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Ferutinin as Ca^{2+} complexone: lipid bilayers, conductometry, FT-IR, NMR studies and DFT-B3LYP calculations.

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Calcium ionophoretic properties of ferutinin were re-evaluated in solvent-containing bilayer lipid membranes. Slopes of conductance-concentration curves suggest that in the presence of solvent in the membrane the majority of complexes consist of single terpenoid molecule bound to one Ca ion. By the contrast, stoichiometry of ferutinin-Ca^{2+} complexes in acetone determined using conductometric method was 2:1. While cation-cation selectivity of ferutinin did not change, cation-anion selectivity slightly decreased in solvent containing membranes. FT-IR and NMR data together with DFT calculations at B3LYP/6-31G(d) level of theory indicate that in the absence of Ca ions ferutinin molecules are hydrogen-bonded at the phenol hydroxyl groups. The variations of the absorption assigned to –OH and –C-O stretching mode suggest that ferutinin interacts strongly with Ca ions via hydroxyl group of ferutinol and carboxyl oxygen of the complex ether bond. The coordination through the carbonyl group of ferutinin was demonstrated by theoretical calculations. Taken together, ferutinin molecules form H-bonded dimers, while complexation of Ca^{2+} by ferutinin ruptures this hydrogen bond due to spatial re-orientation of the ferutinin molecules from parallel to antiparallel alignment.

Introduction

Ionophores are organic molecules that facilitate ion transport across hydrophobic barriers, such as artificial and biological membranes. They form lipid-soluble complexes with ions and, depending on the nature and geometry of the coordination site, may present with different ion specificities. Considerable interest to ionophores that selectively transport calcium is maintained by the fact that calcium is largely recognized as a second messenger in cellular activation. In this regard, the antibiotics A23187, ionomycin and X537A that express such selectivity have been widely used in the study of calcium-dependent cellular processes.[1-3]

Recently, calcium ionophoretic properties have been reported for a group of naturally occurring compounds – sesquiterpenoids. It has been shown that complex ethers of sesquiterpene alcohol ferutinol with aromatic substituting groups (ferutinin, tenuferidin and ferutidin) but not with aliphatic groups (lapidin and lapiferin) are capable of electrogenic transfer of divalent cations across artificial bilayer lipid membranes, cell plasma membranes and membranes of mitochondria in dose-dependent manner.[3-6] Steady interest to ferutinin is maintained due to the fact that it has estrogen-like activity[7], it has been used as a molecular tool to induce electrogenic Ca-influx in mitochondria[8], as a substance affecting bone mineralization[9] and as an agent inducing apoptosis in cancer cells[10]. Most of the above physiological effects of ferutinin could be accounted for by the increased membrane permeability to calcium. This increased membrane permeability has been associated with a formation of stoichiometric complexes between cations and sesquiterpenes. Ferutinin exhibited strongest ionophoretic properties and it has been suggested that maximum 2-3 molecules of this sesquiterpene participate in transmembrane diffusion of 1 or 2 calcium ions. Tentative model suggested a complex between one Ca ion and two molecules of ferutinin. In this model, Ca^{2+} interacts electrostatically with the hydroxyl oxygen of ferutinol and the carboxyl and the carbonyl oxygen of n-oxybenzoic acid, forming four strong and two weak electrostatic bonds with two closely placed molecules of ferutinin.[5] However some electrostatic bonds in this model fell outside the limits of expected 2.1-2.8 Å range of coordination distances for calcium ion.

Characteristics of ion transport by ionophores depend on the membrane properties[11], while these properties are significantly affected in the presence of organic solvent.[12,13] Solvent increases hydrophobicity of membrane proper by reducing availability of amphiphilic lipid molecules; solvent significantly increases membrane thickness; the torus with solvent serves as a vibration absorber improving the membrane stability and, thus, membrane may resist stronger solution stirring. Finally, non-polar solvent molecules accumulated in the torus insulate and seal membrane edge area decreasing electrical leakage.[14] All the above membrane effects of solvent significantly modulate electrical signals induced by ion carriers.[11,15]

The goal of the present study was to re-evaluate ionophoretic properties of ferutinin in solvent-containing bilayer lipid membranes. We have also determined exact stoichiometry of ferutinin-Ca complexes using conductometry titration method, and identified structural groups of ferutinin participating in the formation of the complex using infrared and NMR spectroscopy as well as by calculations at B3LYP/6-31G(d) level of theory.

Results and discussion

Planar lipid bilayers

Typical current recordings obtained in the presence of 0.25 mM of $CaCl_2$ and 10 μM of ferutinin added to both sides of bilayers made by Mueller-Rudin technique[16] are shown in the Figure 1A. Steady-state current across modified membranes was achieved only after switching off stirring in bilayer chamber. This contrasts the observations obtained on solvent-free membranes, where membrane conductance reached steady-state only after 10-20 minutes of continuous stirring.[4,5] The explanation of this behavior is increased partitioning of ion carrier into solvent containing membrane during stirring, because there is no equilibrium in its concentration between bulk solution, unstirred layers and the membrane with the Plateau

border.[11] Moreover, such effect is expected to be more apparent for small area membranes.

