Thermodynamics of the acid dissociation of BOPTA. Determination of equilibrium constants by means of ^{13}C NMR spectroscopy

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BOPTA, (9*R*,*S*)-2,5,8-Tris(carboxymethyl)-12-phenyl-11-oxa-2,5,8-triazadodecane-1,9-dicarboxylic acid, is a chelating agent whose gadolinium complex can be used as a magnetic resonance imaging contrast agent (MRI-CA) specific for the liver. The stepwise deprotonation constants for BOPTA were determined from a series of ^{13}C NMR measurements by means of the new computer program HYPNMR and also from potentiometric titration data by means of the program HYPERQUAD. The first three stepwise protonation constants obtained by the NMR method are in very good agreement with those determined by potentiometry. In very acidic solutions the NMR method gave more reliable results because the glass electrode is susceptible to interference at low pH.

The enthalpy changes for the protonation reactions have also been measured by microcalorimetry. The first two protonation reactions are exothermic, indicating that protonation occurs on amine nitrogen atoms, while the following protonation steps, being athermic, involve carboxylate groups.

BOPTA, (9*R*,*S*)-2,5,8-Tris(carboxymethyl)-12-phenyl-11-oxa-2,5,8-triazadodecane-1,9-dicarboxylic acid, H_5L, is an acyclic polyaminopolycarboxylic ligand whose gadolinium complex can be used as a magnetic resonance imaging contrast agent (MRI-CA) specific for the liver (Fig. 1).[1] The effectiveness of the complex ion $[GdL]^{2-}$ for MRI-CA is due to the high magnetic moment (7.94 BM †) of Gd(III),[2] the effect of which is to greatly reduce the relaxation times of nuclei in its close proximity.

For *in vivo* applications a paramagnetic MRI-CA should be a thermodynamically stable and kinetically inert complex in order to minimise the concentration of the highly toxic Gd(III) ion; it should be efficient in water proton relaxation and it should exhibit organ-specificity.[3,4] For these reasons, much current research relating to gadolinium MRI-CAs is aimed at finding suitable ligands for various *in vivo* MRI applications.

Linear polyaminopolycarboxylic acids are good candidate ligands.[5] The thermodynamic stability of the complexes formed by these ligands depends on factors such as the pK_a values of the carboxylic and amino groups, the distances between donor atoms and the size and flexibility of the molecule.[6] Values of the acid dissociation constants, pK_a, are usually obtained from potentiometric titration data by means of sophisticated computer programs able to handle multiple simultaneous equilibria.[7] The potentiometric titration method is relatively cheap and fast but it is difficult to apply in environments such as biological fluids because of the presence of other substances containing acidic or basic groups. Thus, results obtained from aqueous solution data may not accurately reflect the behaviour of a complex *in vivo*. This is a general problem concerning all substances that have acidic and/or basic groups (*e.g.* amino acids, peptides, drugs, metabolites, *etc*.).

† One Bohr magneton = 9.274 02 × 10^{-24} J T^{-1}.

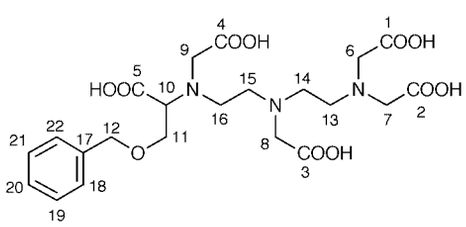

Fig. 1 BOPTA molecule with numbering used in NMR assignment.

NMR spectroscopy, although relatively expensive, can be applied directly in conditions similar to those *in vivo*. The variation of ^{13}C or ^{15}N chemical shifts with pH can be used to determine the pK_a values of a polyprotic molecule even in the presence of other acidic/basic substances.[8–16] Until recently, procedures[17,18] developed for the determination of stability constants from NMR data have generally been of limited application for two main reasons: *i*) the algorithms employed could only be used on simple systems; *ii*) NMR data could not apparently deliver stability constants with the same precision that potentiometric determination can achieve. [Attempts to derive pK_a values theoretically from NMR spectra have been performed.[1] In our opinion the basis for those calculations (empirical shielding constants for protons as a function of the distance from a charged site and empirical rules for amino-acid resonance assignments) is not precise enough to be used in a molecule like BOPTA].

