpressed in these cell lines.

Because such activities may be seen as involving alterations in the transport of calcium ions, the purpose of this study was to determine by ^1H and ^{13}C NMR the complexation ability of **1** and **2** with Ca^{2+}, at several concentrations of $Ca(SCN)_2$ in acetone at 25 °C, and to assess the specific binding by different portions of the molecules. Furthermore, this study was expected to shed some light on the conformational changes undergone by these acetogenins in the presence of metal ligation.

[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1997.

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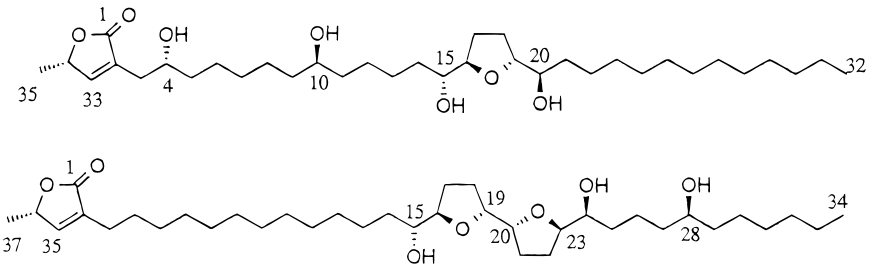

Figure 1. Annonacin (**1**) (top)and squamocin (**2**) (bottom).

Table 1. Cytotoxic Activity on Human Cancer Cell Lines (IC_{50} in μg/mL) (ref 2)

	Fogarty	PC3	MCF7S	SKOV3	HT29
annonacin (**1**)	>10	6.1	4	10	>10
squamocin (**2**)	2.2	2	1.7	1.7	3.9

Experimental Section

Chemicals. Annonacin (**1**) and squamocin (**2**) were isolated in our laboratory from *Annona muricata* and *A. reticulata*, respectively. $Ca(SCN)_2$ was purchased from ICN Ltd. and was freeze-dried and stored over P_2O_5 prior to use. All solvents were spectral grade and purchased from Merck ($CDCl_3$ 99.8%, acetone-d_6 99.8%, D_2O 99.8%). The solvents without purification were added to the unsealed NMR sample tubes, which were cleaned prior use as usual for such studies.

Spectroscopic measurements. Routine 2D NMR experiments (HMQC, HMBC, COSY DQF, HOHAHA) were run under standard conditions at 400 MHz. Stoichiometric amounts of $Ca(SCN)_2$ were used for the relaxation time studies. ^{13}C NMR measurements were performed at 100 MHz on a Bruker ARX 400 spectrometer equipped with a quadrature phase detection system. Longitudinal relaxation time (T_1) measurements were performed at 100 MHz using the inversion recovery technique (at $T = 298 \pm 0.1$ K). A pulse delay time of 16 s and 10 different pulse intervals between 0.005 and 10 s were used for each individual measurement. For each pulse interval 300 scans were used in order to obtain an acceptable signal-to-noise ratio. T_1 values were determined by a linear least-squares, two-parameter fit of the experimental data. The samples (0.1 M acetogenin (= host) and 1 equiv of salt (= guest) in 1 mL of solvent) were prepared in an unsealed degassed NMR tube, which was cleaned by base−acid treatments.

Calculations of Association Constants. The equilibrium constant for a 1:1 host−guest complex between an acetogenin and Ca^{2+} may be given as

$$K_s = [\text{acetogenin}-Ca^{2+}]/[\text{acetogenin}][Ca^{2+}] \qquad (1)$$

with [acetogenin−Ca^{2+}] = concentration of the complex for a given acetogenin concentration, [acetogenin] = concentration of free acetogenin, and [Ca^{2+}] = concentration of free cation. Equation 1 may be solved by introducing the initial concentration:

$$K = [\text{acetogenin}-Ca^{2+}]/([\text{acetogenin}]_0 - [\text{acetogenin}-Ca^{2+}])([Ca^{2+}]_0-[\text{acetogenin}-Ca^{2+}]) \quad (2)$$

with $[\text{acetogenin}]_0$ = initial concentration of acetogenin and $[Ca^{2+}]_0$ = initial cation concentration.

