Nuclear Magnetic Resonance Studies of the Protonation Sequence of Some Oxaaza Macrocyclic Compounds

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The sequences of protonation of a series of oxatriaza and dioxadiaza macrocyclic compounds have been studied by ^1H and ^{13}C n.m.r. spectroscopy. It is shown that Sudmeier and Reilley's quantitative approach to derive sequences of protonation suffers from several limitations when applied to these (and other) polyaza macrocycles due to the lack of suitable models, but the indications directly provided by the corresponding potentiometric and n.m.r. titration curves may complement the information required for this purpose. The results obtained suggest that the 12-membered oxatriaza and dioxadiaza macrocycles adopt different and more compact conformations when the nitrogen atoms are derivatized (*e.g.* with methyl or acetate groups); this results in stronger electrostatic repulsions of consecutive protonated nitrogen atoms of the ring, leading to changeable base strength and to protonation–deprotonation steps that produce 'anomalous' n.m.r. titration curves. In the case of the N-acetate derivatives, the interpretation of the trends is further complicated by the possibility of formation of $CO_2^- \cdots HN^+$ intramolecular hydrogen bonds. The data now available make it possible to predict approximate values for the protonation constants of the various basic centres for the 12-membered polyoxapolyaza macrocycles and, consequently, the corresponding percentages of protonation at different pH values.

N.m.r. spectroscopy is a useful technique to study the protonation sequence of polyfunctional compounds, which is necessary for the correct interpretation of complexation reactions at the microscopic level. It has been used to investigate the protonation behaviour of linear polyaminocarboxylates[1–4] and polyaminopolymethylenephosphonates,[5,6] as well as of N-functionalized macrocyclic compounds, *e.g.* *N*-carboxylate tetra-aza,[7,8] triaza,[9] and diaza[10] macrocycles and *N*-methylenephosphonate tri- and tetra-azamacrocycles.[11]

The most commonly used quantitative approach to derive the protonation sequences is that of Sudmeier and Reilley,[1] which is based on the magnitude of the shifts of covalently bound hydrogen atoms due to protonation of basic centres in their vicinity. An approximate relationship is proposed between the observed shift of the i non-labile proton ($\delta_i^{obs.}$) of a methylenic group and the protonation fraction (f_j) of the various neighbouring basic groups j, the contributions of these different basic sites being additive, equation (1) where δ_i^0 is the intrinsic

$$\delta_i^{obs.} = \delta_i^0 + \sum_{j=1}^{N} C_{ij} f_j \qquad (1)$$

$$\delta_i^{obs.} - \delta_i^0 = \Delta\delta_i$$

shift of the fully deprotonated form of the ligand; the shielding constants, C_{ij}, represent the $\Delta\delta_i$ value of the methylenic proton i caused by full protonation of a given basic site j, N is the number of basic sites and f_j is usually expressed as the percentage of protonation.

On the other hand, if α_j is the number of equivalent sites of protonation j and n the number of moles of acid added per mole of ligand, one has equation (2).

$$n = \sum_{j=1}^{N} \alpha_j f_j \qquad (2)$$

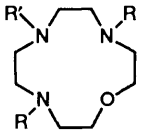
The resolution of simultaneous linear equations (1) and (2) applied to a ligand gives the protonation fraction f_j, provided that the shielding constants are previously known from model compounds. The first, and more frequently difficult, problem to overcome is to find suitable model compounds.

Indeed, the additivity property implied by equation (1) only holds in a polyfunctional molecule if the various substituents maintain constant average orientations throughout the whole pH range.[1] Effects such as long-range shielding of magnetically anisotropic groups, *e.g.* carboxylates,[1] or/and long-range electric field effects of protonated amine groups locked in low-energy conformations,[9] may enhance the non-additivity of the contribution of the different basic centres in equation (1).

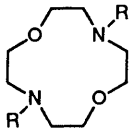
Previous studies of the protonation sequences of several cyclic complexones (cyclic polyaminocarboxylic ligands) have shown that the parent cyclic (simple or methylated) polyamines are not so good model compounds[8,9,11,12] as one might expect. While it is true that conformation effects of the macrocyclic ring of the amine due to repulsion effects (charged protonated nitrogen atoms at a short distance in the macrocycle) or intermolecular hydrogen bonds are likely to be the most important effects, the formation of hydrogen bonds between protonated nitrogen atoms and carboxylates and between protonated carboxylates and non-protonated nitrogen atoms can also alter the protonation shifts experienced by different non-labile protons and may result in conformational changes which affect the signals. This limits the use of amines as models, but in favourable cases the analysis of the titration curves of the cyclic complexone itself may provide other useful indications which allow good estimations of the shielding constants.

Being aware of the limitations of this method, in the present work we extend our previous studies of the protonation sequence of N-acetate polyazamacrocycles to the N-functionalized oxaaza macrocyclic compounds L^1—L^5.