Recently, the program HYPNMR has been applied to determine the 4 protonation constants of spermine, 1,5,10,14-tetraazatetradecane, from NMR data.[18] ^{1}H and ^{13}C chemical shifts were measured in D_2O over a pD range between 6.0 and 12.0. HYPNMR has been also used for the determination of macroscopic protonation constants of organic polyphosphate anions from ^{31}P NMR titration data.[19] The values and precision

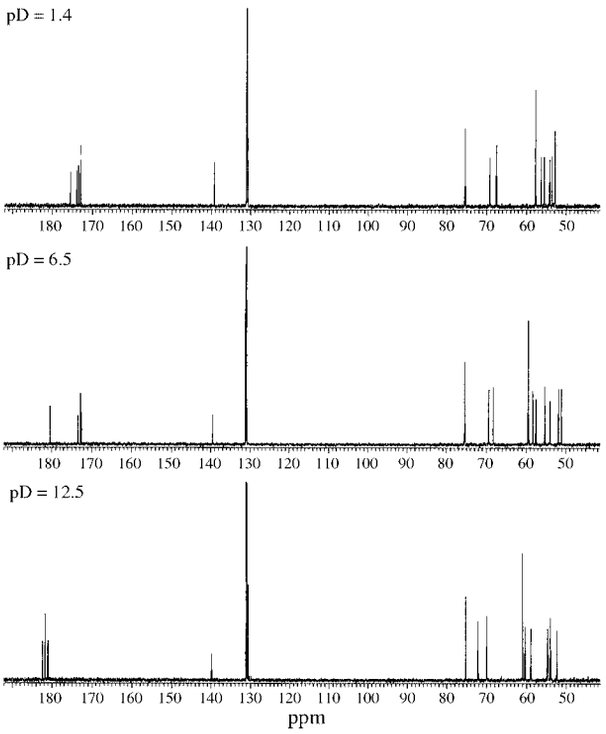

Fig. 2 ^{13}C NMR spectra of 60 mM solutions of BOPTA at 298 K in D_2O at various pD values. Chemical shifts are given relative to TSP, used as external standard.

of the equilibrium constants obtained by means of these procedures were shown to be comparable to those obtained from potentiometric titration data.

The present work has two principal aims: *i*) the determination of the deprotonation constants of BOPTA in D_2O solution at 298 K using ^{13}C chemical shift measurements as an alternative to potentiometric methods; *ii*) the definition of the behaviour of BOPTA in environments where potentiometry cannot be used in order to obtain more realistic information about the *in vivo* stability of this MRI-CA. The first aim was achieved but the second is more difficult to reach and it is still the subject of investigation. The main problem concerns the pH range that can be tolerated by living systems: in effect this is limited to between about 4.5 and 8 and in this range changes of chemical shifts are rather small; we are attempting to devise new methodology to apply to these systems.

Enthalpy changes have been determined by calorimetry and thence entropy changes have also been derived. These data, together with the NMR results, furnish interesting information regarding the microscopic protonation sequence of the ligand.

Results and discussion

In the one dimensional proton-decoupled ^{13}C NMR spectra (Fig. 2) assignment of all 14 resonances was not practicable for the following reasons: *i*) empirical rules for calculating shielding constants as a function of the distance[20] from a charged site have too much uncertainty to be used in a molecule like BOPTA; *ii*) empirical rules which hold true for amino acids[21] could not be used for C6, C7, C8 and C9 because of the presence of five carboxylic groups and three nitrogen atoms; *iii*) any attempt to assign the C13, C14, C15 and C16 resonances using the empirical rules for polyamines[22] was also difficult; *iv*) theoretical calculations of chemical shifts were not considered because they had previously failed for polyamines,[23] a class of molecules that are generally easier to characterise than polyaminopolycarboxylic acids.