Results and Discussion

Before the complexation studies were carried out, assignment of the resonance peaks of **1** were confirmed by several 2D experiments, both in $CDCl_3$ and acetone-d_6. Then, we verified that the NMR spectrum of the per(trimethylsilyl) ether of annonacin was not affected by addition of $Ca(SCN)_2$. Complexation studies of annonacin (**1**) were performed in acetone-d_6 at room temperature. The ^1H NMR data showed that several resonance peaks of **1** were shifted downfield as the salt $Ca(SCN)_2$ was added (see Figure 2). This indicated that the exchange process between the free compound and the cation complex is rapid on the NMR time scale. Even at much lower temperatures (for instance at −90 °C), the exchange process is too fast to be noticed by direct observation of decreasing resonance peaks and the appearance of new ones. Therefore, stoichiometry of the complex [**1**−Ca^{2+}] could not be determined by simple observation of the NMR signals. Nevertheless, the chemical shift variations for several protons (assignments were confirmed by 2D NMR experiments) provided curves from a plot of induced shifts *versus* equivalents of guest added (Figure 2), which allowed the maximal chemical shift changes ($\Delta\delta_{max}$) and the observed complex stability constants (K_s) to be determined. Indeed, because the observed shifts of the peaks correspond to the average chemical shifts of the free molecule and the complex, it is not possible to know both the concentration of the complex and the concentration of the free molecule. Therefore, for the calculation of the K_s we postulated that at 98% of the $\Delta\delta_{max}$, 98% of the total acetogenin is complexed. Then, the curve indicates the corresponding number of equivalents of Ca^{2+} added and thus consequently the corresponding concentration, which when inserted in eq 2 allows us to determine the observed K_s. For the first series of experiments, we report the observed variations of chemical shifts for characteristic protons as percentages of maximum changes as a function of the molar fraction ([Ca^{2+}]/[**1**]); see Table 2. It is interesting to note that for H-4 and H-10 $\Delta\delta_{max}$ is attained at molar fraction 0.2, whereas for H-34, H-16, -19, H-15, -20, and H-33 the maximal variation is reached at 0.5. The $\Delta\delta_{max}$ values (in ppm) are also interesting to note, since the highest values are obtained for H-16, -19 and H-15, -20 (0.306 and 0.369, respectively) whereas $\Delta\delta_{max}$ is 0.022 for H-4 and 0.011 for H-33 and H-34. We should point out that in a second series of experiments (see Table 3) a slight difference of $\Delta\delta_{max}$ was observed although within 10% of the previous values (e.g., $\Delta\delta_{max}$ H-16, -19 = 0.306 *vs* 0.325 ppm).

The observed K_s values for annonacin (**1**) were obtained following the induced chemical shifts of characteristic protons such as H-16/H-19 and H-34 at 98% $\Delta\delta_{max}$, which correspond to the same number of equivalents of Ca^{2+} added and thus the corresponding concentration, which was inserted in eq 2. The results are summarized in Table 3.

Examination of Table 3 shows that the K_s obtained from either $\Delta\delta$ series (for each resonance signal) are generally in good agreement with each other (within 10%) and indicates that compound **1** is a strong Ca^{2+} ligand.

