^1H and ^{13}C Nuclear Magnetic Resonance Studies on X-537A (Lasalocid-A)–Calcium Complexes: Observation of a Sandwich Complex

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Studies on the conformational and binding characteristics of the ionophoric antibiotic X-537A (lasalocid-A)–calcium ion complexes have been carried out in deuteriated acetonitrile (CD_3CN) using proton and carbon-13 nuclear magnetic resonance (^1H and ^{13}C n.m.r.) spectroscopy. Detailed analysis of the salt-induced chemical shifts at various X-537A to calcium concentration ratios indicated that X-537A forms charged complexes with calcium with 2:1 and 1:1 stoicheiometries. The conformational model for the complex based on the n.m.r. data showed that the calcium ion is preferentially bound to one end of the molecule, which is binding to three oxygen atoms, the other end (the salicylic acid part) being relatively free. In the 2:1 (sandwich) complex, the calcium ion is sandwiched between two X-537A molecules with three oxygen atoms binding to it from each molecule.

X-537A (lasalocid-A) (Figure 1), first reported by Berger *et al.*,[1] is a carboxylic ionophore and has been employed extensively in the study of biological systems by virtue of its effective interaction with them, basically through its ion-transporting capability. Because of its wide range of biological applications (particularly in the understanding of the role of calcium ion in physiological systems) and of its complexing ability with various cations, this molecule has been the subject of extensive biological and physico-chemical studies.[2-11] However, studies on the conformational aspects of the X-537A–calcium complex have been very limited. Recently, we have shown, using circular dichroism (c.d.), that X-537A forms stable complexes with calcium with different stoicheiometries, depending on the salt concentration.[12] In this paper, we report the studies on ^1H and ^{13}C n.m.r. of X-537A–calcium complexes in acetonitrile. We obtained very different conformations for these complexes as compared with free X-537A. Our results also indicated that X-537A predominantly forms charged complexes with stoicheiometries of 2:1 and 1:1, suggesting that transmembrane calcium transport by this ionophore could be co-mediated by both types of complexes.

Experimental

Salt free X-537A was extracted from the sodium salt of X-537A (Sigma Chemical Co., USA) by the previously described procedure.[12] CD_3CN was obtained from Stohler Isotopes. A calculated amount of vacuum-dried (P_2O_5) calcium perchlorate (from Alpha Inorganics, USA) was added to a CD_3CN solution of X-537A (of known concentration) for the preparation of a stock solution containing the ionophores and Ca^{2+} in a ratio of $R = 1:15$. For titration experiments, solutions of intermediate stoicheiometries were obtained by gradually mixing the stock solution with appropriate quantities of free X-537A solution of exactly equal ionophoric concentrations. Spectra of the samples were recorded at 270 MHz (^1H n.m.r.) and 67.89 MHz (^{13}C n.m.r.) on a Bruker WH-270 FT n.m.r. spectrometer at the Sophisticated Instruments Facility, Bangalore.

Results

The 270 MHz ^1H n.m.r. spectra of free X-537A and its calcium complex in CD_3CN are shown in Figure 2. Using spin-decoupling techniques extensively, and comparing with the assignments reported in other solvents,[10,11,13,14] the signals of free X-537A in CD_3CN were assigned. For assignments in the calcium complex, along with the spin decouplings, changes in the signals of free X-537A on the gradual addition of calcium perchlorate were monitored. The final assignments for the free and the complexed X-537A are shown in Figure 2. The two low-field doublet signals belonging to H(4) and H(5) at *ca.* 7.2 and 6.7 p.p.m. remained virtually unshifted during the salt addition (not shown in the figure). The ^1H n.m.r. chemical shifts for the free and the calcium complex of X-537A are given in Table 1. Coupling constants of most of the coupled protons did not change during complexation.

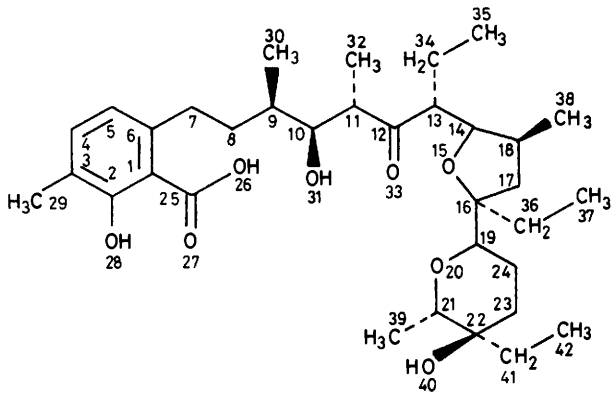

Figure 1. Structure and numbering scheme of X-537A

The ^{13}C chemical shifts for various signals of the free and the calcium complex are listed in Table 2. The signal assignments for free X-537A in CD_3CN were based on the reported assignments for the ionophore in $CDCl_3$.[14,15] Chemical shift changes on salt addition were followed to give the final assignments for the calcium complex.