Some ^{1}H and ^{13}C chemical shifts, in particular those pertaining to the benzyl-oxa chain, could be assigned unequivocally using standard rules. Other resonances were assigned on the basis of scalar couplings observed in the 2-dimensional HMQC $^{1}H/^{13}C$ spectra which were measured at each of the pD values used for the 1-dimensional spectra; a typical HMQC spectrum is shown in Fig. 3. By using various evolution times it was possible to identify both direct and long-range scalar couplings and thus to characterise the heteronuclear correlations: these are shown in Table 1. The remaining unassigned signals could then be associated with particular nuclei. A detailed assignment of the ^{13}C chemical shifts is given in Table 2: values marked with an asterisk are too close to each other at the particular pD to be separately identifiable.

In order to compare the results obtained with the NMR/HYPNMR method with the basicity constants calculated from the potentiometric data, account must be taken of the differences between D^+ and H^+. A correction was made by use of the semiempirical formula (1).[24]

$$\log K_{(H_2O)} = \log K_{(D_2O)} - 0.63 \pm 0.07 \qquad (1)$$

The logarithms of values for the stepwise deuteron basicity constants of L^{5-} obtained by HYPNMR processing of the ^{13}C

Table 1 $^{1}H/^{13}C$ correlation deduced from HMQC spectra performed on 60 mM solutions of BOPTA in D_2O at different pD values. Correlations with protons inside brackets were detectable only at pD values for which proton resonances of H13, H14 and H16 were distinguishable

	Direct correlation	Long range correlation
C1, C2		H6, H7
C3		H8
C4		H9
C5		H10, H11
C6, C7	H6, H7	H13
C8	H8	H14, H15
C9	H9	H10
C10	H10	H9, H11
C11	H11	H10, H12
C12	H12	H11
C13	H13	H6, H7, H8, (H14)
C14	H14	H8, (H16)
C15	H15	H9, H10, (H15)
C16	H16	H12
C17	H17	H12
C18, C22	H18, H22	H12
C19, C21	H19, H21	H12
C20	H20	H12

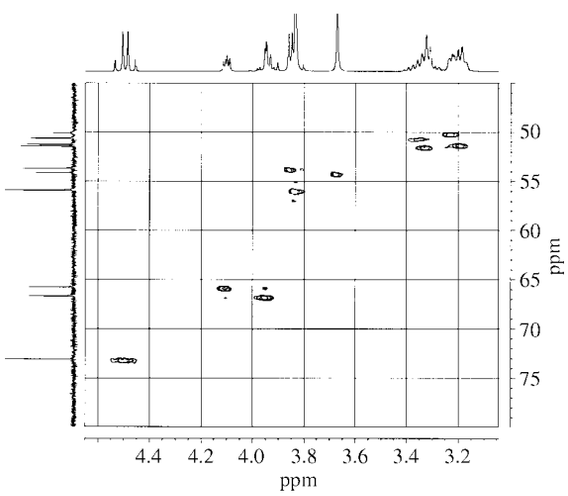

Fig. 3 $^{1}H/^{13}$ HMQC spectra of a 60 mM solution of BOPTA at 298 K in D_2O at pD = 1.81; chemical shifts are given relative to TSP, used as external standard. Correlations are reported in Table 1.

