^{13}C Relaxation Studies of the Structure and Flexibility of the Carboxylic Ionophore Lasalocid A

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Carbon-13 spin lattice relaxation times (T_1) were determined for all the protonated carbon atoms of the carboxylic ionophore lasalocid A in non-hydroxylic ($CDCl_3$) and hydroxylic (CD_3OD) media. In $CDCl_3$, the motions of the protonated backbone carbon atoms C-4 to C-24 (inclusive) are all equally restricted. The correlation time for overall molecular reorientation, τ_{eff}, calculated from an average NT_1 value of 565 ms, is 8.6×10^{-11} s, where N is the number of attached protons. The carbon atoms in the side chains are more mobile than those in the backbone. The carbon backbone in CD_3OD is characterized by segmental motion at the aromatic end, as evidenced by increased NT_1 values for C-4, C-5, C-7, and C-8. The remainder of the backbone appears to be rigid and to have a τ_{eff} virtually identical with that observed for the entire backbone in $CDCl_3$. These results are discussed in terms of the mechanism that has been proposed for ion complexation and transport by carboxylic ionophores in biomembranes.

The spin-lattice relaxation rates, cross-relaxation rates, and nuclear Overhauser effects (n.O.e.s) of the ^{13}C nuclei in natural products and biological macromolecules have been used to make structural assignments, to determine internuclear and hydrogen-bonding distances,[1,2] and to relate structure to mobility.[3–7] This last correlation has been possible because (*a*) the relaxation of the ^{13}C nucleus is generally dominated by dipolar interactions with directly bonded protons,[8,9] and (*b*) the distance between the ^{13}C nucleus and its directly bonded proton(s) is known to be 1.09 Å,[10] and to a first approximation is considered to be independent of conformation.[3] The relationship between the frequency of reorientation of the ^{13}C–^{1}H dipolar relaxation vectors relative to the magnetic field, H_0, and the observed T_1 value permits the calculation of overall tumbling rates for rigid molecules and the detection of intramolecular motion in flexible molecules.[11] Although the foregoing principles must be applied with care, and have required modification in particular cases, they have provided chemists with simple probes of molecular structure and flexibility. As a consequence, the chemical interpretations of biological functions have been forced to involve flexibility as well as structure.

This paper reports a ^{13}C n.m.r. relaxation study of the carboxylic ionophore lasalocid A (**1**)[12] in hydroxylic (CD_3OD) and non-hydroxylic ($CDCl_3$) solvents. Carboxylic ionophores transport ions across biomembranes by an electrically silent exchange-diffusion process.[13] Various cation-inclusion complexes and the free acid of (**1**) have been examined by *X*-ray crystallography,[14] ^{1}H n.m.r.,[15] and c.d.,[16–18] but the structural information derived from these studies does not adequately explain the kinetics and thermodynamics of the complexation–decomplexation and translocation steps[19] involved in the transport of ions from a polar phase across a bilayer containing an apolar interior. One approach to determining the effects that membrane microenvironments of varying polarity have on ionophore conformation is to examine the conformational equilibrium and dynamics of (**1**) in solvents that mimic both the polar environment in which an ion is captured and released, and the apolar environment in which the ionophore must insulate the captured ion. By providing quantitative measurements of the motion of the backbone and side chains of (**1**) in both hydroxylic and non-hydroxylic environments, this study provides a detailed insight into the role structural dynamics play in the membrane transport process and provides the first direct experimental evidence for the existence of mobile hinge bonds[20] in (**1**).

Experimental

A mixture of the sodium and potassium salts of lasalocid A (**1**) was obtained from Sigma (cat. no. L-9127). To produce the free acid, the salt mixture (5 g) was dissolved in methylene dichloride (200 ml) and washed with 10% aqueous HCl (3 × 200 ml) The organic layer was then washed with thrice-distilled, deionized water (2 × 200 ml) and flash-evaporated. The crude acid (**1**) was recrystallized from ethanol and dried *in vacuo* to give a product that melted at 109—110 °C (lit.,[12] 100—109 °C). Flame emission photometry indicated that the sample contained <0.1% of the sodium or potassium salt.