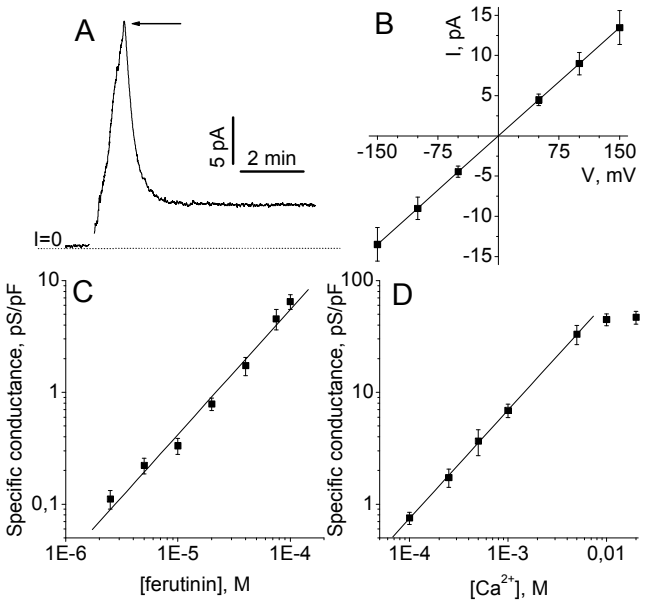

FIGURE 1. Steady-state currents across lipid bilayer membranes modified by ferutinin. A. Representativerecording for a membrane bathed by 10 μM of ferutinin, 250 μM $CaCl_2$, 10 mM TRIS, pH 7.4. Applied voltage was + 50 mV. Dot line represents zero current level. Arrow indicates switching off the stirring. B. Current-to-voltage curve for a membrane modified by 10 μM of ferutinin. Data are Mean±SE, n=5. Line represents linear regression to the data. C and D – dependence of ion conductance of modified membranes on the concentration of ferutinin and calcium ion, respectively. C. Concentration of $CaCl_2$ was 250 μM. Line represents linear regression fit to the data. D. Concentration of ferutinin was 40 μM. Line represents linear regression fit to the data.

The I/V relationship was linear in the range of ±150 mV (Fig 1B). This observation suggests that the presence of solvent in the membrane does not modify complex formation of ferutinin with calcium at the membrane-water interface compared to solvent-free membranes and that the diffusion of this complex through the hydrophobic barrier remains the limiting step in the ion translocation.

Figures 1C and 1D show dependences of membrane conductance on the concentration of terpenoid or Ca ions, respectively, in double logarithmic scale. The conductance of modified membranes showed clear saturation effect at high concentrations of $CaCl_2$ (above 10 mM, Fig 2D). The slope of BLM conductance plotted against concentration of terpenoid or conducted ion reflects the number of components (terpenoid molecules or calcium ions, respectively) in the conducting complex. The calculated values of the slopes were 1.12±0.06 (n=5) in case of ferutinin-dependence of BLM conductance. This number exceeds 1 and suggests that more than one terpenoid molecule may participate in the complex. However, under these experimental conditions it might seem that the majority of complexes consist of single terpenoid molecule. Corresponding slope value for Ca ion dependence of BLM conductance for ferutinin-modified membranes was 0.97±0.01 (n=5). This value indicates that only one Ca ion is bound by the terpenoid.

Other transport properties of ferutinin were studied using ion selectivity measurements. First, anion-cation selectivity was evaluated in the presence 3-fold gradient of $CaCl_2$. Under conditions when *cis* compartment contained 5 mM and *trans* compartment contained 15 mM $CaCl_2$ solution, the estimated zero current potentials was 9.4±0.7 mV (n=5). This value is close to Nernst equilibrium potential for calcium ions for this ionic gradient (12.6 mV) and indicates substantial cation selectivity of ferutinin. Next, selectivity for different physiological cations was determined. The values of zero-current potentials obtained when *trans* compartment of the bilayer chamber was washed with 10 mM $CaCl_2$ and *cis* compartment contained either 10 mM $MgCl_2$ or 20 mM of salts of NaCl or KCl were 3.0±0.4 mV, 12.6±0.9 mV and 10.7±1.2 mV, respectively. According to these data and calculation using Goldman equation and Eq 2, relative permeability sequence of ferutinin-modified membranes remains $P_{Ca}(1)>P_{Mg}(0.79)>P_K(0.52)\sim P_{Na}(0.46)$.

Conductometric study

In order to reveal the precise stoichiometry of permeating complexes, we undertook conductivity measurements of Ca ion solutions in acetone in the presence of ferutinin. The representative titration curve is shown in the Fig. 2. The change in the slope of the curve occurs at a molecular ratio of 2 ferutinin / 1 Ca ion and this suggests that ferutinin forms complexes with Ca ions with a general formula L_2Ca^{2+}. The logarithm of ferutinin-Ca complex stability constant was 3.56±0.17 (n=4). This value is comparable with LgK=3.81 that was reported for well-known Ca-ionophore A23187.[17] However, it should be noted that current data were obtained with different method.

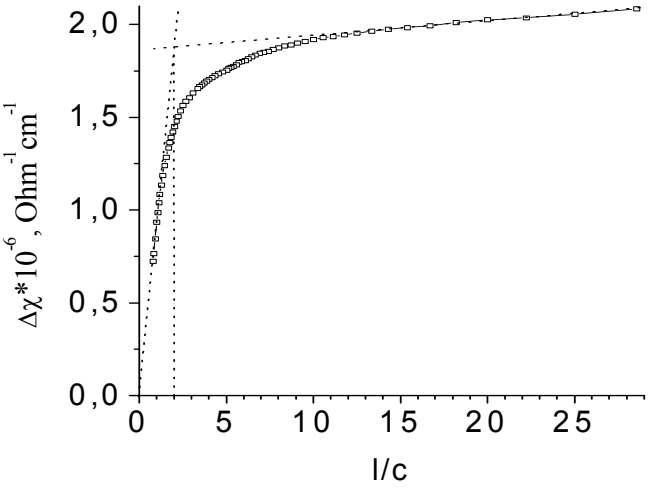

FIGURE 2.Conductometry titration curve of ferutinin solution in acetone by $CaCl_2$ in acetone. Representative of 4 experiments curve is shown. Change in solution conductivity (Δχ) is plotted against the molecular ratio Ferutinin/Ca^{2+}, l/c. Dot lines represent linear approximations on the two slopes of the titration curve.