Table 2 ^{13}C chemical shifts (δ_C/ppm) of BOPTA solutions at 298 K in D_2O at different pD values. All chemical shifts are given relative to TSP, used as external standard. * Interchangeable assignments

pD	C1	C3	C4	C5	C6	C8	C9	C10	C11	C12	C13	C14	C15	C16
1.81	172.95	173.41	175.42	173.85	57.64	56.21	55.530	67.48	69.14	75.41	53.43	52.77	54.12	52.77
2.30	172.88	174.00	175.03	173.63	58.11	56.47	55.92	67.96	69.03	75.41	53.73*	52.54	53.69*	52.90
2.37	172.81	174.16	174.89	173.52	58.24	56.52	56.04	68.09	68.96	75.41	53.78	52.44	53.60	52.95
2.68	172.60	174.74	174.38	173.15	58.70	56.60	56.46	68.56	68.69	75.41	54.10	52.07	53.13	53.13
2.90	172.53	175.01	174.13	172.96	58.90	56.60	56.66	68.76	68.54	75.41	54.24	51.89	52.88	53.23
3.21	172.48	175.45	173.74	172.66	59.21	56.58	57.00	69.09	68.30	75.41	54.44	51.61	52.48	53.36
3.59	172.49	175.72	173.57	172.53	59.35	56.58	57.16	69.24	68.18	75.40	54.53	51.48	52.28	53.42
4.05	172.53	176.32	173.37	172.38	59.51	56.75	57.37	69.43	68.05	75.40	54.68	51.34	52.05	53.52
5.85	172.64	179.97	173.31	172.33	59.31	58.00	57.38	69.28	68.08	75.36	55.15	50.95	51.73	53.94
6.17	172.66	180.15	173.32	172.35	59.31	58.07	57.39	69.28	68.10	75.36	55.18	50.95	51.73	53.96
6.87	172.73	180.26	173.40	172.41	59.29	58.11	57.39	69.27	68.13	75.35	55.16	50.95	51.74	53.95
7.05	172.80	180.24	173.46	172.47	59.31	58.13	57.41	69.28	68.16	75.36	55.15	50.98	51.77	53.92
7.51	173.00	180.10			59.34	58.10	57.43	69.29	68.26	75.35	55.05	51.05	51.84	53.84
9.30					60.07	57.44	58.17	69.69		75.25				
9.64					60.23	57.38	58.32	69.77	71.47	75.24	52.18	53.35	54.31	51.28
9.90					60.28	57.40	58.37	69.79	71.60	75.23	52.08	53.45	54.43	51.17
10.28	180.41	175.32	181.66	179.85	60.35	57.52	58.44	69.81	71.77	75.22	52.07	53.58	54.53	51.12
11.78	181.438	179.89	182.289	180.697	60.64	59.57	58.73	69.79	72.03	75.18	53.40	53.85	54.58	52.02
12.42	181.602	180.822	182.349	180.822	60.69	59.98	58.78	69.78	72.06	75.18	53.89	53.68	54.57	52.22
12.89	181.642	181.071	182.363	180.847	60.70	60.09	58.78	69.77	72.05	75.17	53.90*	53.75*	54.56*	52.26

Table 3 Logarithms of the stepwise deuteron basicity constants of BOPTA determined at 298 K in D_2O by means of ^{13}C NMR data processed using the computer program HYPNMR

Equilibrium	log K
$L^{5-} + D^+ \rightleftharpoons DL^{4-}$	11.17 ± 0.01
$DL^{4-} + D^+ \rightleftharpoons D_2L^{3-}$	8.81 ± 0.02
$D_2L^{3-} + D^+ \rightleftharpoons D_3L^{2-}$	4.81 ± 0.03
$D_3L^{2-} + D^+ \rightleftharpoons D_4L^-$	2.57 ± 0.06
$D_4L^- + D^+ \rightleftharpoons D_5L$	2.2 ± 0.2