The objective is to determine the protonation sequences of the compounds L^3 and L^4, for which one has the parent amine L^1 and its methylated derivative L^2 as possible models for the determination of the shielding constants, besides the indications given by potentiometric and n.m.r. titration curves of L^3 and L^4 themselves. As commented below, it was found useful to



L^1 R,R' = H
L^2 R,R' = Me
L^3 R = CH_2CO_2H, R' = H
L^4 R,R' = CH_2CO_2H



L^5 R = CH_2CO_2H
L^6 R = H

consider also compound L^5, interesting in its own sake and as a model to derive shielding constants to be used in the study of compounds L^3 and L^4. The parent amine of L^5 (compound L^6) was synthesized as a necessary intermediate.

Experimental

Synthesis and Characterization of the Ligands.—The cyclic amines L^1 and L^6 were synthesized and isolated as the tri- and di-hydrobromide forms, respectively, following previously described procedures.[13] The methylated amine L^2 was obtained by refluxing L^1·3HBr with formic acid and formaldehyde for 24 h.[14] The pure product was obtained in 74% yield by vacuum distillation as an oil, and was characterized by ^1H n.m.r. spectroscopy and potentiometric titration.

The ligand L^5 was synthesized by refluxing the cyclic amine L^6 (0.007 mol) in the dihydrobromide form with ethyl bromoacetate (0.015 mol) and triethylamine (0.029 mol) in absolute ethanol (20 cm^3) for 24 h. The mixture was cooled, filtered, concentrated, and poured into chloroform (20 cm^3). The chloroform extracts were evaporated and the solid residue was hydrolysed in water (20 cm^3) for 18 h. The refluxed mixture was extracted with three portions of chloroform (25 cm^3); the aqueous layer was concentrated to almost dryness. Addition of ethanol gave a white precipitate which was collected by filtration. The dihydrobromide salt was obtained by acidification with 5 mol dm^{-3} HBr and recrystallized from ethanol–water. Yield: 57%. M.p. = 286—288 °C (Found: C, 31.60; N, 6.20; H, 5.10. Calc. for $C_{12}H_{24}Br_2N_2O_6$: C, 31.90; N, 6.20; H, 5.35%).

The ligand L^3 was prepared by condensation of the cyclic amine L^1 (0.006 mol) with monochloroacetate (0.018 mol) in aqueous basic solution. The temperature was kept between 40 and 60 °C and the pH below 8.5. After the reaction the pH was adjusted to 2.0 with 5 mol dm^{-3} hydrochloric acid. The reaction mixture was concentrated and the precipitated inorganic salts were filtered off. The hydrochloride salt of the product was recrystallized from ethanol–water. Yield: 25%. M.p. = 189—192 °C (Found: C, 36.45; H, 6.80; N, 10.25. Calc. for $C_{12}H_{26}Cl_3N_3O_5$: C, 36.15; H, 6.55; N, 10.55%).

The ligand L^4 was prepared by a previously reported procedure.[15]

Equipment.—For the potentiometric measurements a CRISON Digilab 517 measuring instrument was used together with an Ingold U1330 glass electrode and a Radiometer K401 saturated calomel reference electrode described previously.[16] Titrations were carried out in a thermostatted cell at 25.0 ± 0.1 °C and the ionic strength of the solutions was kept at 0.10 mol dm^{-3} with KNO_3 or NMe_4NO_3.

Solutions of the ligands (0.01 mol dm^{-3}) for n.m.r. measurements were made up in D_2O (99.8 atom % deuterium from Merck) and the pD* [operational pD (value measured with a pH meter standardized with conventional aqueous pH 4 and 7 buffers)] was adjusted by adding DCl or CO_2-free KOD or CsOD. The pD* was measured using a pHM64 Digilab Radiometer (or a Crison micropH 2002) instrument fitted with a combined Ingold 405 M3 microelectrode, calibrated with two aqueous standard buffers at pH 4 and 7.

Usually pD = pD* + 0.40 (refs. 17 and 18) and pK_D values determined from the pD in D_2O are related linearly to pK_H values, determined from the pH in water.[19]

Solutions of the free ligands were titrated to the basic form in a n.m.r. tube and in all cases sodium 3-(trimethylsilyl)-propanesulphonate was used as internal reference. The ionic strength was not strictly constant.

Proton n.m.r. spectra were recorded at 100 MHz and the probe temperature in a JEOL JNM 100PTF spectrometer coupled to a JEOL 980A computer (for compounds L^1, L^2, and L^5). For compounds L^3 and L^4 the spectra were recorded at 300 MHz and at the probe temperature in a Bruker CXP 300 spectrometer coupled to an Aspect 2000 computer. Two-dimensional ^1H–^{13}C chemical shift correlation spectra were obtained with the conventional pulse sequence optimized for one-bond correlation, $\Delta_1 = 4$ ms, in a Bruker AM-360 instrument operating at 90.6 MHz for ^{13}C. Each experiment took about 10 h and the ^{13}C spectra are referenced as above.