Spectroscopic analysis of the ferutinin and the ferutinin/Ca^{2+} complex

Figure 3 shows the FT-IR spectra of ferutinin and the ferutinin Ca^{2+} complex taken as KBr pellets. Since ferutinin is an ester of ferutinol alcohol with 4-hydroxybenzoic acid, the characteristic ν_{O-H}, ν_{C-O} and $\nu_{C=O}$ absorption bands are expected in the FT-IR spectrum of ferutinin. The strong and broad band was observed in the 3500-3200 cm^{-1} region with a maximum at 3266 cm^{-1}. This band is associated with the stretching vibration of the O-H group. It is known that phenols have more acidic properties than alcohols due to higher polarization of phenol hydroxyl group. Therefore, the band associated with the stretching vibration of the phenol hydroxyl is

more intense than that of alcohol hydroxyl. Consequently, it is reasonable to accept that in the IR spectrum of ferutinin the band centered at 3266 cm^{-1} is dominated by the contribution of phenol hydroxyl group. The broadening of the ν_{O-H} is due to intermolecular interaction, which results in weakening of the O-H bond[18]. This indicates that ferutinin molecules are bound by hydrogen bonds. In addition there is also a weak band at 3592 cm^{-1} which arises from the free OH stretching vibrations. Alcohols and phenols can also be characterized by C-O stretching vibrations that usually appear between 1300 and 1050 cm^{-1}. Spectrum of ferutinin reveals strong bands at 1164, 1115 and 1100 cm^{-1} due to C-O stretching (ν_{C-O}). In the carbonyl stretching region, the intense and broad band at 1698 cm^{-1} was observed. This band revealed an irregular shape, which is due to some overlapping bands, most probably that of benzoic group which contributes at 1685 cm^{-1} and 1680 cm^{-1} provided same plane positioning. It is likely that carbonyl group is involved in intermolecular interactions with OH groups. Furthermore, the spectrum shows an intense band at 1274 cm^{-1}. It was assigned to the –CO-C-O stretching of the aromatic ester moiety.

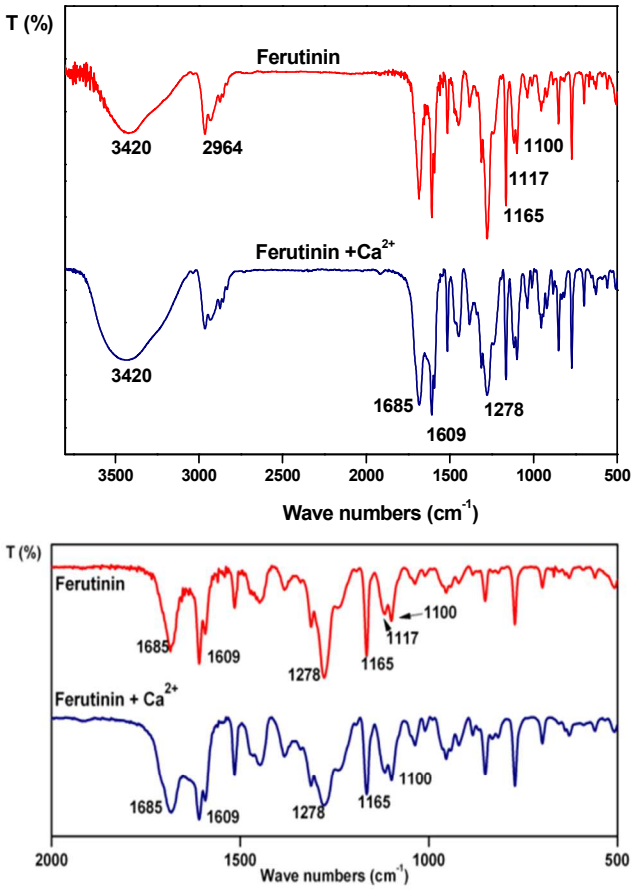

FIGURE 3. Infrared spectra of ferutinin and Ca^{2+}ferutinin complex run as KBr pellets. Insert below shows expansion of the spectra between 2000 and 500 cm^{-1}.

It is worth mentioning that the groups that donate proton (O-H) and groups that accept proton (C=O and O-H) are sensitive to an interaction such as a hydrogen bonding or formation of metal complexes. Formation of hydrogen bond between phenol and ester moieties causes typical changes in the free OH and C=O bands such as bordering and shifting. These effects make IR spectroscopy a sensitive tool for detection of intermolecular interactions. We therefore compared IR spectrum of the ferutinin Ca^{2+} complex with that of ferutinin alone. It was observed that the spectrum of Ca^{2+} complex is largely dominated by the contribution of the ligand. For these two spectra relevant similarities between the location of the ν_{O-H}, $\nu_{C=O,}$ and ν_{C-O} bands were observed. However, an increase in intensities of theν_{O-H} and ν_{C-O} bands for Ca^{2+} complex as compared with ferutinin, was noticed. The ratio of the integrated intensities of O-H and C-H stretching bands ($I_{\nu O-H}/I_{\nu C-H}$) was calculated for ferutinin and ferutinin Ca^{2+} complex. These ratios amount to 0.98 and 1.57 for ferutinin and for ferutinin-Ca^{2+} complex, respectively. It means that the intensity of ν_{O-H} band increased in comparison with intensity of ν_{O-H} of the free ligand ferutinin, upon complex formation. This suggests that the hydroxyl groups of ferutinin are involved in the intermolecular interaction with the Ca^{2+} ion. Moreover, the intensity of ν_{C-O} band at 1100 and 1115 cm^{-1} for ferutinin Ca^{2+} complex was higher than the C-O band intensity of the ferutinin. The ratio ν_{C-O} /ν_{C-H} calculated for ferutinin and ferutinin Ca^{2+} complex amounts to 1.27 and 1.49, respectively. It means that intensity of skeletal C-O band was increased upon complex formation. The spectra of ferutinin and Ca^{2+} complex show also an intense $\nu_{C=O}$ absorption at 1698 cm^{-1}. It arises from the stretching vibration of the ester carbonyl group. The C=O absorption in the spectrum of complex moiety showed the same band pattern as C=O absorption of ligand alone. It suggests that carbonyl group is only weakly involved in complex formation between ferutinin and Ca^{2+} ion. On the basis of IR study it seems most likely that divalent Ca^{2+} interacts with both the hydroxyl and carboxyl groups.