Discussion

Stoicheiometry of the Complex.—The salt-induced changes in the ^1H and ^{13}C n.m.r. chemical shifts show stabilizing tendencies beyond R *ca.* 1:0.5 (X-537A:Ca^{2+}). A trial Scatchard plot[16] of the $\Delta\delta$ changes with the change in concentration ratio range (up to $R = 1:2.5$) for the H(21) proton and C(12) carbon signals (the signals that showed maximum $\Delta\delta$ changes in ^1H and ^{13}C titration experiments) were non-linear suggesting that the complex is not of a simple $R = 2:1$ stoicheiometry. A four-parameter, K_1, K_2, $\Delta\delta_1$, and $\Delta\delta_2$, computer fit[17] was tried [$XH + Ca^{2+} \rightleftharpoons XH{\cdot}Ca^{2+}$; $2XH + Ca^{2+} \underset{k_2}{\rightleftharpoons} (XH)_2{\cdot}Ca^{2+}$, k_1 and k_2 are dissociation constants; $\Delta\delta_1$ and $\Delta\delta_2$ are limiting $\Delta\delta$ values of the

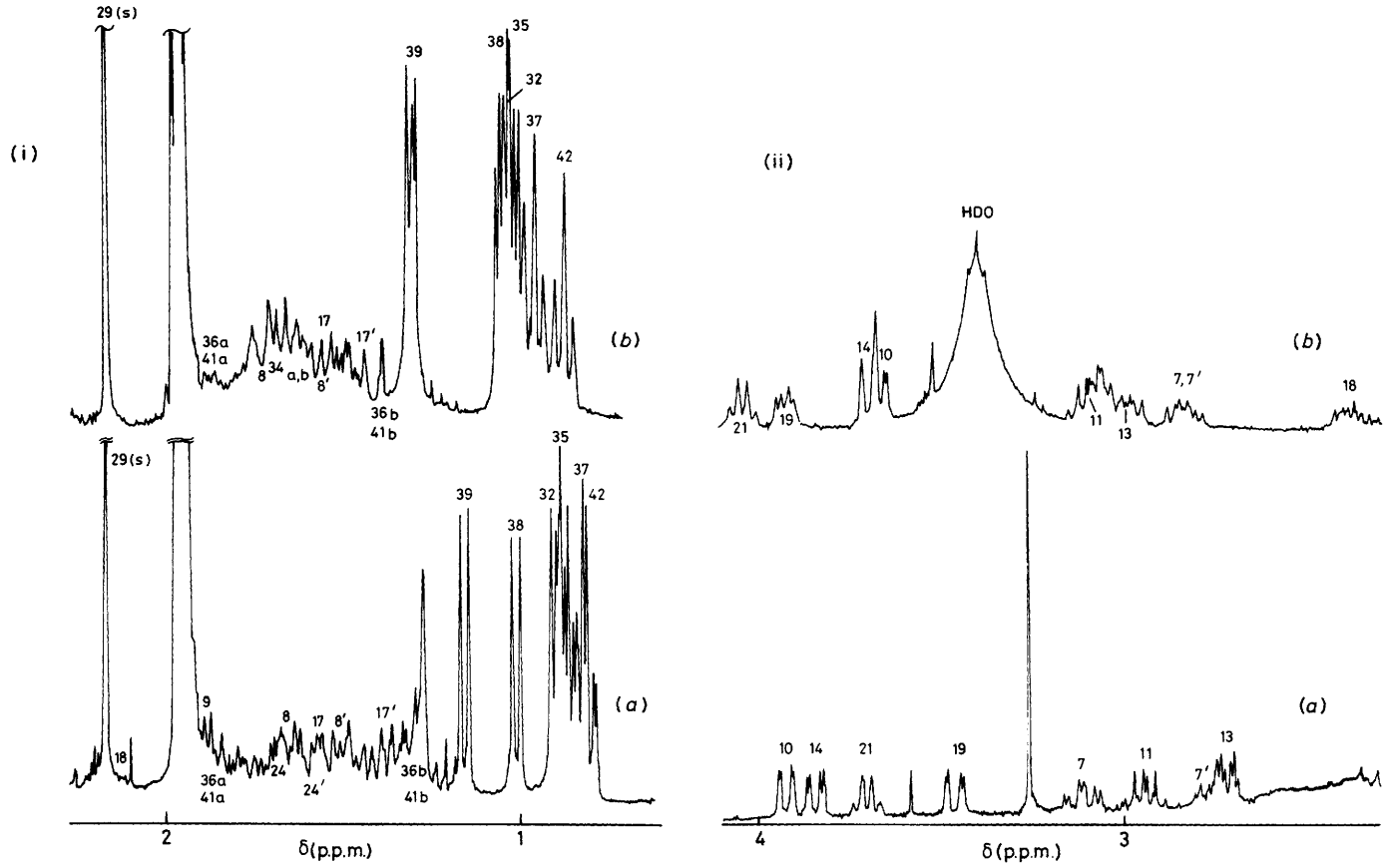

Figure 2. 270 MHz ^{1}H n.m.r. spectra of: (*a*) free X-537A and (*b*) its calcium complex (1:2.5) in CD_3CN at 25 °C; [X-537A] *ca.* 15mM. (i) High-field region and (ii) low-field region

Table 1. ^{1}H N.m.r. chemical shifts (p.p.m.) for free X-537A and its calcium complex in CD_3CN (25 °C). [X-537A] = 15mM; [X-537A]:[Ca^{2+}] = 1:2.5

	H(4)	H(5)	H(10)	H(14)	H(21)	H(19)	H(7a)	H(11)	H(7b)	H(10)	H(29)	H(18)	H(39)	H(38)	H(32)	H(35)	H(37)	H(42)
Free X-537A	7.24	6.73	3.95	3.87	3.73	3.46	3.11	2.94	2.78	2.71	2.16	2.12	1.15	1.00	0.89	0.85	0.78	0.71
Calcium complex	7.26	6.76	3.76	3.80	4.16	4.04	2.78	3.13	2.80	3.08	2.17	2.37	1.27	1.01	1.00	0.93	0.79	0.76

Table 2. ^{13}C N.m.r. chemical shifts (p.p.m.) for free X-537A and its calcium complex in CD_3CN (30 °C). [X-537A] = 53mM; [X-537A]:[Ca^{2+}] = 1:2.5

