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SPECIAL ISSUE PAPER

**Chiara Frassineti · Lucia Alderighi · Peter Gans
Antonio Sabatini · Alberto Vacca · Stefano Ghelli**

Determination of protonation constants of some fluorinated polyamines by means of ^{13}C NMR data processed by the new computer program HypNMR2000. Protonation sequence in polyamines

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Abstract The pK_a values of 6-fluoro-4,8-diazadodecane-1,12-diamine (6-fluorospermine) (1), 6,6-difluoro-4,8-diazadodecane-1,12-diamine (6,6-difluorospermine) (2), 6-fluoro-4-azaoctane-1,8-diamine (6-fluorospermidine) (3) and 6,6-difluoro-4-azaoctane-1,8-diamine (6,6-difluorospermidine) (4) in D_2O solution have been determined at 40 °C from ^{13}C NMR chemical shifts data using the new computer program HypNMR2000. The enthalpies of protonation of compounds 1–4 and the parent amines spermine (5) and spermidine (6) have been determined from microcalorimetric titration data. The values of $\Delta H°$ were used to derive basicity constants relative to 25 °C. The NMR data have been analysed by two different methods to obtain information on the protonation sequence in the polyamines 1–5. The protonation sequence for spermine is related to its biological activity.

Keywords ^{13}C NMR · Fluorinated compounds · Polyamines · Protonation constants · Protonation enthalpies · Protonation sequence

Abbreviations *PKC* Protein kinase C · *PS* Phosphatidylserine · *VB* Microsoft Visual Basic

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C. Frassineti
Dipartimento di Scienze Biomediche,
Università degli Studi di Modena,
via Campi 287, 41100 Modena, Italy

P. Gans
Department of Chemistry, The University of Leeds,
LS2 9JT Leeds, England

L. Alderighi · A. Sabatini · A. Vacca (✉)
Dipartimento di Chimica, Università degli Studi di Firenze,
via della Lastruccia 3, 50019 Sesto Fiorentino, Italy
e-mail: alberto.vacca@unifi.it

S. Ghelli
SPIN, via Tamagno 3, 42048 Rubiera (Reggio Emilia), Italy

Introduction

Aliphatic polyamines are generally present as metabolites in prokaryotic and eukaryotic cells where they play an important role in various cell functions [1, 2, 3] such as cell growth, division, and differentiation but also apoptosis and cell death [4, 5]. It has also been shown that polyamines modulate cell membrane functions, interact with ion channels [6, 7] and stimulate or inhibit the activity of several enzymes [1] such as Protein Kinase C (PKC), which is involved in the control of cell proliferation [8, 9, 10]. The functionality of a polyamine within the cell is mainly determined by its interactions with the lipid bilayer of cell membranes [1]. The interactions are mainly electrostatic and are strongly dependant on the number and position of the electrical charges of a protonated form of the polyamine [11]. Knowledge of the equilibrium constants for protonation of each nitrogen atom (the so-called microconstants) allows the protonation sequence of the polyamine to be determined so that the distribution of protonated sites on the polyamine can be calculated at the relevant pH. This information will provide insight into the mechanism, at the molecular level, by which the polyamines interact with liposomes and cell membranes. These interactions are basis of important biological functions, such as the activation process of PKC which is triggered by its association with the acidic phospholipid phosphatidylserine (PS) in the cell membrane. The activation process is modulated (inhibited) by the presence of polyamines, especially spermine.

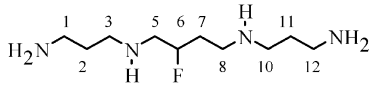
Fluorinated derivatives of naturally occurring polyamines are of interest because they have similar chemical structures, but different protonation constants due to the electron withdrawing effects of the fluorine atoms. A comparison of the protonation sequences of naturally occurring and fluorinated polyamines should assist in understanding what the structural requirements are for optimal function in nature.

It is important that protonation constants should be determined under experimental conditions which are similar to those in which the molecules exhibit biological activity.