To gain insight into participation of the carbonyl groups, we have also performed measurements of IR spectra of ferutinin and Ca^{2+}/ferutinin complex taken in $CHCl_3$ solution. However the two spectra were quite similar and did not show any changes (Fig 4b lower panel).

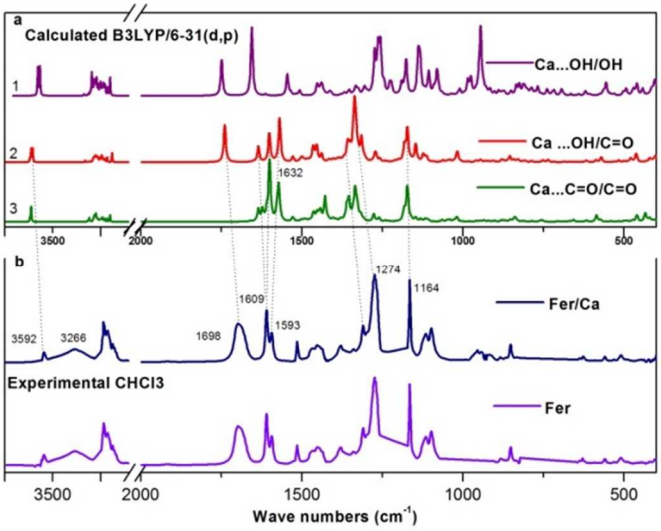

FIGURE 4. (a) Calculated and (b) experimental ($CHCl_3$) IR spectra of ferutinin and ferutinin/Ca complex. Complex **1** (pink line), complex **2** (red line), complex **3** (green line).

We then decided to approach the problem theoretically. First, we hypothesized that ferutinin undergoes a tautomerisation upon complex formation. Such metal ion coordination-induced tautomerisation has been described for several different complexes[19-21]. Then, in the case of enol-keto tautomerisation, Ca^{2+} coordination by ferutinin occurs via ester O-C oxygen and two OH groups.

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Table 1. The energies of the optimized molecular structures and experimental and calculated IR frequencies.

	Energy [Hartree]	$\nu_{C=O}^{ester}$	$\nu_{C=O}^{ketone}$	$\nu_{C=C}$	ν_{C-O}^{ester}	ν_{OH}^{Ph}	$\nu_{OH}^{Alc.}$
		[cm^{-1}]					
Ferutinin 1a (enol)	-1157.27327127	1764(297)		1688(2) 1634 (146)	1252 (423)	3672(49)	3669(9.5)
Ferutinin 1b (keto)	-1157.25132705		1691 (105)	1688(2) 1654 (832) 1618 (746)			3673(25) 3167(1919)
Complex 1 $(FER1a)_2Ca^{2+}$ (-OH /-OH)	-2991.7435516	1836 (216) 1737 (414) free				3657(127)free 3635 (175) as.	3663 (33)free 3653 (64) as.
Complex 2 $(FER1a)_2Ca^{2+}$ (-OH/-C=O)	-2991.8466679	1738(507) free 1602(321)as.		1690 (2)	1319 (216)	3654 (165) free 3639 (172) as.	3672 (27)free
Complex 3 $(FER1a)_2Ca^{2+}$ (-C=O/-C=O)	-2991.8870097	1601(942)as.		1687 (2) 1636 (205)		3657 (117) free	3673(26)free
Experimental ($CHCl_3$) - FER		1609		1698	1274	3592 free 3266 as	
Experimental ($CHCl_3$)-FER/Ca^{2+}		1609		1698	1274	3592 free 3266 as	
Experimental (KBr)-FER		1685		1609	1278	3422	
Experimental (KBr)-FER/Ca^{2+}		1683		1609	1278	3432	
$(FER1a)_2$ h-bonded in acetonitril	-2314.61070775	1725 (449) 1721 (428)		1708 (5)	1261 (289)	3662(170)free 3487(967) as.	3632 (148)free 3631 (153) as
$(FER1a)_2$ H-bonded in water	-2314.61131190	1708 (458) 1720 (444)		1708 (5) 1707 (4)	1260(913) 1257(271)	3661(174) free 3485(980) as	3632 (151) free 3631 (156) as
$(FER1a)_2$ non H-bonded in water	-2314.60556849	1721 (476)		1708 (4.7) 1707 (5)	1258 (824)	3658(129) free 3657(144) as	3631 (162) free 3629 (163) as
$(FER1a)_2Ca^{2+}$ in water	-2992.04183955	1645(362) 639 (322) 1617 (358) 1615(1417)		1703 (2.8) 1702 (2.2)	1298(1024) 1290(829)	3658(147) free 3657(152) as	3649 (174) free 3644 (102) as
$(FER1b)_2Ca^{2+}$ in water	-2991.84357913			1709 (2) 1740 (2)			