Calculations.—The protonation constants were obtained from the potentiometric titrations using the Superquad program.[20] The fractions of protonation were obtained from the experimental n.m.r. titration curves by the combination of equation (2) and m equations of type (1) obtained for the m n.m.r. ligand peaks. The set of simultaneous linear equations was solved using a multiple linear regression program.[8]

Table 1. Protonation constants[a] of N-substituted cyclic oxa (or dioxa) tri (or di) azamacrocycles and of the parent compound oxatriazacyclododecane. Values obtained from potentiometric titrations

Ligand	log K_1	log K_2	log K_3
L^{1b}	10.109 ± 0.003	8.525 ± 0.004	1.56 ± 0.04
L^{2b}	10.973 ± 0.004	8.286 ± 0.009	1.67 ± 0.03
$L^{3c,d}$	11.24 ± 0.02	6.02 ± 0.04	2.94 ± 0.05
$L^{4c,e}$	11.61 ± 0.03	7.70 ± 0.05	4.05 ± 0.07
L^{5c}	9.558 ± 0.006	7.47 ± 0.02	2.10 ± 0.02

[a] Defined as stepwise proton association constants: $K_i = [H_iL]/[H_{i-1}L][H^+]$. [b] Values were determined at 25 °C, $I = 0.10$ mol dm^{-3} KNO_3. [c] $I = 0.10$ mol dm^{-3} NMe_4NO_3. [d] log $K_4 = 1.4 \pm 0.1$. [e] From ref. 15. log $K_4 = 2.8 \pm 0.1$, log $K_5 < 2$.

Results and Discussion

Potentiometric Determinations.—Table 1 summarizes the protonation constants of the ligands studied, calculated from potentiometric titrations, together with the corresponding values for some of these ligands reported previously.[15] The data show that the two amines L^1 and L^2 have one weakly basic nitrogen atom, corresponding to the third protonation step (log $K_3 = 1.56$ and 1.67, respectively), as with other triaza and tetra-aza macrocyclic compounds.[7–9] The same can be observed for the two polyaminocarboxylic acids L^3 and L^4, but for the last two compounds one cannot say, based only on the macroscopic constants, which centre is being protonated for $n > 2$: the carboxylate groups, the last nitrogen atom, or both at the same time. The following n.m.r. spectroscopy study attempts to clarify these points.

N.M.R. Studies on the Model Compounds. Calculation of the Shielding Constants.—The titration curves of the two amines L^1 and L^2 are shown in Figures 1 and 2, together with their respective proton n.m.r. spectra at the specified pD* values. The analysis of these spectra is described below.

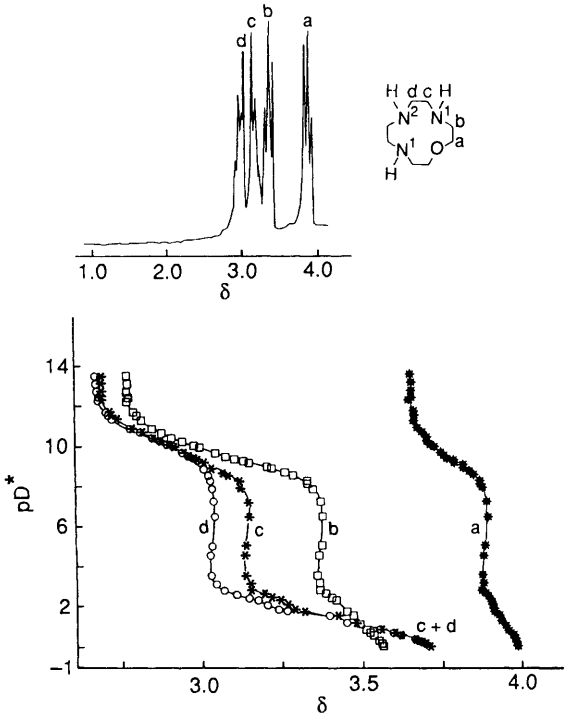


Figure 1. Proton n.m.r. spectrum of L^1 at pD* 4.52 and the titration curves δ as a function of pD*