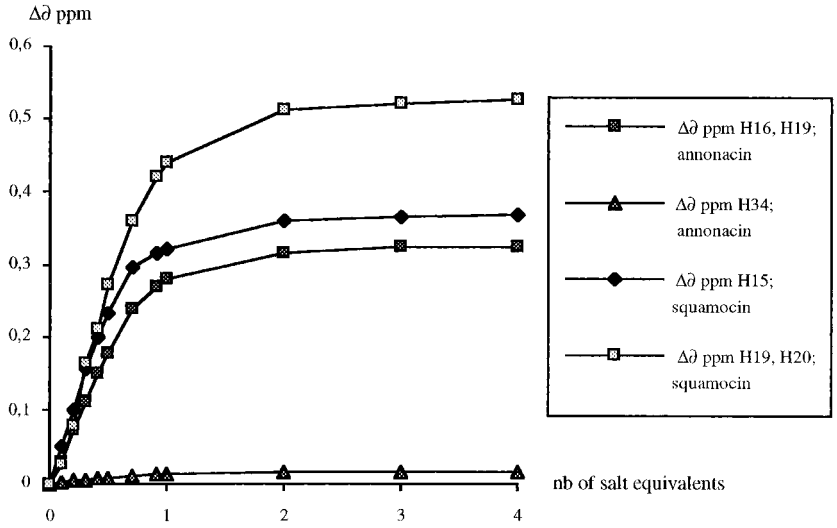

Figure 2. ^{1}H NMR titration curves from a plot of induced shifts of characteristic protons of **1** and **2** *versus* equivalents of guest added.

Table 2. Chemical Shifts for Characteristic Protons and the Percent of Maximum Changes as a Function of the Mole Fraction ([Ca^{2+}]/[1])

[Ca^{2+}]/[**1**]	δ H-34[a] (δ/Δδ$_{max}$, %)	δ H-10[b] (δ/Δδ$_{max}$, %)	δ H-16,19[c] (δ/Δδ$_{max}$, %)	δ H-4[d] (δ/Δδ$_{max}$, %)	δ H-15,20[e] (δ/Δδ$_{max}$, %)	δ H-33[f] (δ/Δδ$_{max}$, %)
0	5.039 (0)	3.505 (0)	3.764 (0)	3.764 (0)	3.352 (0)	7.366 (0)
0.1	5.042 (27)	3.506 (0)	3.802 (12)	nd	3.417 (18)	7.367 (9)
0.2	5.046 (64)	3.516 (**100**)	3.994 (75)	3.786 (**100**)	3.620 (73)	7.371 (45)
0.4	5.047 (73)	3.516 (100)	4.023 (85)	3.786 (100)	3.655 (82)	7.374 (73)
0.5	5.050 (**100**)	3.516 (100)	4.070 (**100**)	3.786 (100)	3.721 (**100**)	7.377 (**100**)
0.7	5.050 (100)	3.516 (100)	4.070 (100)	3.786 (100)	3.721 (100)	7.377 (100)
0.9	5.050 (100)	3.516 (100)	4.070 (100)	3.786 (100)	3.721 (100)	7.377 (100)

[a] $\Delta\delta_{max} = 0.011$. [b] $\Delta\delta_{max} = 0.011$. [c] $\Delta\delta_{max} = 0.306$. [d] $\Delta\delta_{max} = 0.022$. [e] $\Delta\delta_{max} = 0.369$. [f] $\Delta\delta_{max} = 0.011$.

Table 3. ^{1}H NMR Complexation Data of 1 and 2 with $Ca(SCN)_2$ at 400 MHz in Acetone-d_6 at 298 K[a,b]

	annonacin (**1**)		squamocin (**2**)
$\Delta\delta_{max}$ H-16, H-19	0.325	$\Delta\delta_{max}$ H-15	0.376
K_s	3996	K_s	2245
$\Delta\delta_{max}$ H-34	0.016	$\Delta\delta_{max}$ H-19, H-20	0.265
K_s	4175	K_s	2335

[a] Δδ are expressed in ppm. [b] K_s are expressed in M^{-1}.

A similar complexation study was then performed with **2** in acetone-d_6 at room temperature. Once again, the stoichiometry of the complex could not be determined by simple observation of the NMR spectra since several resonance peaks of **2** were shifted downfield as the Ca-$(SCN)_2$ salt was added (see Figure 2). However, by direct observation of the curves from a plot of induced chemical shifts *versus* equivalents of guest added (following the characteristic induced chemical shifts of protons H-19, -20 and H-15), we could determine both the maximal chemical shift changes ($\Delta\delta_{max}$) and the observed complex stability constants (K_s) at 98% $\Delta\delta_{max}$, as above (see Figure 2). Here again, observed K_s obtained from either set of Δδ are generally in good agreement with each other (within 10%).