Carbon-13 spectra were taken at 75 MHz with a Varian XL-300 Advance n.m.r. spectrometer. Samples contained 25 mmol of the acid (**1**) in $CDCl_3$, CD_3OD, and $CDCl_3$–CD_3OD mixtures. At this concentration, (**1**) existed as a monomer in both $CDCl_3$ and CD_3OD.[21] All measurements were made at 25 °C.

Carbon-13 assignments in $CDCl_3$ agreed with those previously published by Seto *et al.*[22] with the exception that the C-23 and C-24 shifts were interchanged. The ^{13}C assignments in methanol were correlated with the assignments in $CDCl_3$ by solvent mixture experiments. The amounts of methanol in the chloroform were 25, 50, and 75%. All assignments were made using DEPT (distortionless enhancement by polarization transfer)[23] and two-dimensional ^{13}C–^{1}H chemical-shift correlation spectroscopy[24] (Figure). The DEPT spectra were run using ($\pi/2$, ^{1}H)—($2J$)$^{-1}$—(π, ^{1}H; $\pi/2$, ^{13}C)—($2J$)$^{-1}$—(θ, ^{1}H; π, ^{13}C)—($2J$)$^{-1}$—(BB, ^{1}H; FID, ^{13}C) pulse sequence and θ values of $\pi/4$, $\pi/2$, and $3\pi/4$. A repetition rate of 2.0 s was utilized with 1 000 scans per experiment. An average $^{1}J(^{1}H, ^{13}C)$ value of 130 Hz was assumed in setting $(2J)^{-1}$. The ^{1}H–^{13}C chemical-shift correlation spectra for the high-field portion of the ^{1}H spectrum (above δ 4.5) were obtained using the pulse sequence ($\pi/2$, ^{1}H)—

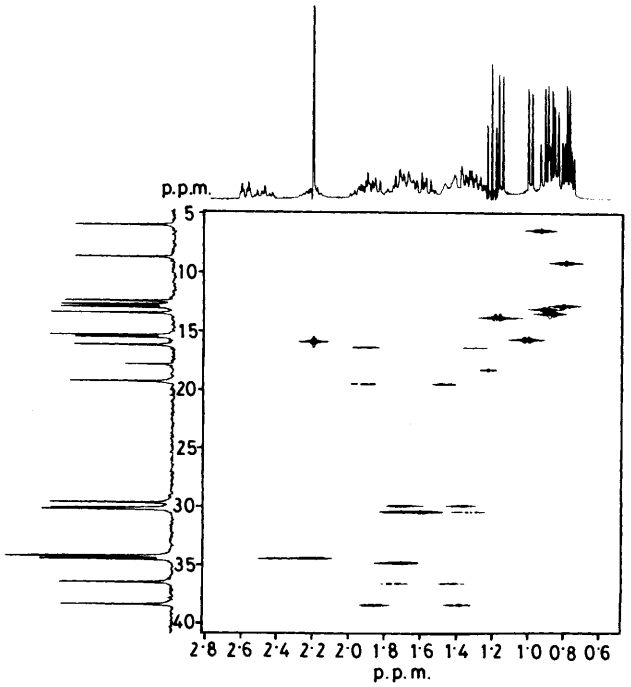
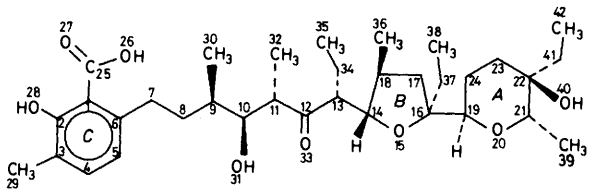