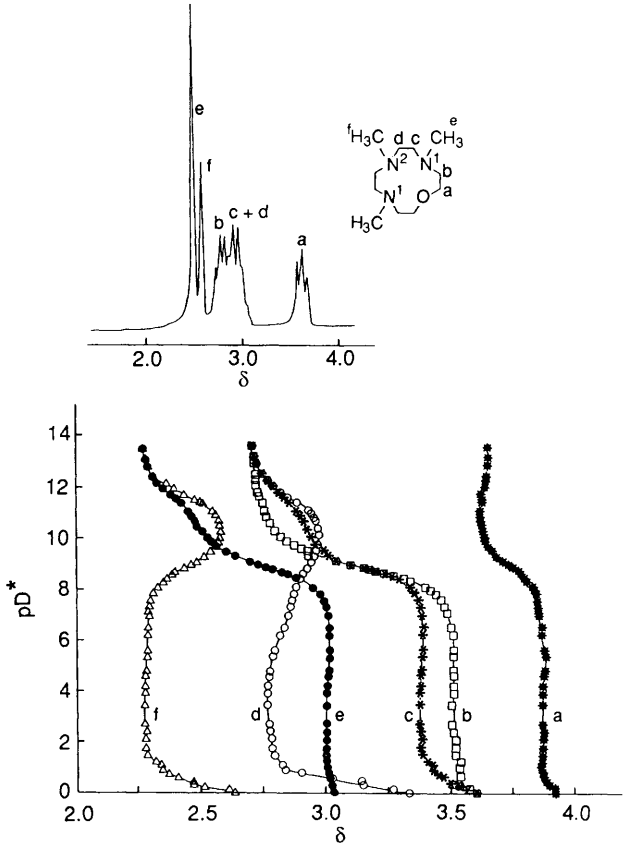


Figure 2. Proton n.m.r. spectrum of L^2 at pD* 10.87 and the titration curves δ as a function of pD*

L^1. The titration curves and the proton n.m.r. spectrum at pD* 4.5 for this amine are shown in Figure 1. The four non-labile methylenic protons of the molecule present different resonances throughout the entire pD* range. The assignment of the resonances was made by double-resonance experiments. Protons a, deshielded by the electronegative oxygen atom nearby, appear as a triplet at lower field. The assignment of the triplet corresponding to protons b was made by irradiation of the resonance of protons a at pD* 0 and 7.25. The smaller chemical shift difference between methylenic protons c and d makes their spectra a more complex A_2B_2 pattern. In this case the resonances were assigned by taking into account the profile of the titration curves. The c methylenic protons are influenced by protonation of both types of nitrogen atoms but to a larger extent that of N^1. Therefore they appear in an intermediate position between protons b and d following the profile of b protons at high pD* and that of d at low pD*.

The titration curve for this compound has the first inflection at high pD* (11.0—7.5) corresponding to two protons added, according to the potentiometric results. All the methylenic protons shift to lower fields, although to a lesser extent for protons d. The opposite is verified for the second inflection (pD* < 3.5) where methylenic protons d exhibit the largest shift. Protonation of the three basic centres of the molecule is almost complete at the end of the titration (as shown by the protonation constants calculated from the n.m.r. titration curves[19]). It therefore appears that the first two protonations occur simultaneously at the three nitrogen atoms but at N^1 to a higher degree (protons b and c exhibit the largest shift); on further addition of acid the largest shift of protons d shows that N^2 is then protonated to a larger extent than the others until almost total protonation is achieved.

The application of Sudmeier and Reilley's method to this compound to determine the shielding constants C_N and $C_{N'}$† is limited to very low pD* values. Indeed, at high pD* values, the first two protons seem to be added to the three basic centres but the relative percentages are difficult to predict. Only at the end of the titration ($n = 3$) it is possible to calculate the shielding constants assuming that all centres are completely protonated. The values obtained are: $C_{N'} = 0.33 \pm 0.01$ and $C_N = 0.72 \pm 0.05$ p.p.m. The larger standard deviation for C_N shows the dispersion of values calculated for the different methylenic groups (for protons b the values are higher than for protons c).

L^2. The spectrum of this compound at pD* 10.9 is shown in Figure 2 together with the titration curve for all resonances in the entire pD* range. The titration curves present some striking differences relative to that of the parent amine L^1; (1) there are two more resonances (e and f) readily assigned to the methyl groups as they are singlets and distinguishable from one another by their relative areas; (2) the last protonation step begins at lower pD* and is still not over at pD* values near zero; and (3) methylenic protons d and f have a very abnormal pattern with the chemical shift moving to higher field between pD* 5 and 10.

The assignment of the protons of the ring was made as described for the parent amine L^1. That of the methylenic protons d is now easier as their titration curve profile is very similar to that of protons f, both being shifted only by protonation of N^2.

The ligand L^2 has a more complicated behaviour than that of the parent amine L^1 because protonation is not complete even at pD* values near zero and at high pD* abnormal effects occur due to competition between nitrogen atoms 1 and 2. Indeed, the first equivalent of acid added (pD* 12.0—10.5) protonates the basic centre 2 (only resonances d and f shift downfield and protons c also exhibit a smaller shift); the second equivalent of acid (pD* 10.5—7.5) protonates nitrogen atoms 1 while N^2 is

† C_N is the shielding constant for the α methylenic protons, $C_{N'}$ for the β methylenic protons, caused by the protonation of a nitrogen atom.