These results are in excellent agreement with those obtained by Sasaki *et al.* for two other annonaceous aceogenins, squamocin G and squamocin H, where K_s with Ca^{2+} were determined by different techniques as 3100 and 5500 M^{-1}, respectively.[5] Furthermore, both results are comparable to those observed for lasalocid A, one of the simplest polyether antibiotics (K_s for Ca^{2+}: 3700 M^{-1}).[11]

In order to better determine the site of complexation of Ca^{2+} in annonacin (**1**) and squamocin (**2**), we studied the ^{13}C NMR longitudinal relaxation times (T_1) of both [**1**−Ca^{2+}] and [**2**−Ca^{2+}] complexes separately. Indeed, this technique allows a convenient means for determining specific binding interactions in different portions of a ligand molecule, and in turn, good information on spatial structural changes in molecular complexes.[12] The T_1 measurements (repeated twice) were performed at 100 MHz in acetone-d_6 and $CDCl_3$ for the free molecule **1** (see Table 4) and in acetone-d_6 for the free molecule **2** (see Table 5). Assignments were again confirmed by 2D experiments (HMBC, HMQC, HOHAHA, COSY DQF, ...). Then the same measurements of T_1 for the 1:1 mixtures of annonacin **1** and $Ca(SCN)_2$ were performed under the same conditions (temperature, concentration, and solvent, see Table 4). The differences between the T_1 values in acetone-d_6 before and after adding the guest ion are expressed as the percentage by which the relaxation times dropped (sign (−)) or increased (sign (+)). For the free molecules, the T_1 relaxation times depend upon molecular mobility and specific motion due to the internal degrees of freedom of the molecules.[12] It is impossible to distinguish these two aspects of T_1, but by comparing the values for **1** observed in $CDCl_3$ and in acetone-d_6 (where most of the T_1 values are higher than in $CDCl_3$) we can deduce that the mobilities in $CDCl_3$ are less than

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Table 4. ^{13}C NMR Relaxation Times for Annonacin (1) and Its [1−Ca^{2+}] Complex

carbon	**1** ($CDCl_3$) δ (ppm)	**1** ($CDCl_3$) T_1 (s)	**1** (acetone) δ (ppm)	**1** (acetone) T_1 (s)	[**1**−Ca^{2+}] (acetone) δ (ppm)	[**1**−Ca^{2+}] (acetone) variation T_1 (%)
1	174.6		174.50			
2	130.79	3.702	131.35			
3	33.01	0.347	34.02	0.706	33.92	−6
4	69.59	0.676	69.72	1.105	69.89	−32
5			38.08	0.640	38.00	−13
6−8						
9	*c*		38.38[a]	0.418	38.10	−27
10	71.43	0.521	71.38	0.693	71.48	−35
11	*c*		38.48[a]	0.515	38.34	−27
12						
13	25.28*b*	0.398	26.44	0.499	26.42	−29
14	*c*		34.02	0.706	33.92	−6
15	73.89	0.447	74.43	0.380	76.52	−56
16	82.40	0.483	83.50	0.409	83.16	−53
17−18			29.26−30.57			
19	82.51	0.512	83.50	0.409	83.16	−53
20	74.01	0.486	74.43	0.380	76.52	−56
21	*c*		34.02	0.706	33.92	−6
22	25.35[b]	0.381	26.57	0.499		
23−29			29.26−30.57			
30	31.70[b]	2.663	32.59	3.78	32.58	*+7*
31	22.47	3.579	23.28	5.07	23.27	*+15*
32	13.91	3.931	14.33	5.67	14.33	*+31*
33	151.84	0.836	151.80			
34	77.88	0.958	78.26	2.16	78.45	−19
35	18.84	0.846	19.30	0.920	19.30	−29

[a] May be interchanged. [b] Postulated. [c] Between 35 and 40 ppm.