In order to supply evidence for preferred geometry, a theoretical calculation was applied. The geometry optimization performed for possible isomers of ferutinin **1a** (enol form) and **1b** (keto-form) being a result of tautomerisation are presented in Table 1. The total energy of **1a** enol form is lower by 13,75 kcal/mol than energy of **1b** keto-form meaning that **1a** enol form is more stable. This difference did not depend on the type of solvent used, while ferutinin had lower energy in polar solvent (see calculations in electronic supplementary information), However, it has been shown that some reactions of metal ions with mixtures containing an excess of the more stable tautomer and a negligible amount of the rare tautomer, can lead to a product containing exclusively the minor tautomer.[22-25] Thus,

calculation of energies of ferutinin tautomers does not exclude possible tautomerisation upon complex formation.

We have carried out the simulation of IR spectra. Calculated spectra of the ferutinin-Ca^{2+} complexes such as complex **1** (interaction between Ca^{2+} and OH/OH groups), complex **2** (interaction between Ca^{2+} and C=O/OH groups) and complex **3** (interaction between Ca^{2+} and C=O/C=O groups) are presented in Figure 4a (upper panel). The vibrational modes are presented in Table 1. It was found that the calculated spectra of complex **2** and **3** in the carbonyl region were bit different from corresponding experimental IR spectra of ferutinin-Ca^{2+} complex. Close inspection of these spectra revealed that the experimental spectrum of ferutinin-Ca^{2+} complex is closest to a superposition of the spectra calculated for complex **2** and **3**. The major difference between calculated and experimental spectrum was observed for complex **1** where the interaction Ca^{2+}-hydroxyl groups were taken into account. It means that Ca^{2+} ion is most likely linked also by carbonyl group. The total energies of complexes (**1-3**) were calculated. These revealed that interaction of calcium ion with two carbonyl groups (complex **3**) is more preferable by 90 kcal/mol than interaction of Ca^{2+} with two hydroxyl groups (complex **1**). Furthermore, the interaction of calcium ion with two carbonyl groups (complex **3**) is more preferable by 25 kcal/mol than interaction of Ca^{2+} with hydroxyl and carbonyl group (complex **2**). One can also note that the interaction of calcium ion through the hydroxyl and carbonyl group (complex **2**) is preferable by 64 kcal/mol than interaction of Ca^{2+} through the hydroxyl groups (complex **1**). These observations support the above suggestion that there is an interaction between ferutinin and Ca^{2+} through the carbonyl groups as well as through alcohol hydroxyl group, while there is apparently no energy possibility for tautomerisation of ferutinin within the complex with calcium. This was confirmed by geometry optimization of hexacoordinated Ca^{2+} ion complexes with two keto- or enol- ferutinin molecules: total energy of enol-form complex is lower by 90,87 kcal/mol (124,40 kcal/mol in water). Theoretical calculations also suggest that two ferutinin molecules form a dimer via hydrogen bond at phenol hydroxyl, since the energy of hydrogen bonded ferutinins is lower by 4.98 kcal/mol (3,60 kcal/mol in water) and this is consistent with our IR spectroscopy observations. Geometry optimization attempts to coordinate calcium ion with only one ferutinin molecule, that would be consistent with apparent stoichiometry observed in bilayer studies, did not return any stable complexes. It should be also noted that no stable geometry was obtained for hexacoordinated Ca^{2+} complex with two parallel ferutinins with or without hydrogen bond at their phenolic hydroxyls as well.

NMR study

The proton and the carbon NMR spectral data as a chemical shifts and difference ($\Delta\delta$) between chemical shifts of ferutinin-Ca(II) complex and pure ferutinin are provided in Table 2. In the ^{13}C spectra the most significant coordination shifts were detected for hydroxybenzoic moiety, which revealed downfield shifts of the resonances attributed to the C-1', C-4'and C-5' ($\Delta\delta$ = 0.093 ppm, 0.087 ppm and 0.182 ppm, respectively). Moreover for the resonance assigned to the C-2' upfield shift ($\Delta\delta$ = -0.058) was observed, very likely due to mesomeric effect. Accordingly, the relevant ^{1}H NMR spectra revealed coordination downfield shifts for the phenolic OH ($\Delta\delta$ = 0.158 ppm) and for the 3'-H ($\Delta\delta$ = 0.024 ppm) resonances, whereas upfield shifts for the resonances 2'-H, 4-OH and 9-H ($\Delta\delta$ = -0.013 ppm, -0.007 ppm and -0.007 ppm, respectively) were observed. It should be noted that the most distinct differences were observed for Ca^{2+} concentration corresponding to the molar ratio Ca^{2+}-ferutinin = 2:1. Other observed coordination shifts were minor range and negligible. These results correlate well with the IR data reported here and suggest participating groups are ester carbonyl (C-1') and ferutinol hydroxyl (4-OH). Large downfield shift for phenolic OH that does not participate in complex formation with Ca^{2+} might be explained by changes in magnetic anisotropy nearby these OH groups. Energy calculations suggest a hydrogen bond between these two groups of two ferutinin molecules. Since the center of benzene rings may act as a strong magnetic shield, we suggest that phenol hydroxyls are shielded when ferutinin forms dimers. In the presence of calcium ions these dimers change spatial orientation from parallel to antiparallel alignment resulting in de-shielding of these hydroxyls reflected in relatively large downfield shift.