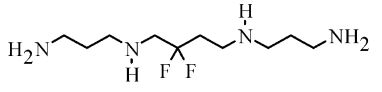
Potentiometry [12, 13, 14] is the most commonly used and the least expensive technique for protonation constant determination, but it cannot be used in real or simulated biological conditions. The same limitation applies to calorimetry, UV/Vis or infrared spectroscopy, and chromatography, though these techniques do provide valuable information concerning the protonation of molecules with many potential protonation sites [15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29]. NMR spectroscopy, on the other hand, though expensive and time consuming, can be used to study complex systems [18, 24, 26, 30, 31, 32, 33, 34] under experimental conditions close to those found in biological macro-aggregates and cells. This is possible because the variation of chemical shift of a substance, as a function of pH, is independent of the presence of other protolytic substances, considered as impurities in this context, as long as the impurities do not interact directly with the substance itself. We have recently shown that macro-protonation constants of spermine obtained from ^{13}C NMR data with the computer program HypNMR [30] were comparable in both accuracy and precision with the values obtained from potentiometric measurements.

We now report the results of a study of the protonation behaviour of the following fluorinated polyamines (structures in Fig. 1): 6-fluorospermine (1), 6,6-difluorospermine (2), 6-fluorospermidine (3) and 6,6-difluorospermidine (4) at 40 °C in D_2O solution in the pD range 3-12. These experimental conditions are the same that will be used in studies of the interactions of polyamines with synthetic liposomes mimicking cellular membranes. The macro-protonation constants have been obtained from ^{13}C NMR data using the new program HypNMR2000 and the protonation enthalpies have been obtained from microcalorimetric measurements. The effects of fluorine substitution on pK_a and $\Delta H°$ values of spermine (5) and spermidine (6) have been examined.

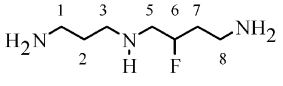
1. 6-monofluorospermine



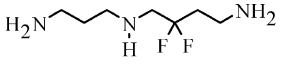
2. 6,6-difluorospermine



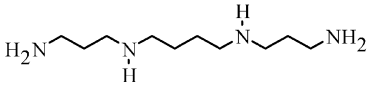
3. 6-monofluorospermidine



4. 6,6-difluorospermidine



5. spermine



6. spermidine

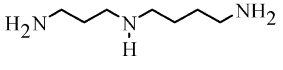


Fig. 1 Structures of the polyamines

Experimental section

Chemicals

Samples (ca. 50 mg) of the hydrochlorides of 6-fluorospermine (1), 6,6-difluorospermine (2), 6-fluorospermidine (3) and 6,6-difluorospermidine (4) were kindly provided by Marion Merrell Dow Research Institute, Strasbourg, France (present address: Hoechst Marion Roussel, Cincinnati, OH, USA.), and were kept in a desiccator at -20 °C over silica gel. Spermine (5) tetrahydrochloride and spermidine (6) trihydrochloride were from Sigma Chemical Co. All these products were used without further purification.

D_2O, KOD, and DCl were obtained from Merck (Germany; Uvasol grade, 99.9%); sodium 3-(trimethylsilyl)propionate-d_4 (TSP) was supplied by Wilmad (U.S.A.). All other chemicals were analytical grade (Merck and Aldrich) and were used as received.

NMR spectra

^{13}C spectra at natural abundance were recorded on a AMX-400 WB Bruker spectrometer, operating at 9.395 Tesla, with a reverse ^{1}H/BB 5 mm probe head. All spectra were acquired at 40 °C (controlled by Eurotherme 3000 VT control unit), with deuterium as lock channel and using TSP (sodium 3-(trimethylsilyl)propionate-d_4) as an internal standard for chemical shifts. Experimental parameters were as follows: base spectral frequency = 100.61 MHz, spectral width = 10,000 Hz (about 100 ppm), 64 K data points in the time domain and 64 K data points in the frequency domain, pulse width = 6.0 µs (about 49° flip angle), acquisition time = 2.16 s, number of scans = 1024. Spectra were acquired using WALTZ-16 pulse sequence for proton decoupling (proton rectangular soft pulse = 95.0 µs). FIDs were processed with enhancement multiplication (line broadening = 1 Hz).