Figure. An absolute value contour plot of the high-field regions of the two-dimensional ^{13}C-^{1}H chemical-shift correlation spectrum of lasalocid A (1) in $CDCl_3$. For reference, the one-dimensional 75 MHz carbon spectrum is plotted along the vertical axis, and the one-dimensional 300 MHz proton spectrum above the horizontal axis. The cross-peaks in the contour plot indicate coherence transfer between a specific carbon atom and its directly bound hydrogen atom *via* the $^{1}J_{CH}$ scalar coupling



(1)

$(\frac{1}{2}t_1)$—$(\pi, {}^{13}C)$—$(\frac{1}{2}t_1)$—(τ_1)—$(\pi/2, {}^{1}H; \pi/2, {}^{13}C)$—$(\tau_2)$—(BB, ^{1}H; FID, ^{13}C, t_2)]. Five hundred and twelve spectra were recorded with 400 scans per spectrum, 2 048 data points in t_2 and a sweep-width of 6 800 Hz in f_1 and 1 267 Hz in f_2. Before Fourier transformation, the time-domain spectra were multiplied by a phase-shifted sine bell function and zero-filled to 1 024 in t_1 and 4 096 in t_2. An average (^{1}H, ^{13}C) value of 130 Hz was used to calculate τ_1 $[(2J)^{-1}]$ and τ_2 $[(3J)^{-1}]$.

Carbon-13 T_1 values for individual resonances were measured from partially relaxed spectra, obtained using a $(\pi$—τ—$\pi/2$—$T)_n$ pulse sequence.[25] The relaxation delay, T, was set to four times the longest T_1. The results were analysed using a two-parameter fit procedure which included an H_1 inhomogeneity factor I.[26] Each T_1 determination included at least ten incremental τ values that ranged from 0.4 to 1.5 T_1. The magnitudes of the ^{13}C-$\{^{1}H\}$ n.O.e.s were determined from the difference in integrated intensity observed for the ^{13}C resonances in ^{1}H noise decoupling and ^{1}H gated decoupling experiments. All samples were degassed by either three freeze–pump–thaw cycles or by bubbling argon gas.

Table. ^{13}C Chemical shift, spin-lattice relaxation, and n.O.e. parameters for all protonated carbon atoms of lasalocid A (1) in $CDCl_3$ and in CD_3OD

	$CDCl_3$				CD_3OD			
C no.	δ^a	$NT_1{}^b$	η^c	% DD^d	δ^a	$NT_1{}^b$	η^c	% DD^d
4	135.0	0.55	1.73	87	135.8	0.71	1.75	88
5	121.3	0.56	1.79	90	122.6	0.70	1.83	92
29	15.6	3.91	1.10	56	15.9	3.89	1.27	64
7	34.3	0.58	1.80	91	35.1	0.69	1.79	90
8	36.8	0.57	1.85	93	38.2	0.68	1.86	93
9	34.6	0.55	1.77	89	35.8	0.53	1.87	94
30	13.4	1.61	1.78	90	12.6	3.20	1.82	92
10	71.9	0.58	1.82	92	76.0	0.57	1.83	92
11	48.8	0.55	1.92	97	49.9	*e*	*e*	*e*
32	12.9	3.24	1.77	89	14.2	2.67	1.93	97
13	55.3	0.57	1.95	98	57.1	0.56	1.78	90
34	16.1	0.63	1.85	93	19.1	0.66	1.77	89
35	12.8	3.23	1.61	81	12.8	3.47	1.65	83
14	83.2	0.55	1.93	97	85.5	0.57	1.72	86
17	38.4	0.53	1.83	92	40.6	0.51	1.86	93
37	30.6	0.66	1.85	93	32.2	0.64	1.77	89
38	9.2	1.99	1.64	83	8.8	3.34	1.90	96
18	34.5	0.56	1.76	89	37.4	0.54	1.75	88
36	15.9	2.40	1.78	90	16.5	2.07	1.67	84
19	70.4	0.58	1.98	100	73.2	0.56	1.84	92
21	75.7	0.59	1.85	92	77.8	0.57	1.91	96
39	13.8	2.10	1.75	88	14.7	2.20	1.82	92
41	30.6	0.55	1.98	100	30.8	0.55	1.71	86
42	6.4	2.87	1.58	79	6.8	3.33	1.67	84
23	19.4	0.56	1.84	93	21.9	0.53	1.85	93
24	30.1	0.56	1.91	96	30.3	0.54	1.85	93