In order to support NMR observations we have carried out calculations of SCF GIAO magnetic shielding in DMSO for geometry-optimized ferutinin (Fer1a), its hydrogen-bonded dimer and for its complex with Ca^{2+} and results of these calculations are shown in Table 2. We have first compared experimental chemical shifts for ferutinin in the absence of Ca^{2+} with those obtained for Fer1a and its hydrogen-bonded dimer. Out of 15 atoms explored, 5 atoms showed overall similar shifts, while 7 of them were much closer to the shifts calculated for hydrogen-bonded dimer. These results provide additional support to the hypothesis of ferutinin dimerization in solutions. Next, the calculations support general trends of changes in the chemical shifts. In particular, they confirm negative shift difference for C-2', which can be attributed to mesomeric/resonance effect, as well as positive shift difference for phenolic hydroxyl group induced by de-shielding due to spatial re-orientation of ferutinins in the complex. However in some instances differences between calculated and experimental shift values are substantial. This concerns particularly moieties, which are involved in the complex formation, namely C-4 and C-1'. We suggest that these differences can be accounted for by the DMSO solvation effect. In fact the calculations treat solvent as a homogeneous medium without taking into account weak local intermolecular interactions, which can effectively average charge distribution around Ca cation and complexation site. The lifetime of these interactions is very short on the NMR time scale so the experimental spectrum might represent an average of DMSO/ferutinin/Ca species involved.[26]

Experimental

Reagents

Ferutinin was kindly provided by Dr. Saidkhodzjaev[27] or purchased from Enzo Life Sciences (Antwerpen, Belgium). All other reagents were from Sigma-Aldrich. Acetone was double distilled and its specific conductivity was $\chi<10^{-7}$ $Ohm^{-1}cm^{-1}$.

Planar lipid bilayers

Membranes were formed at room temperature (20-23 °C) using Mueller-Rudin technique[16] by passing a bubble from a pipette tip, prewetted with a membrane-forming solution of azolectin (25 mg/ml in n-octane), over an aperture of 0.2 mm in diameter drilled in a 0.05 mm wall of delrine cup. Currents were measured using a conventional current-to-voltage converter based on an OPA-121 (Burr-Brown, Tucson, AZ , USA) operational amplifier with a 1 Gohm feedback resistor. The current-to-voltage converter was connected to the *trans* compartment (0.8 ml) of bilayer chamber using a Ag-AgCl electrode and 3 M KCl-3% agar bridge. Thus the *trans* side made virtual ground. The *cis* compartment (0.6 ml) was connected to a voltage source. Membrane formation was monitored by increase in capacitive current (calibrated with a known capacitor) to triangle pulses (10 V/s) from a function generator. Membranes with a specific capacitance of 0.48-0.64 μF/cm² and basal conductance of <10 pS were considered satisfactory for experiments.

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Table 2. Calculated and experimental ^{1}H and ^{13}C NMR chemical shifts values (ppm) for the ferutinin and its Ca(II) complex. The chemical shifts were calculated by the B3LYP/6-31(d,p) method.

Atoms	Experimental ^{1}H and ^{13}C NMR data		Calculated NMR SCF GIAO magnetic shielding in DMSO-d6			
	^{1}H NMR	Δδ	FER1a Hartree: -1157.28952736	$(FER1a)_2$ H-bonded Hartree: -2314.61096880	$(FER1a)_2Ca^{2+}$ Hartree: -2992.03813489	Δδ
phenolic OH	10.250	0.158	4.42	7.08	7.61	0.53
3'-H	7.752	- 0.013	7.94; 7.34	7.45; 7.27; 7.08	7.55; 7.45	0.10; 0.18
4'-H	6.836	0.024	6.52; 6.24	6.47; 6.33; 6.16	6.60; 6.39	0,13; 0.06
9-H	5.504	- 0.007	5.41	5.35; 5.32	5.41; 5.14	0.06; -0.18
4-H	5.055	- 0.007	4.83	4.89; 4.49	4.81; 4.74	-0.08; 0.25
	^{13}C NMR					
C-4 C_{alif}-OH	84.61	0.064	77.97	79.54; 79.35	86.41; 84.06	6.86; 4.71
C-8 C=C	133.33	0.053	128.36	123.85; 123.77	122.16; 121.55	-1.69; -2.22
C-9 C=C	124.70	0.058	114.29	114.95; 114.48	115.44; 115.42	0.49; 0.94
C-11 C-CH3	35.94	0.052	30.08	34.81; 35.11	34.98; 34.67	0.17; -0.44
C-12 C-CH3	17.64	0.052	9.51	10.66; 12.01	11.08; 11.91	0.42; -0.10
C-15 CH3	20.20	0.056	18.29	20.53; 20.51	20.75; 20.19	0.22; 0.32
C-1' C=O	164.65	0.093	158.56	151.36; 151.35	164.74; 162.32	13,38; 10.97
C-2' C=C	121.49	-0.058	109.28	111.85; 109.42	109.39; 105.84	-2.46; -3.58
C-4' C=C	115.12	0.087	101.95	101.51; 101.43	103.28; 102.54	1,64; 1.11
C-5' C_{ar}-OH	161.56	0.182	144.38	146.37; 143.21	147.28; 145.91	0.91; 2.7

Currents were monitored on an oscilloscope and/or a computer display. Current records were acquired at the rate of 100 Hz using TL-1 interface and Axotape software (Axon Instruments).

Ferutinin was kept as 10 mM stock solution in ethanol and final concentration of ethanol in the bathing solutions did not exceed 1%. Steady-state currents through the membranes were measured at least at 5 different applied voltages. Slope conductance was estimated by the approximation of the data to Ohm's law expressed as G=I/V, where G is conductance, I is measured steady-state current and V is applied voltage. In order to minimize variations in experimental data, membrane current was normalized by the corresponding membrane capacitance.