The pH meter was calibrated in terms of deuterium ion activity and the samples were prepared as described previously [30]. 16 to 21 sample solutions were prepared for each of the fluorinated polyamines 1–4, the analytical concentration of the polyamine being close to 20 mM and the pD ranging from 3.2 to 12.4.

For each polyamine the first solution was prepared in a polypropylene Eppendorf tube by dissolving approximately 5 mmol of the hydrochloride in 0.50 cm^3 of D_2O. A small amount of TSP (few mm^3 of a 0.1 mol dm^{-3} D_2O solution) was also added. The pD of the solution was set by addition of a few mm^3 of KOD or DCl solution. The solution was then transferred into a 5 mm NMR tube for measurement of the spectrum. The solution was then transferred back (at least 95% recovery) and pD was measured again. Agreement of the pD values before and after measurement of the nmr spectrum was within 0.02 pD units. A known volume (a few microlitres) of KOD or DCl were then added and the sequence – measure pD, record spectra, measure pD, add base or acid – was repeated 15-20 times. Great care was taken to minimise solution losses and to obtain precise measurements of volumes, pD and spectra.

No attempt was made to keep the ionic strength of the solutions, I, constant as is usually done in order to make the quotient of activity coefficients into a constant quantity. In fact the activity coefficients calculated with the Davies equation [35]:

$$\log \gamma_i = -Az_i^2\left[I^{1/2}/(1 + I^{1/2}) - 0.3I\right] \quad (1)$$

did not vary significantly over the range of ionic strengths (between 0.1 and 0.2 mol dm^{-3}) used for the NMR measurements. In particular, the activity coefficient values for the deuterium ion, $-0.11 < \log \gamma < -0.13$, is effectively constant so that the activity of the deuterium ion, a_D, can be converted into concentration, $[D^+]$, by means of the simple formula:

$$-\log\left[D^+\right] = \mathrm{pD} - 0.12 \quad (2)$$

NMR data analysis.

The determination of the equilibrium constants of 1-4 was carried out using the computer program HypNMR2000. The chemical shifts of all the peaks present in the ^{13}C NMR spectra of 1 and 3 were used in the refinement calculations. For compounds 2 and 4 the signals due to the quaternary C_6 carbon atoms were ignored due to their having a low signal to noise ratio. It is usual for C atoms with no hydrogens attached to them to give noisy ^{13}C NMR signals. For the other ^{13}C signals the precision on the chemical shift measurements was estimated to be ±0.002 ppm. The total number of experimental values for each system ranged from 91 to 189.

^{1}H NMR data could not be included in the calculations, as the assignment of the peaks, which is an essential part of the computational process, proved to be impossible on account of the wealth of fine structure and extensive overlap. ^{19}F NMR spectra were not collected, principally because it was expected that the signal from a fluorine atom in position 6 of the polyamine chain would be sensitive only to changes in the degree of protonation of a single secondary nitrogen and hardly at all to the others; this information would not be sufficient for a calculation of all the protonation constants.

Calorimetric measurements

The enthalpies of reaction were determined in 0.15 mol dm^{-3} NaCl solution at 25 °C by means of an automated system composed of a Thermometric AB thermal activity monitor (model 2277) equipped with 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 $\pm 2\times10^{-4}$ K. The microcalorimeter was checked by determining the enthalpy of reaction of strong base (NaOH) with strong acid (HCl) solutions. The value obtained, -56.7(2) kJ mol^{-1}, is in good agreement with literature values [36]. The enthalpies of protonation of the polyamines 1-6 were determined as previously reported for different compounds [37]. In a typical experiment, a 0.1 mol dm^{-3} NaOH solution was added in about 20 successive steps (15 µl) to a solution containing the polyamine hydrochloride (1.3 cm^3, 0.05 mol dm^{-3}, ionic strength adjusted to 0.15 mol dm^{-3} by NaCl) until the polyamine was completely deprotonated (pH ca. 11). Corrections for the heats of dilution were applied. Titrations (about 20 data points) were performed at least in duplicate for each system.