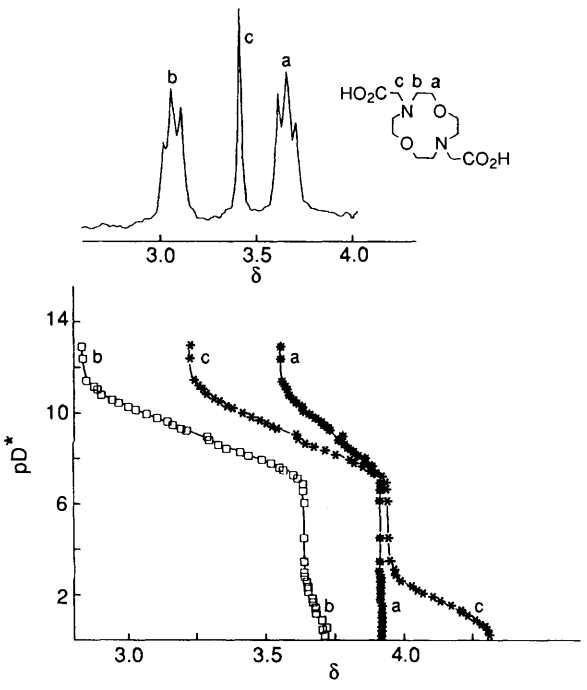


Figure 3. Proton n.m.r. spectrum of L^5 at pD* 10.0 and the titration curves δ as a function of pD*

progressively deprotonated (resonances a—c and e increase in chemical shift but d and f move in the opposite direction). Centre 2 starts to be protonated again only at pD* < 1. In this respect it is interesting to stress that while in amine L^1, with secondary nitrogen atoms only, the conformation of the ring is such that the first two protons added at high pD* seem to be distributed amongst the three basic centres, in the methylated amine L^2 the first proton added is localized on the nitrogen atom opposite to the oxygen atom of the ring but migrates when the second proton starts to be added (then a redistribution of charges occurs, leading to preferential protonation of the other two nitrogen atoms).

Under these conditions it is possible to determine C_{ij} values for L^2 by considering that at pD* ≈ 7 the nitrogen atoms 1 are 100% protonated and N^2 is not protonated, as the titration curve suggests. The calculated values are $C_{N'} = 0.23 \pm 0.01$ and $C_N = 0.73 \pm 0.03$ p.p.m. At low pD* it is impossible to determine these values, since the protonation is not complete.

It is clear that these compounds do not fulfil the necessary requirements to be taken as models. Other oxapolyaza macrocycles[21] are not adequate since the ring sizes are different and the same may be said of the tetra-aza macrocycle compounds studied before[8] which have only nitrogen atoms in the ring. A better model ought be a 12-membered macrocycle, with nitrogen and oxygen atoms in the ring and substituted N-carboxylates, but with a simple n.m.r. titration curve with well defined protonation stages. As will be seen, L^5 seems to satisfy these conditions.

L^5. Figure 3 shows the titration curve and the ^1H n.m.r. spectrum of L^5 at pD* 10.0. The assignment of the resonances is straightforward. The spectra exhibit three resonances over the entire pD* range: one singlet c and two triplets a and b, the first at low field. From the profile of the titration curves and the protonation constants it is easily seen that at high pD* (7.5—12.0) only the two nitrogen atoms are protonated; the two carboxylate groups are protonated at lower pD* and from the shape of the n.m.r. titration curves in Figure 3 it appears that for pD* values near zero the protonation is complete since the chemical shift tends to remain the same. Assuming that protonation is indeed complete, the shielding constants calculated at four pD* values lead to the following values, considering $C_{CO_2^-}$ as constant and equal to 0.20 p.p.m. ($C_{CO_2^-}$ is the shielding constant for the methylenic protons resulting from the protonation of a carboxylate group).

pD*	*n*	$C_{N'}$/p.p.m.	C_N/p.p.m.
9.3	1	0.35 ± 0.01	0.71 ± 0.06
6.0	2	0.36 ± 0.01	0.77 ± 0.04
1.75	3	0.36 ± 0.01	0.84 ± 0.02
0.0	4	0.37 ± 0.01	0.88 ± 0.01

All the values increase from high to low pD*. The C_N values determined for methylenic protons b in the chain linking the oxygen atom are higher than for the others (c), but the difference between them decreases at lower pD* values. It therefore appears that all the C_N values are functions of pD*: $C_N = 0.88 - 0.02pD^*$ and $C_{N'} = 0.37 - 0.005pD^*$. If these shielding constants are compared with those calculated for L^2 in the corresponding range of pD* one can verify that the values of C_N are similar but the values of $C_{N'}$ are considerably higher (0.35 compared to 0.23) although both were determined from methylenic protons a, near the oxygen atom of the ring.

It is recognized that the shielding constants determined by this procedure are not very accurate since it is necessary to postulate complete protonation of the carboxylates at low pD. Although this appears to be justified by the n.m.r. titration curves, it cannot be absolutely demonstrated and some doubts remain (see Conclusion).

Determination of Protonation Functions of Ligands L^3 *and* L^4.—*Ligand* L^3. In Figure 4 the titration curves of this ligand and its n.m.r. spectrum at pD* 9.3 are presented. The spectrum exhibits, over almost the entire pD* range, four triplets and one singlet with the same area showing that the molecule has five different types of methylenic protons.