Cation-anion selectivity of modified BLMs was measured in the presence of 3-fold concentration gradient of $CaCl_2$ (5 mM/15 mM, *cis/trans*). Zero current potential (V) was taken as the potential that must be applied to the membrane in order to reach zero transmembrane current, equal to that of a symmetrical system with zero mV applied potential. Cation transference number t^+ was calculated using following equation[28]:

$$t^+ = (V/2E_{Ca})+0.5 \qquad \text{eq. 1,}$$

where V is zero current potential and E_{Ca} is the Nernst potential for calcium ions.

The cation-cation selectivity of modified BLMs was evaluated from values of zero current potential in bi-ionic systems. The

Goldman equation was used to estimate the ratio of BLM permeability coefficients for divalent cations. In case of bionic conditions with di- and monovalent cations we applied the Lewis equation[29] as follows:

$$V=(RT/F)ln(4P'_{Ca}[Ca^{2+}]_o/P_X[X^+]_i) \quad \text{eq.2,}$$

where P'_{Ca} is $P_{Ca}/[1+exp(VF/RT)]$; V is the zero current potential; P_{Ca} and P_X are permeability coefficients for calcium and monovalent cations, respectively; and R, T and F have their usual thermodynamic meanings. All zero current potentials were corrected for liquid junction potentials (LJ). The actual potential V is related to experimentally measured value, V_e, as:

$$V=V_e-(E^o_L-E^i_L) \quad \text{eq. 3,}$$

where E^o_L and E^i_L represent the LJ potentials of the *trans* and *cis* electrodes, respectively, in both cases being taken as that of bathing solution with respect to electrode solution. The values of LJ potentials were calculated using the generalized Henderson Liquid Junction Potential Equation[30]. Ion activities were used in all calculations and the molar activity coefficients at given concentration were obtained from Lide.[31]

Solution conductivity measurements

Solution conductivity was measured using conductometer Redelkis OK 102/1 (Budapest, Hungary) at room temperature. The constant of the experimental chamber was determined using standard aqueous KCl solutions and it did not depend on the volume in experimental chamber in the range of 20-40 ml. In the course of experiment, terpenoids were dissolved in 20 ml of acetone directly in the chamber and resulting solution (10^{-4} M) was titrated with 10^{-3} M $CaCl_2$ solution in acetone followed by measurements of conductivity. Since titration curves of ferutinin do not show a plateau, the complex stability constant was calculated using following equation[32]:

$$K=\frac{\frac{\Delta\chi}{\delta}\cdot 10^3}{\left(\tilde{n}-\frac{\Delta\chi}{\delta}\cdot 10^3\right)\cdot\left(l-\frac{\Delta\chi}{\delta}\cdot 10^3\right)} \quad \text{eq. 3,}$$

where K is the complex stability constant; $\Delta\chi$ is the change in solution conductivity; c and l are concentrations of calcium and ferutinin, respectively; δ is a coefficient that determines concentration of the complexed ion in solution. The value of δ was calculated from the data obtained at different l/c ratios close to value of 2 by equalization of the right parts of the equation.

Infra-red spectra

The FT-IR spectra were recorded using a Nicolet Magna IR 550 Series II Spectrometer equipped with KBr beam splitter and DTGS detector. A spectral resolution of 2 cm^{-1} was used. Samples were measured as KBr pellets and $CHCl_3$ solution. Briefly, 10 mMoles of ferutinin were dissolved in 1 ml of EtOH and then dried. In case of ferutinin-Ca^{2+} mixture, 5 mMoles of $CaCl_2$ in 5.5 µl of EtOH were added to ferutinin solution and then dried.

NMR

The ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectra were measured on a Bruker Avance II 400 spectrometer in DMSO-d_6 solution at room temperature. Chemical shifts are reported in ppm on δ scale and referenced to the solvent resonances (2.50 ppm for ^1H and 39.50 ppm for ^{13}C).[33]

Theoretical Calculations

The calculations were performed with the Gaussian 09 sets of codes.[34] The B3LYP hybrid density functional was applied which combines Becke's three-parameter nonlocal exchange potential with the nonlocal correlation functional of Lee, Yang and Parr.[35] The Pople style basis set B3LYP/6-31G(d,p) was used. The geometries of samples were fully optimized. On the basis of optimized structures, vibrational frequencies were calculated. The results of optimizations correspond to energy minima since no imaginary frequencies were detected. The computed frequencies were multiplied by the uniform factor 0.98 to obtain a good estimate of the experimental results and to eliminate known systematic errors related to the anharmonicity[36]. The NMR chemical shifts were calculated by the B3LYP/6-31(d,p) method in the GIAO approximation. The NMR chemical shifts (^{13}C or ^1H) were calculated as the differences of isotropic shielding constants with respect to the TMS reference. To account for solvent (DMSO) effects, the standard Polarisable Continuum Model (PCM) was employed.