The enthalpies of protonation were determined from the calorimetric data by means of the computer program AAAL [38]. The procedure involves the calculation of the concentration of each species on the basis of a set of "known" protonation constants such that the total enthalpy can be partitioned between the individual protonation equilibria. However, since the protonation constants for 1-5 were determined at 40 °C both the constants and enthalpies had to be adjusted to 25 °C by an iterative procedure. The first set of $\Delta H°$ values was used to correct the protonation constants to 25 °C, using the van't Hoff equation. New $\Delta H°$ values could then be calculated and the constants adjusted again, repeatedly, until the values became self-consistent.

HypNMR2000The computer program HypNMR [30] was written for the DOS operating system as a set of stand-alone FORTRAN programs. This program has now been enhanced and adapted to run as a single entity under the Windows operating systems. The stability constant refinement part is based on the original FORTRAN program, but now compiled with a 32-bit compiler for additional precision. This program reads an input file and creates an output file. The data is prepared with a Visual Basic (VB) program that runs as a typical Windows application. The VB program supports various types of data input and includes an interactive manual fitting routine for the purpose of finding initial stability constant estimates. Once reasonable agreement is found between observed and calculated chemical shifts the data may be saved to a file and the FORTRAN program is called to read that file and perform the stability constant refinement. At each refinement iteration the chemical shifts of the individual species, δ, are calculated by linear least-squares using the current set of stability constants.

The output file can also be read by the VB program. This file contains information not only on the refined stability constants but also on all the species' concentrations, calculated chemical shifts, etc. The refined stability constants can be substituted for the initial values and then the fit can be examined visually using the manual fitting routine.

HypNMR2000 has no restrictions on the number of stability constants, the number of reagents, the number of resonant nuclei recorded, the number of data points and so forth. The data may include pH values, as in the present work, or not, as would be the case in a study of complexation equilibria in aqueous or non-aqueous solution. Further details are available at http://www.chem.leeds.ac.uk/People/Peter_Gans/hypnmr.htm (case-sensitive).

Results

Chemical structures of the compounds 1-6 are shown in Fig. 1. The carbon atoms are numbered following Baillon et al. [39]. Observed ^{13}C chemical shift profiles as a function of pD are illustrated in Fig. 2. The signals were assigned on the basis of the relative positions of the peaks, on a previous assignment of ^{13}C NMR spectra of spermine [30] and on the observation that carbon resonances C_5 and C_7 are split by ^{19}F on adjacent carbon atoms with a relatively small $^{2}J_{CF}$ coupling constant. C_5 and C_7 present as doublets in the mono-fluoro compounds 1 and 3 ($^{2}J_{CF}$ = 19–20 Hz) and as triplets in the difluoro-compounds 2 and 4 ($^{2}J_{CF}$ = 23–24 Hz).

The most deshielded signal, assigned to C_6, appears as a doublet for compounds 1 and 3 with a relatively large $^{1}J_{CF}$ coupling constant (169–170 Hz). The C_6 signal in compounds 2 and 4, where this carbon atom has no hydrogen atoms attached, has a very low S/N ratio. The shift is around 120 ppm; excessively long spectral acquisition times (collection of a very large number of transients) would have been needed to obtain more precise values. Ambiguities may still remain regarding the assignments of C1 and C12, C2 and C11, C3 and C10.

Calculated chemical shifts of the non-equivalent ^{13}C nuclei in D_iL^{i+} (I = 0–4) are given in Table 1.