The assignment of the singlet to the protons of the acetate groups is immediate. The methylenic protons a are deshielded by the nearby ether oxygen atom and were assigned to the triplet at lower field. Irradiation of this signal (at pD* 13.59, 10.39, 3.96, and 0.93) allowed the assignment of the adjacent methylenic b protons. The methylenic protons d are not easily assigned as a pronounced shielding effect is not shown upon addition of the second equivalent of acid and long-range proton–proton nuclear Overhauser enhancement effects are difficult to observe for small non-rigid molecules. However, the assignment of the methylenic carbon resonances was possible by a two-dimensional ^1H–^{13}C shift correlation experiment. Resonances a and d are easily assigned as they appear well separated from the other methylenic carbon resonances. The resonances d appear at 45.04 p.p.m. at pD* 3.97 and 42.73 p.p.m. at pD* 10.12.

The analysis of the titration curve for this ligand is not straightforward. In the range pD* 13.0—10.5 only the c and d resonances move downfield, which can be ascribed to the protonation of N^2. There is also a slight shift of resonance e which can probably be explained by a hydrogen-bond association involving carboxylate 3 and the protonated N^2. At pD* 8.0—5.0 all resonances but d shift downfield, meaning that the nitrogen atoms 1 are being protonated. For pD* < 5 the trend is difficult to explain; the methylenic protons d move to low field, hence one wonders whether N^2 is completely protonated at high pD* or not; on the other hand, the methylenic protons a—c shift to high field in the range pD* 5.0—2.0 as if the N^1 atoms are being deprotonated, but shift again to low field below pD* ≈ 2.

The protonation fractions of this compound were calculated

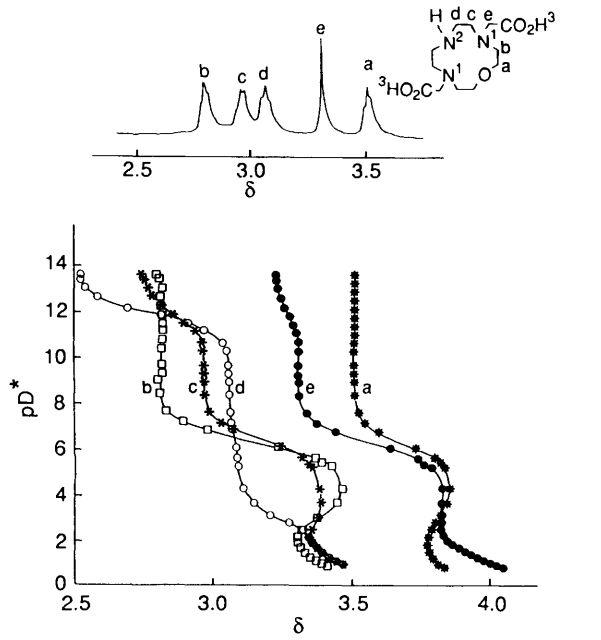


Figure 4. Proton n.m.r. spectrum of L^3 at pD* 9.3 and the titration curves δ as a function of pD*

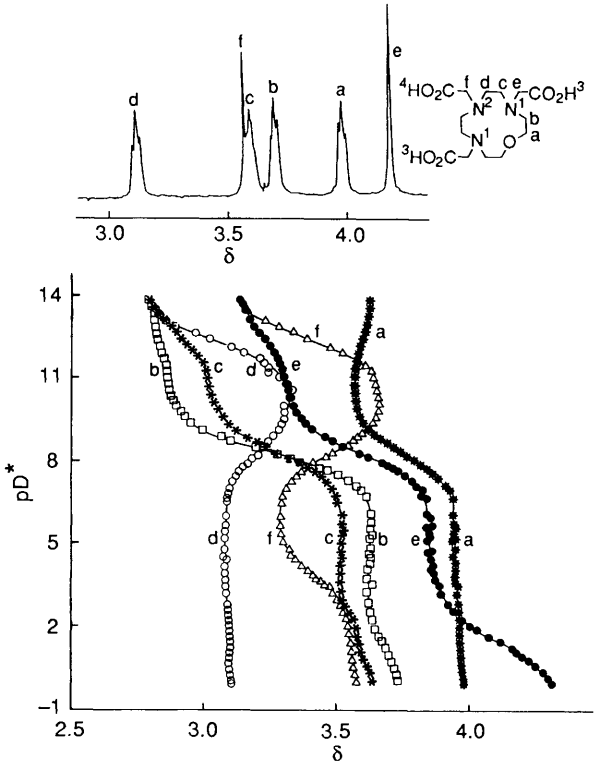


Figure 5. Proton n.m.r. spectrum of L^4 at pD* 1.23 and the titration curves δ as a function of pD*

using the C_N and $C_{N'}$ values obtained in the previous case and $C_{CO_2^-}$ = 0.20 p.p.m. (Table 2). According to the results the sequence of protonation of compound L^3 seems to be as follows: N^2 opposite to the oxygen atom in the ring is the first to be protonated, but there appears to be some degree of protonation of the carboxylates, in agreement with the shape of the titration curves for protons e at $n = 1$, which may be due to their involvement in hydrogen bonding with the protonated nitrogen atoms N^2. Further acidification (to $n = 2$) results in preferential protonation of nitrogen atoms 1 with partial deprotonation of N^2. These two simultaneous effects result in a constant shift value for methylenic protons d. The third proton binds preferentially to the carboxylate groups, while some degree of deprotonation occurs at nitrogen atoms 1 with some protonation of N^2, and the fourth binds preferentially to the carboxylate groups and to N^2. At pD* values near zero the carboxylates groups seem to be completely protonated and the nitrogen atoms remain partially deprotonated.