Table 4 Comparison between the potentiometric and ^{13}C NMR pK_a evaluations. Standard deviations in parentheses

log K_1	log K_2	log K_3	log K_4	log K_5	T/°C	Method
10.86(3)	8.29(1)	4.34(1)	2.78(1)	2.23(1)	25	[a]
10.76(2)	8.17(2)	4.31(2)	2.69(2)	2.18(2)	25	[b]
10.71(3)	8.27(3)	4.35(2)	2.83(2)	2.07(5)	20	[c]
10.51(1)	8.17(2)	4.19(3)	1.94(6)	1.5(2)	25	[d]

[a] Potentiometric method, 0.1 mol dm^{-3} $TMANO_3$. [b] Potentiometric method, 0.15 M NaCl. [c] Potentiometric method, 0.1 M KCl (ref. 1). [d] ^{13}C NMR method.

NMR chemical shifts are given in Table 3. Derived protonation constants are given in Table 4, together with results obtained from potentiometric data from three different ionic media. A comparison of the results obtained from NMR and potentiometric data shows that: *i*) the precision of the calculated constants is comparable for the two techniques and; *ii*) while the first three protonation constants evaluated using the NMR/HYPNMR method are in very good agreement with those obtained by potentiometric determination, K_4 and K_5 values are somewhat different. A possible cause of these discrepancies may lie in the very acidic pD range of the first five solutions used for the ^{13}C studies. No correction for liquid junction potential was applied to the potentiometric data. It may be noted that there is very good agreement between the protonation constants obtained using the two ionic media 0.15 M $TMANO_3$ and 0.15 M NaCl. This means that there is no significant interaction between BOPTA and the Na^+ ion even in the alkaline pH range, where the ligand exists in the form L^{5-}.

The calculated concentrations of the species $D_nL^{(5-n)-}$ (n = 0–5), derived using the stability constants given in Table 3, are shown in Fig. 4 as a function of pD; the ^{13}C NMR chemical shifts for these species are given in Table 5.

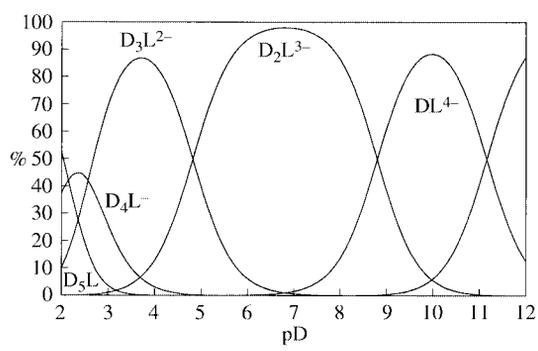

Fig. 4 Distribution diagram of the species formed in the system BOPTA (H_5L)-deuterium ion in D_2O as a function of pD at 298 K.

An analysis of the way the chemical shifts vary with pD could, in principle, yield micro-constants, *e.g.* individual constants for the formation of the 5 species of formula $D_nL^{(5-n)-}$ (n = 1–5). In the present case, however, the complete protonation sequence could not be determined from the NMR data. Nevertheless, interesting information about ligand protonation reactions is furnished by the enthalpy and entropy changes determined for these proton transfer processes. As shown in Table 6, protonation of L^{5-} is principally promoted by favourable entropy contributions concomitant with the charge neutralisation which occurs when a positively charged proton is added to a negatively charged anion. This effect decreases with decreasing ligand charge, *i.e.* with increasing ligand protonation, so that the entropy contribution decreases in successive protonation stages. The first two protonation reactions are also promoted by favourable, but less important, enthalpy terms; the next protonation reactions are almost athermic ($\Delta H° \approx 0$).