The stepwise (deuteron) protonation constants are defined as in

$$D_{i-1}L^{(i-1)+} + D^{+} \rightarrow D_iL^{i+}\ ;\ K_i = \frac{\left[D_iL^{i+}\right]}{[D^{+}]\left[D_{i-1}L^{(i-1)+}\right]}, \quad (4)$$

(L=polyamine) and values of log K_i are given in Table 2. These values can be converted into proton basicity constants by applying the semiempirical formula due to Dagnall et al. [40]:

$$\log K_{(H_2O)} = \log K_{(D_2O)} - 0.63 \quad (5)$$

The pK_a values for stepwise de-protonation are numerically equal to the log K values as defined above. These are called macro-constants and as such are the sum of all the micro-constants relating to tautomers having the same stoichiometry.

The protonation enthalpies of compounds 1–5 were obtained by the iterative procedure described above. The iterative process was not needed for spermidine (6) as the

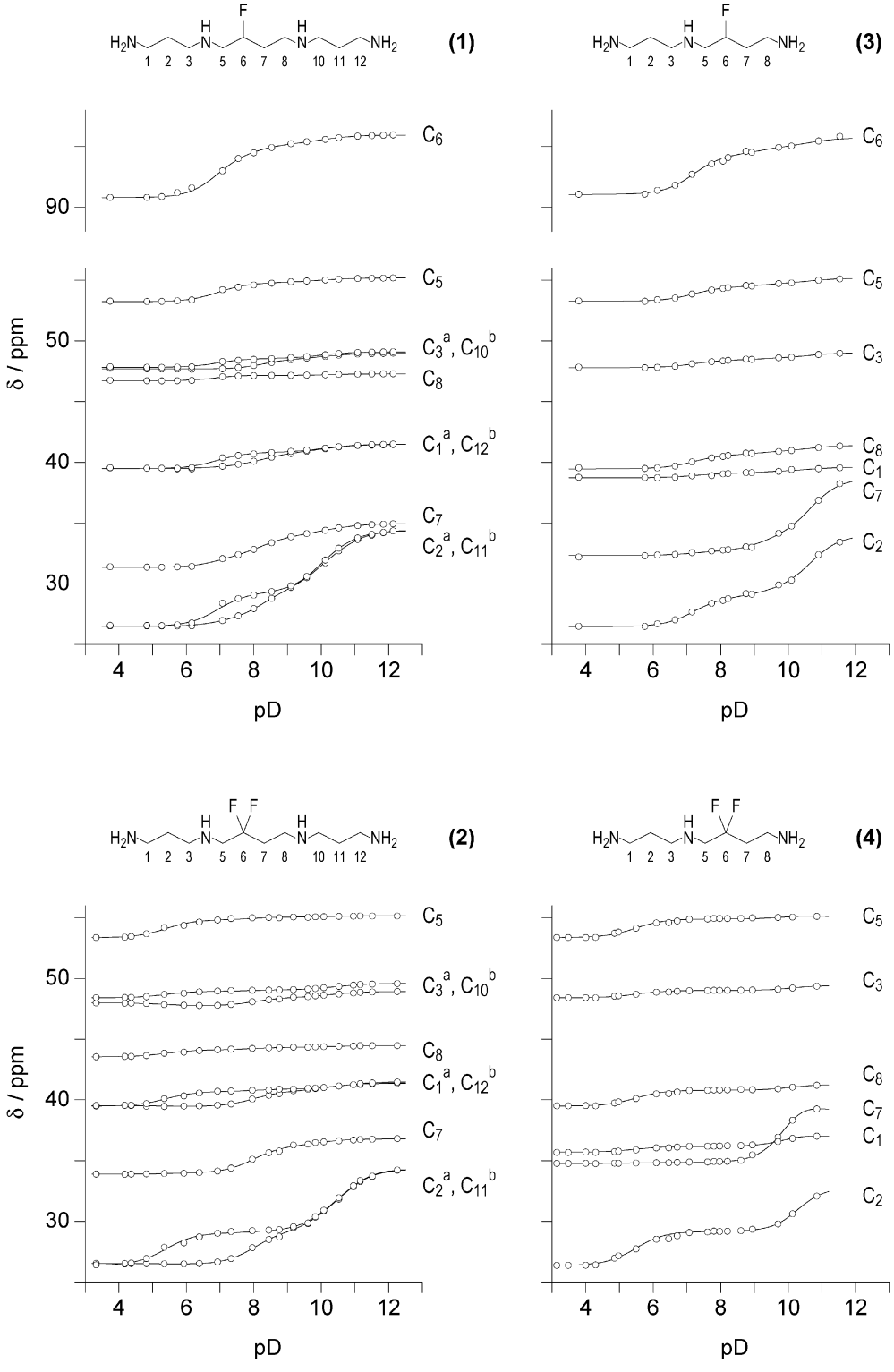


Fig. 2 Experimental ^{13}C NMR chemical shifts (δ, ppm) as a function of pD at 40 °C: 6-fluorospermine (1), 6,6-difluorospermine (2), 6-fluorospermidine (3) and 6,6-difluorospermidine (4). The *solid lines* are drawn by joining calculated values, obtained using the refined equilibrium constants and the individual chemical shifts shown in Tables 1 and 2. The superscipts a and b for the pairs of nuclei with very similar chemical shifts (C1, C12; C2, C11; and C3, C10) refer to the *upper* and *lower lines*, respectively

basicity constants used for the calculation of the enthalpies were based on published values [39] related to measurements at 25 °C.

Discussion