	C(12)	C(25)	C(2)	C(6)	C(4)	C(3)	C(5)	C(1)	C(16)	C(14)	C(21)	C(19)	C(10)	C(22)	C(11)	C(13)	C(23)
Free X-537A	215.5	174.1	162.0	145.1	136.0	124.7	122.5	112.1	86.8	85.2	77.8	75.0	72.4	72.2	55.9	49.1	39.9
Calcium complex	230.7	173.1	160.7	144.0	135.8	124.5	122.3	111.6	91.3	85.8	78.7	76.6	69.6	76.2	55.3	51.4	36.4

two individual species of complex]. This gave a best-fit for 1:1 and 2:1 coexisting complex species with k_1 = 1.820 mM and k_2 = 0.053 mM; $\Delta\delta_1$ = 0.30 p.p.m. and $\Delta\delta_2$ = 0.39 p.p.m. with $\Delta\delta_{RMS}$ = 0.02 p.p.m. for the H(21) proton signal and almost comparable values for the C(12) carbon signal. These values are in good agreement with the corresponding values reported from c.d. studies.[12] Figure 3 shows the two component curves for the two species (1:1 and 2:1) for the H(21) proton signal. It is clear that the 2:1 complex is preferred at lower salt concentrations, while at higher concentrations the 1:1 complex also forms in comparable amounts.

Conformation of the Complex.—A comparison of the chemical shifts of H(4) and H(5) (sensitive to the state of deprotonation of carboxylate group[9,10]) in $CDCl_3$[9] and methanol[10] with those in CD_3CN (Table 1) suggests that X-537A is more likely to exist as the XH form rather than as the dissociated charged (X^-) species in CD_3CN. In fact, addition of a few drops of DCl (0.1N) to X-537A in CD_3CN had little effect on the chemical shifts of H(4) and H(5). This is consistent with the u.v. and c.d. results.[12] Further, a comparison of chemical shift differences between H(7a) and H(7b) signals, which reflect the flexibility of the salicylic head with respect to the rest of the