Table 2. Protonation fractions (%) for L^3

n	f_1	f_2	f_3
1	3 ± 3	73 ± 4	11 ± 3
2	77 ± 6	34 ± 9	6 ± 6
3	61 ± 6	60 ± 9	59 ± 6
4[a]	67 ± 6	76 ± 9	95 ± 6

[a] Only indicative, since full protonation by four protons cannot be inferred from the n.m.r. titration curve down to pD* 0.87.

The shift to high field of protons a and b at pD* 3.5—2.0 seems to be due to deprotonation of centre 1 in favour of 2. In the same pD* range the resonance e has a constant chemical shift in spite of the protonation of carboxylate groups. This may again be due to the compensatory effect of the simultaneous deprotonation of nitrogen atoms 1 (*cf.* Table 2).

Ligand L^4. Figure 5 shows the titration curve and the ^1H n.m.r. spectrum of compound L^4 at pD* 1.23. The titration was carried out with CsOD since this ligand forms complexes with K^+ that are stable enough to cause broadening of the resonances at high concentrations of added base.

The spectrum of compound L^4 presents six different resonances for almost the entire range of pD*; at pD* > 13 the pattern changes and only three different peaks can be distinguished. Resonances e and f are singlets with area ratio 2:1 and correspond to the methylenic protons of the acetate groups. All the other resonances are triplets with the same area and correspond to the different methylenic protons of the macrocycle ring. The assignment of the ring methylenic protons was carried out using the methods described for the methylated amine L^2. It was also possible to assign the methylenic proton resonances of this compound by a two-dimensional ^1H–^{13}C shift correlation experiment. At pD* 1.02 the methylenic carbon resonances d appear at 50.46 p.p.m.

The n.m.r. titration curve of this ligand is very similar to that of the methylated cyclic amine L^2 but very different from that of amine L^1. Here, again, the first equivalent of acid added to the completely deprotonated ligand binds to N^2 opposite to the ring oxygen between pD* 12.5 and 10.5, but resonances b and e shift slightly to low field, which may indicate some degree of protonation of nitrogen atoms 1 or their participation in hydrogen bonding. At pD* ≈ 10 the resonances a—c and e start moving to low field (protonation of the nitrogen atoms 1) up to pD* ≈ 6 and, at the same time, resonances d and f shift in the opposite direction, indicating that the macrocycle prefers a conformation with the positive charges on the two nitrogen atoms more distant from each other, hence N^2 is deprotonated. Below pD* 6.0 and till the end of the titration, resonance d exhibits a constant chemical shift, an indication that N^2 does not protonate again till pD* ≈ 0. Between pD* 6.0 and 3.5 resonance f shifts to low field, indicating protonation of carboxylate 4; at lower pD* values protonation of carboxylate groups 3 occurs with a net deshielding of resonance e, although a slight shift of resonances b and c probably corresponds to some protonation of nitrogen atoms 1.

The titration curve of this compound is easy to interpret and Sudmeier and Reilley's method is not necessary to derive the

Table 3. Protonation fractions (%) for L^4

n	f_1	f_2	f_3	f_4
1	8 ± 4	73 ± 9	11 ± 7	−11 ± 9
2	97 ± 4	7 ± 8	−5 ± 8	10 ± 6
3	95 ± 3	−9 ± 5	9 ± 7	101 ± 13
4*	98 ± 2	−9 ± 4	55 ± 5	104 ± 9
5*	103 ± 4	−12 ± 5	96 ± 8	113 ± 15

* Only indicative, since full protonation for four and five protons added cannot safely be inferred from the n.m.r. titration curves, although the profile of resonance e in Figure 5 supports this hypothesis.

protonation sequence; however it is useful to test the model and to help clarify the protonation sequence in the first part of the curve. Using the same set of constants as in the previous case, one obtains rather anomalous results, particularly for the percentage of protonation of the carboxylates 4 which exceeds 160% for $n = 5$.

Obviously, the set of shielding constants is inadequate for this case, particularly the value of $C_{CO_2^-}$. Since the difference relative to the previous case is the acetate group on N^2, we have calculated $C_{CO_2^-}$ for this carboxylate assuming complete protonation for $n = 5$: $C_{CO_2^-} = 0.40$ p.p.m.† The results obtained using this value and $C_{CO_2^-} = 0.20$ p.p.m. for the other carboxylate are shown in Table 3.

With the values of Table 3 it can easily be seen that for $n = 1$ there is slight protonation of the nitrogen atoms 1 and extensive protonation of N^2. For $n = 2$, the centre 2 is almost completely deprotonated in favour of nitrogen atoms 1 which are near 100% protonated. For $n = 3$ the carboxylate group linked to deprotonated N^2 is protonated and for $n = 4$—5 the other two carboxylate groups are protonated. The atom N^2 remains deprotonated till pD* values near zero.