Conclusions

In the present study, calcium permeability measurements in artificial lipid membranes modified by ferutinin were performed in solvent-containing lipid bilayers, in contrast to solvent-free bilayers used in previous studies[4,5]. We therefore compared ion permeability parameters. The absolute membrane conductance amplitudes for ferutinin were lower than those published earlier for solvent-free membranes.[4] We estimated that in the presence of 40 µM ferutinin and 5 mM Ca in the bathing solution, solvent-containing membranes generated approximately 10,000 nS/cm² of conductance (this study), while it was ~20,000 nS/cm² in solvent-free membranes[4]. However, it should be noted that the actual planar bilayer surface in solvent-containing membranes compared to that of solvent-free membranes is smaller due to the presence of lipid torus. Consequently, this underestimates specific conductance. If, by the contrast, the membrane permeability was normalized by the corresponding membrane capacitance, we obtained closer values of ion permeability: 33.2±6.5 Siemens/Farad and ~42 Siemens/Farad[4] for solvent-containing and solvent-free membranes, respectively. Still, it is clear that both methods of calculation reveal a difference in absolute ion permeability of membranes formed by different techniques in the presence of ferutinin. This difference is consistent with the observation of lower rates of carrier-mediated ion transport in solvent-containing membranes compared to solvent-free membranes.[11]

We also observed small changes in ion selectivity of ferutinin. While cation-cation selectivity of ferutinin did not change, cation-anion selectivity slightly decreased in solvent containing membranes. In solvent-free membranes, ferutinin generated 12.5 mV of potential difference in the presence of three-fold $CaCl_2$ gradient, which indicated ideal cation selectivity.[4] In the present study, ferutinin generated only 9.4±0.7 mV in analogous conditions. Corresponding cation transference number equals 0.87 and indicates that in solvent-containing membranes approximately 13% of ion current is carried by chloride.

The results on the stoichiometry of the complex of ferutinin with calcium ion support previously published studies.[4] While lipid bilayer measurements do not precisely indicate a formation of a complex with exact number of sesquiterpene molecules, they suggest that more than one molecule may participate. Nonetheless, in solvent containing membranes slope of dependence of membrane conductance on ferutinin concentration pointing at apparent number of ferutinin molecules in the complex was 1.12±0.06 (this study), while in solvent-free membranes it was 2.5±0.3.[4] It should be noted, however, that determination of stoichiometry based on bilayer conductance measurements assumes that the concentration of complexes in the membrane is proportional to the bulk concentrations of the ligand and the ion. However, partitioning of

nonpolar molecules to the membrane might not be linear function of the bulk concentration, it is influenced by the membrane properties (presence or not of the solvent, quantity of torus lipids, etc), by the presence of unstirred layers near membrane and thus to a big extend by the stirring of the bathing solutions (see for example Fig1A). Therefore these results can give only a general idea of the binding between ionophore and the ion. There are no indications for concentration dependence of stoichiometry, because our solution conductivity measurements were done with 100 micromolar concentration of ferutinin as in some bilayer studies and it showed undeniably 2:1 (ligand:ion) binding. In addition, our calculations show that 1:1 binding is not possible for several reasons. First, coordination number of calcium ions is 6, thus there should be six coordination bonds in the complex, while there are only four ligand oxygens available in one ferutinin molecule. Binding 2:2 is also not possible for the same reason. Forced DFT-B3LYP energy calculations of 1:1 complexes with four-coordinated calcium ion, which was shown to be possible, returned complexes with broken bonds, thus these complexes are not stable.

Simultaneous analysis of infrared spectra and NMR spectra of ferutinin in addition to theoretical energy calculations suggest a disposition as follows. In the absence of calcium ions, ferutinin forms dimers coordinated by hydrogen bond at phenolic hydroxides. Coordination of Ca^{2+} induces spatial re-orientation of ferutinin molecules from parallel to antiparallel alignment and thus disruption of hydrogen bond at phenolic OH groups. These groups find themselves on the opposite sides of the molecular complex too far away from the coordination site and cannot participate in complex formation with Ca^{2+}. Tautomerisation of ferutinin does not take place and hexa-coordination of Ca^{2+} occurs via two ferutinol hydroxyls, two carbonyls and two carboxyl groups (Figure 5). These six oxygens align to form a prismatic coordination site for calcium ion and the lengths of coordination electrostatic bonds are within the range of 0.236-0.265 nm, which is consistent with the expected range of coordination bonds for calcium ions.[37]

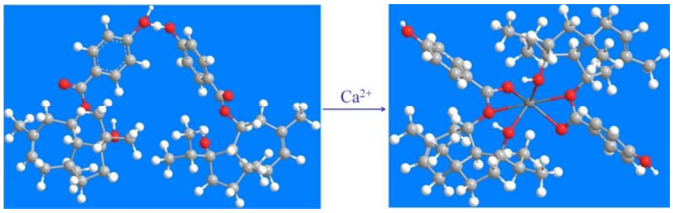

FIGURE 5. Complexation of Ca^{2+} by two initially H-bonded ferutinin molecules.

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Notes and references

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Electronic Supplementary Information (ESI) available: NMR experimental and calculated spectra, optimized molecular structures in 3D and their calculated energies. See DOI: 10.1039/b000000x/

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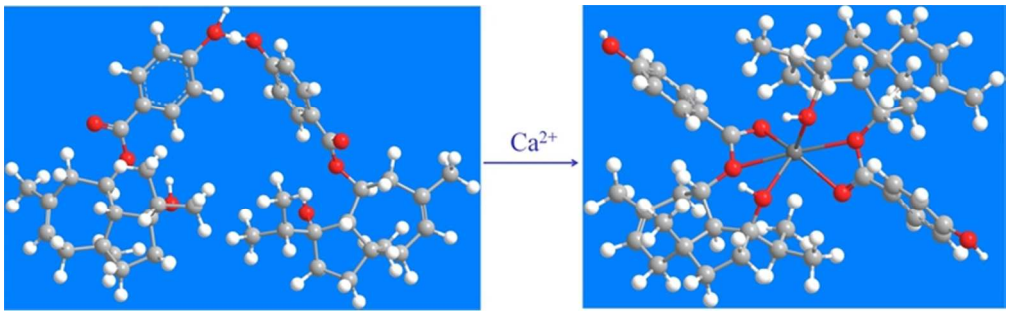

Spatial re-orientation of two initially H-bonded ferutinin molecules upon Ca^{2+} complexation
66x20mm (300 x 300 DPI)