It is well known that protonation of amine nitrogen atoms is an exothermic reaction, while protonation of carboxylate groups is accompanied by small enthalpy changes. On this basis the thermodynamic data derived for the stepwise protonation of BOPTA (Table 6) indicate that the first two steps involve, for the most part, protonation of the amine nitrogen atoms while the remaining three steps mainly involve protonation of the carboxylate groups. With this information the ^{13}C NMR data given in Table 2 can be further interpreted in terms of a protonation sequence, that is, those micro-constants which are the major contributors the macroscopic equilibrium constants

Table 5 Refined values (ppm) of ^{13}C NMR chemical shifts for the various species of BOPTA at 298 K in D_2O[a]

Nucleus	L^{5-}	DL^{4-}	D_2L^{3-}	D_3L^{2-}	D_4L^{-}	D_5L
C1	181.66(2)	180.51(2)	172.65(1)	172.44(2)	172.77(3)	173.13(2)
C3	181.20(2)	174.40(6)	180.37(6)	175.69(2)	173.86(8)	173.01(6)
C4	182.38(2)	181.85(2)	173.30(1)	173.34(2)	175.50(3)	175.55(2)
C5	180.87(2)	179.97(6)	172.33(6)	172.35(2)	174.06(7)	173.89(6)
C6	60.71(2)	60.34(2)	59.29(1)	59.55(2)	57.88(2)	57.39(1)
C8	60.13(2)	57.18(5)	58.15(6)	56.48(2)	56.93(7)	55.86(6)
C9	58.80(2)	58.43(2)	57.39(1)	57.36(2)	55.52(2)	55.40(1)
C10	69.78(2)	69.83(6)	69.27(6)	69.45(2)	67.71(8)	67.23(6)
C11	72.06(2)	71.88(2)	68.09(1)	68.03(2)	69.51(2)	69.06(1)
C12	75.17(2)	75.22(5)	75.36(6)	75.41(2)	75.41(6)	75.41(6)
C13	53.93(2)	51.71(2)	55.21(1)	54.61(2)	53.61(3)	53.25(1)
C14	53.77(2)	53.66(5)	50.92(6)	51.35(2)	52.88(7)	52.85(6)
C15	54.57(2)	54.65(2)	51.70(1)	52.09(2)	54.21(3)	54.23(1)
C16	52.30(2)	50.87(7)	53.99(6)	53.47(2)	52.78(5)	52.69(6)

[a] Values in parentheses are estimated standard deviations on the last significant digit.

Table 6 Thermodynamic parameters for the protonation of BOPTA in 0.1 mol dm^{-3} $TMANO_3$. Standard deviations in parentheses

Reaction	$-\Delta G°$/ kJ mol^{-1}	$-\Delta H°$/ kJ mol^{-1}	$T\Delta S°$/ kJ mol^{-1}
$L^{5-} + H^+ = HL^{4-}$	61.95(4)	18.8(2)	43.1(2)
$HL^{4-} + H^+ = H_2L^{3-}$	47.28(4)	12.9(2)	34.4(2)
$H_2L^{3-} + H^+ = H_3L^{2-}$	24.77(4)	2.3(2)	22.5(2)
$H_3L^{2-} + H^+ = H_4L^{-}$	15.86(4)	0.6(3)	15.3(3)
$H_4L^{-} + H^+ = H_5L$	12.72(4)	−0.5(4)	12.2(4)

can be identified. This protonation sequence is in good agreement with previous results.[1] The first atom of L^{5-} to be protonated is the nitrogen atom in the centre of the molecule. This is consistent with the β-shift effect[25] which has been observed in the protonation of polyamines and which consists of an up-field shift of the ^{13}C signals of carbon atoms (in this case C16 and C13) in the β-position with respect to a protonated amine group. In the species H_2L^{3-} the two added protons are localised mainly on the terminal nitrogen atoms, a disposition which minimises the electrostatic repulsion between the two positive charges. The third proton goes principally onto the carboxylate group C3. It has been suggested[1] that the central nitrogen atom also contributes to proton binding. However, as the enthalpy change for the third protonation step is almost zero it is unlikely that nitrogen protonation makes a significant contribution.