[a] Chemical shifts in p.p.m. relative to Me_4Si. [b] The product of the number of attached protons (N) and the observed T_1 value. Estimated maximum error in T_1 is $\pm 5\%$. [c] ^{13}C-$\{^{1}H\}$ nuclear Overhauser enhancement factor. [d] Percentage contribution of dipolar relaxation to total relaxation mechanism. [e] Accurate calculation of T_1 was not possible owing to overlap with CD_3OD signal.

Results and Discussion

The NT_1 values for all the protonated carbon atoms of lasalocid A (1) in $CDCl_3$ and CD_3OD are given in the Table. Extraction of meaningful motional information from the measured T_1 values requires evaluation of the contributions from individual relaxation mechanisms. The contribution of dipole–dipole relaxation to the total relaxation mechanism of each carbon atom, % DD, is given in the Table for both solvents. All of the protonated carbon atoms, with the exception of C-29, are either completely or predominantly relaxed by ^{13}C-$\{^{1}H\}$ dipolar interactions (*i.e.* $T_1{}^{DD}$). For C-29, significant relaxation occurs by another mechanism, presumably spin rotation. The relationship between observed T_1 values and the effective correlation time for isotropic overall molecular motion, τ_{eff}, when $T_1 = T_1{}^{DD}$ and $\omega_C\tau_C \ll 1$ is given by equation (1),[8,27] where N is the

$$1/T_1^{DD} = N\hbar^2\gamma_C^2\gamma_H^2[r_{CH}^{-6}]\tau_{eff} \qquad (1)$$

number of attached hydrogen atoms, γ_C and γ_H are the magnetogyric ratios of ^{13}C and ^{1}H, respectively, r_{CH} is the ^{13}C–^{1}H internuclear distance (1.09 Å), and $\hbar$ is Planck's constant divided by 2π.

In the non-hydroxylic solvent chloroform, all the protonated carbon atoms on the main backbone of (1), C-1 to C-25 (inclusive), have virtually identical NT_1 values. The range is 530 ± 21 to 595 ± 30 ms with a mean value of 565 ms. The backbone can therefore be considered rigid and rotating isotropically with τ_{eff} [calculated from equation (1)] 8.6 ×

10^{-11} s. Increases in the NT_1 values of C-30, C-32, C-34, C-35, C-36, C-37, C-38, C-39, and C-42 arise from increased motion in these side chains analogous to that seen in the side chains of the cyclic peptide gramicidin S.[28] It should be noted that since dipolar relaxation is dominated by motions of frequencies similar to the ^{13}C Larmor precession frequency, the possibility of extremely fast or extremely slow segmental backbone motion cannot be precluded.[11,28]

Two intramolecular hydrogen bonds, O(26)–HO(31) and O(26)–HO(40), have been shown to exist in non-hydroxylic media[21] and to close lasalocid A (1) into a macrocycle. Like many other carboxylic ionophores, (1) has a high binding affinity and transport capacity for protons.[29] Therefore, the protonated ionophore in non-hydroxylic media is probably best described as a proton-inclusion complex which is structurally analogous to the cation-inclusion complexes formed with alkali metal ions. It is clear from the NT_1 values in chloroform that in non-hydroxylic media this inclusion complex represents a truly rigid structure in which the heteroatoms that act as ligands are locked into a spatial arrangement dictated by the conformation of the carbon backbone. So, although (1) is not a true macrocycle, in non-hydroxylic media it is functionally equivalent to the rigid inclusion complexes characteristic of the covalently closed macrocycles such as the macrotetrolide nactins,[30] crown ethers,[31] and cyclic depsipeptides.[32]