Table 5. ^{13}C NMR Relaxation Times for Squamocin (2) and Its [2−Ca^{2+}] Complex

carbon	**2** ($CDCl_3$) δ (ppm)	**2** (acetone) δ (ppm)	**2** (acetone) T_1 (s)	[**2**−Ca^{2+}] (acetone) δ (ppm)	[**2**−Ca^{2+}] (acetone) variation of T_1 (%)
1	173.9	173.00		173.00	
2	134.25	133.95		133.95	
3	25.14	25.75	1.708	25.73	−20
4−13	29−30	25−30		27−33	
14	33.33	34.47[c]	0.487	33.40	−58
15	74.08	74.31	0.717	76.56	−52
16	83.25	83.87	0.709	83.84	−51
17−18	28.32−28.86	25−30		27−33	
19	82.20	82.77[b]	0.760	83.59	−55
20	82.47	82.63[b]	0.709	83.31	−42
21−22	28.86−24.83	25−30		27−33	
23	82.76	83.93	0.678	82.98	−49
24	71.37	73.08	0.692	72.77	−54
25	32.50	34.15[c]	0.604	33.40	−58
26	21.99	22.88	0.626	22.94	−55
27	37.30	38.55[a]	0.780	38.47	−39
28	71.74	71.43	1.197	71.70	−46
29	37.46	38.51[a]	0.862	38.28	−42
30−31	25.62−29.57	25−30		27−33	
32	31.81	32.69	2.619	32.67	−28
33	22.59	23.32	3.838	23.31	−24
34	14.05	14.39		14.41	−11
35	148.82	150.78	3.639	151.03	−30
36	77.37	78.02	4.197	78.18	−29
37	19.15	19.47	2.124	19.48	−27

[a] −*c*Values with the same superscript may be interchanged.

in acetone because of the higher viscosity of chloroform (at 25 °C: $\eta_{chloroform} = 0.542$ cp, $\eta_{acetone} = 0.316$ cp).

For the molecular complex [**1**−Ca^{2+}], the relaxation times for all signals in the ^{13}C NMR spectrum were measured under the same conditions as above, but comparisons were only made with specific signals. As expected, complexation occurs around the hydroxyls and the THF moiety of the molecule, and indeed, the corresponding T_1 of the *CH*OR dropped significantly (55%). More interestingly, the γ-lactone appears to be involved in the binding, since diminished T_1 values were observed for C-34 and C-35 of annonacin (**1**) (20% and 30% less, respectively).[13] In contrast, the increase of the longitudinal relaxation times for the alkyl chain carbon atoms (C-30−C-32) of annonacin (**1**) (7%, 15%, 31%, respectively) is in accord with the non-involvement of such carbon atoms in the binding[12] with Ca^{2+} and, therefore, with an increasing degree of freedom of this part of the molecule. From these data, it is reasonable to assume that in acetone-d_6 the stoichiometry of the [**1**−Ca^{2+}] complex must be 1:1. It is relevant to point out that, from the three-dimensional coordinates of protein crystal structures available in the Protein Databank,[14] calcium-binding sites consist of six to eight metal-bound oxygen atoms. Since annonacin (**1**) contains seven oxygens, we

(13) Compounds with amide functions have been shown to complex bivalent ions such as calcium; see, for instance: Raban, M.; Burch, D. L.; Hortelano, E. R.; Durocher, D. *J. Org. Chem.* **1994**, *59*, 1283−1287.

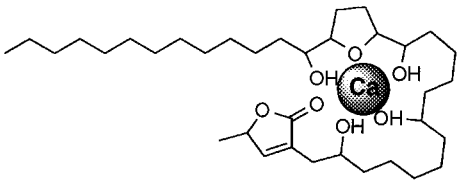

Figure 3. Schematic view of [**1**−Ca^{2+}].

can tentatively suggest a schematic view of the [**1**−Ca^{2+}] complex in order to rationalize these NMR data (Figure 3). It is also noteworthy that, despite considerable efforts, we could never obtain any suitable crystal of this [**1**−Ca^{2+}] complex for X ray studies in order to confirm this structure in the solid state.