Conclusion

The results presented here show that ^{13}C NMR spectrometry combined with HYPNMR processing can be applied to the determination of pK_a values in systems where polyprotic equilibria exist. Although the NMR/HYPNMR method is more expensive in terms of time and equipment costs than the potentiometric method, the NMR technique is better suited to systems containing several acidic and basic substrates and it should give good results in those complicated physico-chemical environments where the potentiometric method would be difficult to use. This suggests that the method may be very useful for the determination of pK_a in biological fluids and so may facilitate detailed investigations in fields such as drug metabolism and drug interaction with cell membranes. Moreover it should be a powerful tool to study the real *in vivo* stability of lanthanide complexes used as MRI-contrast agents.

Experimental

Materials

The BOPTA ligand was synthesized as described previously.[1] HCl, NaOH, $(CH_3)_4NOH$ (TMAOH), NaCl and buffers were purchased from Merck KGoA, Darmstadt, Germany; $(CH_3)_4NNO_3$ ($TMANO_3$) was purchased from Fluka, Buchs, Switzerland; KOD and DCl were purchased from Aldrich Chimica, Milano, Italy. Sodium 3-(trimethylsilyl)propionate-d_4 (TSP) was supplied by Wilmad (USA). All these materials were used without further purification.

NMR measurements

Natural abundance ^{13}C spectra were recorded on an AMX-400 Bruker spectrometer, operating at 9.395 T, with a reverse ^1H/BB 5 mm probehead, using TSP as external reference. All spectra were acquired at 298 K (controlled by Eurotherme 3000 VT control unit) with deuterium as the lock channel. The experimental parameters for the ^{13}C nucleus were as follows: base spectral frequency = 100.61 MHz, spectral width = 15091.5 Hz (150 ppm), 64 K data points in the time domain and 64 K data points in the frequency domain, pulse width = 6 μs (90° flip angle), acquisition time = 2.16 s, number of scans = 1024. Spectra were acquired using a WALTZ-16 pulse sequence for proton decoupling. FIDs were processed with enhancement multiplication (line broadening = 1 Hz).

The experimental parameters employed to record the proton spectra were as follows: base spectral frequency = 400.132 MHz, spectral width = 2800 Hz, 32 K data points in the time domain and 32 K data points in the frequency domain, pulse width = 9 μs. FIDs were processed without filtering.

The experimental parameters used to record the $^1H/^{13}C$ HMQC spectra were as follows: spectral width in ^1H dimension (F_2 dimension) = 2800 Hz (7 ppm), spectral width in ^{13}C dimension (F_1 dimension) = 18110 Hz (180 ppm), 2 K data points in F_2 and 512 data points in F_1 dimension, proton pulse width = 10.6 μs (90° flip angle), carbon pulse width = 6 μs (90° flip angle), acquisition time = 0.367 s, number of scans = 16, number of dummy scans = 32. Evolution delay was fixed at 0.052 s which enabled the contemporaneous observation of both direct and long range ^1H–^{13}C scalar correlation. FIDs were processed after doubling the number of points by zero filling in the F_1 dimension and with SIN function (shifted by π/2) filtering in both dimensions. The FT was performed in magnitude mode. All spectra were recorded using 60 mM solution of BOPTA in D_2O. The chemical shifts of all peaks were used as data points.

$^{13}C\{^1H\}$ NMR spectra were recorded at 20 different pD values, ranging between 1.81 and 12.89. The chemical shifts of all 14 signals forming the BOPTA spectra were employed as raw data to give a total of 261 data points in all (Table 2).

Calibration of the pD-meter

A Metrohm 691 pH-meter with a Metrohm combined glass

electrode was calibrated at 298 K in terms of hydrogen ion concentration $c_H = 10^{-pH}$, using two buffers (pH = 4.00 and pH = 7.00). The pD was calculated by means of the empirical relationship pD = (meter reading) + 0.40.[23] The pD of each sample was varied by addition of small measured quantities, of the order of a few microliters, of a concentrated solution of KOD or DCl to 1.0 ml of a 60 mM stock solution of BOPTA. The concentrations of the resulting solutions were calculated by assuming additivity of volume.