Increases in the NT_1 values observed for C-4, C-5, C-7, and C-8 in methanol indicate that changes in structure and mobility have occurred at the aromatic end of the molecule (Table). Direct comparison of NT_1 values in methanol with those in chloroform is reasonable since the viscosity of methanol at 25 °C is 547 mP and that of chloroform is 542 mP, a difference of less than 1%.[33] The NT_1 values for the remainder of the backbone carbon atoms, C-9 to C-24, are virtually identical, with a mean of 552 ms, suggesting that this portion of the molecule is rigid. The τ_{eff} value calculated from the mean NT_1 is 8.5×10^{-11} s. The similarity of this τ_{eff} value to that calculated for chloroform as solvent indicates that there cannot be a gross change in molecular volume, and that a large increase in the molecular mass of the C-9 to C-24 fragment due to a high degree of solvent association is unlikely.

If intramolecular motion occurs at a rate equal to or greater than that of overall molecular motion, the τ_{eff} value calculated by using equation (1) will be a function of both the correlation times for overall molecular reorientation (τ_{mol}) and internal motion (τ_{int}).[34] When internal motion is absent or extremely slow, as appears to be the case in $CDCl_3$, $\tau_{eff} = \tau_{mol}$; when motion is very fast ($\tau_{int}/\tau_{mol} \ll 1$), $\tau_{eff} = (1/9)\tau_{mol}$.[3,25,34] The mean τ_{eff} value calculated for C-4, C-5, C-7, and C-8 in methanol is 6.8×10^{-11} s. This value is much less than the theoretical limit of 9.6×10^{-12} s expected if internal motion is extremely fast. The attenuated motion may be due to the mass of the 3-methylsalicylate ring at the end of the short chain and/or the anchoring of the C-1 carboxy group into the solvent matrix *via* hydrogen bonding. Motion in the side chains is also attenuated in both solvents, probably as a result of steric crowding. There is, however, a general increase in the NT_1 values of the side chains in going to the hydroxylic solvent.

Conclusions.—Circular dichroism and computer modelling studies have suggested that there are rotations around several hinge bonds, concomitant with the breaking of the intramolecular hydrogen bonds, which facilitate conversion of the rigid cyclic conformation of (1) into an acyclic flexible conformation.[16,35] Bonds that have been postulated to act as hinges are C(6)–C(7), C(7)–C(8), C(8)–C(9), C(11)–C(12), and C(12)–C(13). When the ionophore–cation complex diffuses to the polar membrane interface, electrostatic factors that stabilize the complex in the apolar membrane interior no longer compensate for the unfavourable steric interactions existing in the cyclic conformation of (1), and the ionophore relieves the strain by rotations around the hinge bonds.[16,36] Conversion into the open conformation, which has a diminished capacity for shielding ionic charge, presumably facilitates the quick release of cations at the polar membrane interface.

The ^{13}C relaxation data presented here confirm that a conformational change occurs in (1) with increasing solvent polarity. In chloroform, the carbon backbone is rigid and can be characterized by a single rotational correlation time. In methanol, intramolecular motion occurs at C-4, C-5, C-7, and C-8. This latter motion is consistent with increased rotational freedom around the C(6)–C(7), C(7)–C(8), and C(8)–C(9) hinge bonds. The remainder of the carbon backbone appears to remain rigid. If rotations do occur around the C(11)–C(12) and C(12)–C(13) bonds, as suggested by the molecular modelling studies, they must be discrete (*i.e.* a rotation from one energy minimum to another). The orientations about the C(11)–C(12) and C(12)–C(13) bonds, and the possible existence of motions that do not significantly affect carbon NT_1 values, are being investigated.

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