T_1 measurements (repeated twice) for the free molecule **2** were performed at 100 MHz in acetone-d_6 (see Table 5) and assignments assured by 2D experiments (HMBC, HMQC, HOHAHA, COSY DQF, ...). Then, similar measurements of T_1 for the 1:1 mixture of squamocin (**2**) and $Ca(SCN)_2$ were performed under the same conditions (temperature, concentration and solvent).

In the case of the [**2**−Ca^{2+}] complex, the relaxation times for all signals in the ^{13}C NMR spectrum were again measured, and specific signals (such as the *CH*OR across the THF skeleton) were observed and compared with those of the [**1**−Ca^{2+}] complex. Unexpectedly, all T_1 values for [**2**−Ca^{2+}] were about twice as large as those measured for [**1**−Ca^{2+}]. Even though the increase of mass from **1** to **2** is about 5%, the observed differences in the T_1 are much greater than 5%, in accord with a significant increase of the mass of [**2**−Ca^{2+}] complex compared to [**1**−Ca^{2+}], suggesting that the stoichiometries of both complexes are different. Furthermore, we also observed a drop in the T_1 for the *CH*OR across the THF skeleton (50−60%) as well as for the C-35−C-37 carbon atoms of the γ-lactone ring (around 30%). For the terminal carbon atoms of the alkyl chain (C-32−C-34), in this case, we also observed a drop in the T_1 (28%, 24%, 11%, respectively), indicating that this part of the molecule is now oriented, possibly because of hydrophobic interactions between two molecules of **2**. These data agree with a 2:1 stoichiometry for such a complex[15] (which now has been confirmed by electrospray-ionization mass spectrometry). Figure 4 suggests a schematic view of this [**2**−Ca^{2+}] complex. Even though a single molecule of squamocin (**2**) bears seven oxygen atoms, all donors are probably not involved similarly in the complexation, and equilibrium between several complexes is probably involved. Once again, unfortunately, we were unable to obtain any suitable crystal of the [**2**−Ca^{2+}] complex for X-ray studies.

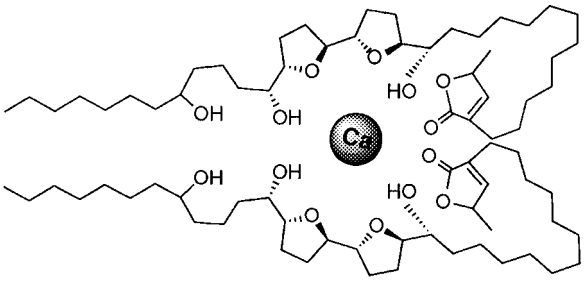

Figure 4. Schematic view of [**2**−Ca^{2+}].

To obtain further information on the closeness of the lactone rings and the THF moieties in both complexes [**1**−Ca^{2+}] and [**2**−Ca^{2+}], ROESY and NOESY experiments were then performed (at different temperatures and in different solvents). Unfortunately, neither NOESY nor ROESY spectra added any useful data regarding the relationships between characteristic protons: e.g., H-35 and H-16 of **1** or **2**, which might indicate that the lactone ring and the tetrahydrofuran parts of the molecule are close to each other. In fact, Ca^{2+} ions might interfere and not allow the direct observation of NOE effects between these specific protons. However, NOE effects have been observed for contiguous methylenic groups.

Conclusion

In conclusion, our NMR results show for the first time that acetogenins of Annonaceae (annonacin (**1**) and squamocin (**2**)), are able to form complexes with Ca^{2+} ions in solution, and furthermore, when they are complexed with Ca^{2+}, they adopt spatial structures different from those of the free molecules. These secondary structures may be seen as the active conformations of the molecules when they interact with their binding sites on the cell membranes. Furthermore, these spatial structures are completely different from the flat plates observed by single-crystal X-ray diffraction on a sample of gigantecin,[16] the only known example of an X-ray structure of a natural acetogenin from the Annonaceae.

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(15) The calculated complexation constant (K_s) for a 1:1 complex of [**2**−Ca^{2+}] should be adjusted for a 2:1 complex, using usual equations, and give a new $K_s = 5 \times 10^6\ M^{-2}$.

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