Potentiometric measurements

Protonation constants of BOPTA in aqueous 0.1 mol dm^{-3} $TMANO_3$ solution at 25 ± 0.1 °C were determined by means of potentiometric titrations. The potentiometric solutions (20 ml, 1×10^{-3} mol dm^{-3}) were acidified with known amounts of HCl in order to study the ligand in the pH range 2.50–11.00 and then titrated in a thermostated cell under a stream of purified nitrogen with 0.2 mol dm^{-3} standard solution of TMAOH, added by means of a 1 ml piston burette. Potentiometric measurements were carried out with a Metrohm E636 potentiometer equipped with a Metrohm glass electrode and a thermostated calomel (KCl saturated) reference electrode. Prior to each potentiometric titration, a calibration of the electrode to measure hydrogen ion concentration ($pH = -\log[H^+]$) was performed by titrating known amounts of HCl with 0.2 mol dm^{-3} TMAOH.

Ligand protonation constants in 0.15 mol dm^{-3} NaCl aqueous solutions were determined by similar procedures by using an Ingold Ag/AgCl combined glass electrode, and 0.1 mol dm^{-3} NaOH as titrant. No correction for liquid junction potential in very acidic solutions was applied.

Potentiometric measurements: data treatment

Calibration data were processed for both ionic media by means of the Gran method.[26] The protonation constants were calculated from the experimental potentiometric data with the computer program HYPERQUAD[27] by treating three titration curves either as a single set or as separate entities without significant variations in the final results.

Computer program HYPNMR

The principles underlying the HYPNMR approach to the calculation of equilibrium constants have been described briefly previously.[7] It is assumed that proton transfer reactions are rapid on the NMR time-scale so that the chemical shift of any one nucleus will be the concentration-weighted average of the chemical shifts of each chemical species in the equilibrium. The concentration of any one species is a function of the equilibrium constant for its formation and the free concentrations of the reagents (*e.g.* ligand and proton). The values of the free concentration of the ligand are obtained by solving the corresponding mass-balance equation with assumed values for the equilibrium constants. The equilibrium constants are determined by non-linear least-squares refinement to optimise the fit between observed and calculated chemical shifts.

HYPNMR has been significantly improved since the original publication. Two new programs have been written, HYPNEDIT and HYPNOUT. HYPNEDIT is a customised editor for the input data and HYPNOUT is used to view and interpret the output from the refinement. In addition, the algorithm has been modified so that either the total hydrogen ion concentration, T_H, or the free hydrogen ion concentration, pH, can be used as input data. The former possibility is analogous to the situation normally obtained in a potentiometric titration. The latter was used in the present work.

Calorimetric measurements

The enthalpy changes for the reactions of ligand protonation were determined in 0.1 mol dm^{-3} $TMANO_3$ solutions by means of microcalorimetric titrations performed with an automated system composed of a Thermometric AB thermal activity monitor (model 2277) equipped with a perfusion-titration device and a Hamilton Pump (model Microlab M) coupled with a 0.250 cm^3 gas-tight Hamilton syringe (model 1750 LT). The measuring vessel was housed in a 25 dm^3 water thermostat which was maintained at the chosen temperature within $\pm 2 \times 10^{-4}$ K. The microcalorimeter was checked by determining the enthalpy of reaction of strong base (TMAOH) with strong acid (HCl) solutions. The values obtained, −13.55(5) kcal mol^{-1} was in agreement with the literature values.[28]

In a typical experiment, a TMAOH solution (0.1 mol dm^{-3}, addition volumes 15 μl) was added to acidic solutions (1.5 cm^3) of the ligands (5×10^{-3} mol dm^{-3}). Corrections for the heats of dilution were applied. The enthalpies of reaction were determined from the calorimetric data by means of a least squares fitting using the AAAL program.[29]

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