It seems, then, that the use of the different values of $C_{CO_2^-}$ provides a satisfactory sequence of protonation, but we must ask why is this necessary, *i.e.* why are the two types of acetate not identical. One possible suggestion is that the carboxylate groups linked to protonated secondary amine groups (possibly involved in hydrogen bonding) have lower $C_{CO_2^-}$ values than those linked to non-protonated secondary amino groups. In the first case, protonation of the carboxylate group involves breaking the $\overset{+}{N}H \cdots {}^-O_2C$ hydrogen bond; in the second case it probably involves forming a $N \cdots HO_2C$ hydrogen bond. In the first case the carboxylate group is already partially neutralized by the charge in the amine group; in the second case, the addition of the proton fully neutralizes the free carboxylates.

The set of values of C_N and $C_{N'}$ used also need some comment. Indeed, if compounds L^3 and L^4 were used to derive the constants by assuming that for $n = 1$ the nitrogen atom N^2 was fully protonated, a value of $C_N = 0.53$ p.p.m. would be obtained, lower than the 0.67 p.p.m. used. However, the resonance of the methylenic protons a of compound L^4 moves upfield, meaning that the deshielding effect of the oxygen atom is being counteracted. The best explanation of this effect is the involvement of the nitrogen atoms 1 in hydrogen bonding with the protonated N^2. Calculations predict that about 20% of the proton on N^2 is distributed between the two nitrogen atoms. If this correction is taken in account, f_{N^2} will not be equal to 1 for full protonation of N^2 but to 0.8, and the recalculated C_N will be 0.68 p.p.m. in good agreement with the value derived from $C_N = 0.88 - 0.02pD^*$.

† In a previous paper dealing with N-carboxylate tetra-aza macrocycles with similar problems we took the value 0.24 p.p.m. for some of the ligands[8] and Letkeman and Westmore[2] used ≈0.28 p.p.m. in a study of triethylenetetranitrilohexa-acetic acid.

Conclusion

The method of Sudmeier and Reilley[1] cannot be considered as generally applicable in quantitative terms to the determination of the protonation sequence of polyfunctional molecules; even for the simple linear polyaminocarboxylates there are limitations. For macrocyclic compounds the additivity property implied by equation (1) is not verified over the entire pD* range as their rings may exhibit several conformational structures and more than one set of shielding constants is necessary. Furthermore, N-substituted macrocycles with protonated functions can introduce new conformational effects and form intramolecular hydrogen bonds that in certain cases may completely change the shielding constants and cause them to vary with the value of pD*.[7–11]

In many cases, *e.g.* those of the present work, these problems can be overcome. First, because the titration curves give information, sometimes enough for the purpose of the work. Secondly, because the compound itself can be used to determine shielding constants at some well defined points, corresponding to protonation of some particular basic centres.

The introduction of one oxygen atom in the ring of the macrocycle modifies the protonation behaviour, as can be seen by comparing L^4 with the similar tetra-aza macrocycle 1,4,7,10-tetra-azocyclododecane-1,4,7,10-tetra-acetic acid (dota).[8] For the tetra-aza macrocycles the sequence is broadly the same: first, two nitrogen atoms are protonated, then the carboxylates, but the remaining nitrogen atoms are not protonated at all or are only slightly protonated. For the corresponding N-methylated amines the last one (or two) nitrogen atoms are only slightly protonated at very low pD* values.

The difference in the case of oxaaza macrocycles arises in the choice of the nitrogen atom to be protonated. Apparently, protonation begins at the nitrogen atom opposite to the oxygen atom; further acidification results in deprotonation of the first nitrogen and preferential protonation of the two nitrogen atoms in opposed positions (as in dota). This situation minimizes the electrostatic repulsion energy in the macrocycle for two reasons: (1) the protonated nitrogen atoms are not adjacent and (2) the possible formation of hydrogen bonds between carboxylate groups and protonated nitrogen atoms helps to stabilize the charge inside the macrocycle.

The results obtained in the present work enable us to justify the values of the protonation constants presented in Table 1. All compounds have two high or fairly high log K values (>6) corresponding to protonation of nitrogen atoms. The protonation of the last nitrogen or of a nitrogen linked to two other previously protonated nitrogen atoms by $-(CH_2)_2-$ corresponds always to a very small value of log K (<2) due to the repulsion of the charged $>\overset{+}{N}$ groups at short distance. Protonation of the carboxylate groups linked to non-protonated nitrogen atoms gives log K values of the order of 4 (the log protonation constant of acetic acid is 4.8), but the protonation of carboxylate groups linked to a positive protonated nitrogen atom corresponds to log K values of the order of 2 or less, due to the effect of the charged $>\overset{+}{N}H$ group and the simultaneous disruption of the hydrogen bonds in which these carboxylate groups are involved.

These conclusions are applicable to other analogous polyaza or polyoxapolyaza macrocyclic compounds.

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