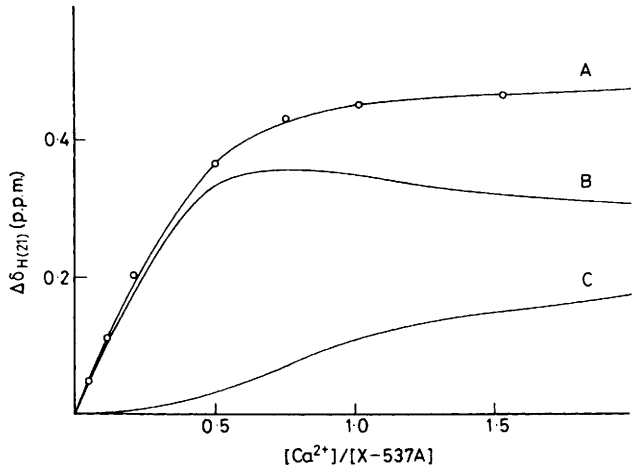

Figure 3. Analysis of the observed ΔδH(21) signal in terms of contributions of component species: A, 4-parameter fit curve and experimental points (○); B, computed contributions from the individual species of the 2:1 complex; and (C) 1:1 computer contributions from the complex

molecule,[10] in $CDCl_3$,[9] methanol,[10] and CD_3CN ($\Delta\delta H_{7a,7b}$ *ca.* 0.93, 0.0, and 0.33 p.p.m., respectively) suggests that the salicylic head part of the molecule is more rigid in CD_3CN than in methanol and more flexible than in $CDCl_3$.

The gradual changes in chemical shift with increasing salt concentration and stabilization beyond the X537A:Ca^{2+} ratio of *ca.* 1:0.5 shows that the chemical shift changes are indeed due to the proximity of the metal rather than steric effects. The large downfield shifts in the proton signals of H(19), H(21), H(13), and H(18) (Δδ *ca.* 0.58, 0.43, 0.37, and 0.25 p.p.m. respectively, Table 1) and in the ^{13}C signals of C(12) (carbonyl), C(16), C(22), and C(19) (Δδ *ca.* 15.2, 4.4, 4.0, and 1.6 p.p.m., respectively, Table 2) for the X-537A–calcium complex compared with free X-537A suggest that the binding site is nearer to the 'tail' portion than to the 'head' (salicylic acid) part of the ionophore. It can be concluded that H(19), H(21), H(13), and H(18) protons are near the binding site and/or directed towards the ion in the calcium complex while H(7a), H(10), and H(14) are either away from the binding site or are directed away from the ion. A large $\Delta\delta H_{7a,7b}$ = 0.93 p.p.m. for X-537A in non-polar solvents was interpreted to be indicative of the rigidity brought about by the 'head carboxy–tail hydroxy' hydrogen bond.[9] On these grounds, the possibility of any 'head to tail' hydrogen bond in the calcium complex in CD_3CN (where $\Delta\delta_{7a,7b}$ is *ca.* 0.33 p.p.m.) can be ruled out and it is reasonable to expect sufficient flexibility in the 'head region' of the molecule. However, with the network of strongly liganding oxygens, *i.e.* O(33), O(15), and O(20) (strong metal–oxygen interaction established by the fact that Δδ are large for the signals of the carbons near to them) the tail portion is expected to be more rigid in comparison with the head portion of the molecule. Further, the fact that practically no changes in the chemical shifts were observed for H(4) and H(5) (sensitive to the deprotonation in the salicylic hydroxy group) and C(4), C(5), and C(1) carbons upon complexation indicates that the salicylic acid part is likely to be away from the site of ion-binding. It also indicates an undissociated form for the ionophore, and hence to a charged complex species for the calcium complex.

Using the Karplus relation[18] some of the dihedral angles were estimated from the available coupling constants for the complex. With the available *X*-ray data,[19] few other dihedral angles were assumed (in the region where changes are expected to be minimal in different metal complexes). Using these

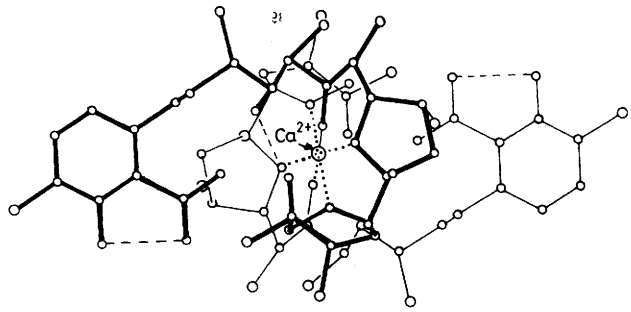

Figure 4. Proposed model for the 2:1 complex of X-537A with Ca^{2+} in CD_3CN

dihedral angles a model for the complex consistent with the observed salt-induced chemical shift changes and information regarding the intramolecular hydrogen bonding will have the Ca^{2+} ion co-ordinated to the three oxygen atoms in the tail part with the salicylic acid part relatively free. The overall conformation and pattern of co-ordination is likely to be the same in the 2:1 complex as in the 1:1 complex, as the titration data showed very little change in the calcium-induced chemical shifts beyond R = 1:0.5 (the data from Figure 2 and Tables 1 and 2 are at an X-537A to Ca^{2+} ratio of 1:2.5, and these data are nearly the same as those observed at a ratio of *ca.* 1:0.5). In the 2:1 complex, the two ionophore molecules are presumably orientated and located identically with respect to Ca^{2+}. The ion is surrounded by a ring of co-ordinating ligands, mainly the oxygens of the carboxy group, the substituted tetrahydrofuran ring, and the substituted tetrahydropyran ring [O(33), O(15), and O(22), respectively] from each of the ionophore molecules. The 2:1 complex as a whole may have two main conformations differing in the orientation of the salicylic heads with respect to each other. In one of these, the two salicylic heads (of the two molecules) are farthest away from each other and in the other the two groups are one above the other, the co-ordination zones of both the molecules overlapping in both the proposed conformations. The choice of a particular conformation between these two is difficult. However, in view of the fact that there are no observable ring current effects on any of the protons, the head over head (or heads nearest) appears to be least reasonable; the relatively more flexible 'salicylic heads' suggest that the conformation from the 2:1 complex is closer to the one with the heads farthest apart. Figure 4 is the suggested model for such a complex.

In conclusion, from the analysis of the n.m.r. data for the calcium complex of the carboxylic ionophore X-537A in the lipophilic solvent, CD_3CN, we have shown the presence of a dimeric charged complex with a conformation in which the ion is bound to one end of the molecule co-ordinating to the carbonyl, tetrahydrofuran, and tetrahydropyran ring oxygens. The transmembrane calcium transport by X-537A is likely to be co-mediated by both equimolar (1:1)- and the sandwich (2:1)-type complexes.

Acknowledgements

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References

1 J. Berger, A. I. Rachlin, W. E. Scott, L. H. Sternbach, and N. W. Goldberg, *J. Am. Chem. Soc.*, 1951, **73**, 5295.

2 A. H. Caswell and B. C. Pressman, *Biochem. Biophys. Res. Commun.*, 1972, **49**, 292.
3 D. H. Haynes and B. C. Pressman, *J. Membr. Biol.*, 1974, **16**, 195.
4 B. C. Pressman, in 'The Role of Membrane in Metabolic Regulation,' eds. M. A. Melmcon and R. W. Hanson, Academic Press, New York, 1972, pp. 149—164.
5 H. Degani and H. L. Friedman, *Biochemistry*, 1974, **13**, 5022.
6 S. R. Alpha and A. H. Brady, *J. Am. Chem. Soc.*, 1973, **95**, 7043.
7 B. C. Pressman, *Fed. Proc. Am. Soc. Exp. Biol.*, 1973, **32**, 1698.
8 M. S. Fernandez, C. H. Celis, and M. Muntal, *Biochim. Biophys. Acta*, 1973, **323**, 600.
9 D. J. Patel and C. Shen, *Proc. Natl. Acad. Sci. USA*, 1976, **73**, 1786.
10 C. Shen and D. J. Patel, *Proc. Natl. Acad. Sci. USA*, 1976, **73**, 4277.
11 S. Chen and C. S. Springer, *J. Bioinorg. Chem.*, 1978, **9**, 101.
12 C. K. Vishwanath and K. R. K. Easwaran, *FEBS Lett.*, 1983, **153**, 320.
13 J. W. Anteunis, *Bioorg. Chem.*, 1974, **5**, 327.
14 J. W. Westley, R. H. Evans, T. Williams, and A. Stampel, *J. Chem. Soc., Chem. Commun.*, 1970, 71.
15 J. W. Westley, D. L. Pruess, and B. G. Pitcher, *Chem. Commun.*, 1972, 161.
16 G. M. Luigi, C. Battazar, P. C. John, C. S. Robert, and C. F. Vuuren, *J. Am. Chem. Soc.*, 1980, **102**, 916.
17 J. Reuben, *J. Am. Chem. Soc.*, 1973, **95**, 3534.
18 M. Karplus, *J. Am. Chem. Soc.*, 1963, **85**, 2870.
19 S. M. Johnson, J. Herrin, S. J. Line, and I. C. Paul, *J. Am. Chem. Soc.*, 1970, **92**, 4428.

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