The thermodynamic functions for stepwise protonation of compounds 1-6 are given in Table 3. The values of the protonation constants for spermine are a little different from the values that we published previously [30] due to

Table 1 Refined values of ^{13}C chemical shifts (ppm) for the various species of the polyamines at 40 °C in D_2O[a]

Compound	Nucleus	L	DL^+	D_2L^{2+}	D_3L^{3+}	D_4L^{4+}
1	C_1	41.49(4)	41.31(4)	40.83(2)	40.77(4)	39.47(2)
	C_2	34.41(3)	32.72(5)	29.58(4)	27.13(3)	26.49(5)
	C_3	49.09(5)	49.00(4)	48.56(4)	48.51(2)	47.81(3)
	C_5	55.18(2)	55.10(3)	54.83(5)	54.66(5)	53.22(4)
	C_6	95.95(4)	95.76(4)	95.23(2)	94.44(5)	90.79(2)
	C_7	34.94(5)	34.61(4)	33.95(5)	32.34(2)	31.34(3)
	C_8	47.29(3)	47.23(5)	47.14(4)	47.20(3)	46.70(5)
	C_{10}	48.99(4)	48.82(2)	48.50(3)	47.68(4)	47.69(4)
	C_{11}	34.37(4)	33.17(2)	29.39(3)	29.11(4)	26.49(4)
	C_{12}	41.46(2)	41.29(3)	40.78(5)	39.72(5)	39.48(4)
2	C_1	41.48(4)	41.05(3)	40.89(3)	40.68(5)	39.48(9)
	C_2	34.32(7)	31.58(4)	29.23(3)	29.04(9)	26.38(5)
	C_3	49.59(3)	49.31(3)	49.05(4)	48.95(3)	48.40(4)
	C_5	55.16(4)	55.09(7)	55.06(4)	54.83(4)	53.35(3)
	C_6					
	C_7	36.78(3)	36.72(3)	36.23(4)	33.98(3)	33.88(5)
	C_8	44.47(3)	44.40(4)	44.33(7)	44.12(3)	43.54(3)
	C_{10}	48.93(7)	48.72(4)	48.42(3)	47.73(8)	47.98(5)
	C_{11}	34.27(3)	31.55(4)	29.13(7)	26.46(4)	26.52(3)
	C_{12}	41.37(4)	41.19(7)	40.71(4)	39.45(4)	39.52(3)
3	C_1	41.39(6)	40.93(4)	40.62(3)	39.46(7)	
	C_2	34.00(4)	29.65(3)	28.9(1)	26.47(3)	
	C_3	49.03(4)	48.60(3)	48.42(6)	47.78(4)	
	C_5	55.15(3)	54.74(6)	54.44(6)	53.26(4)	
	C_6	95.7(1)	95.00(7)	94.35(5)	91.06(7)	
	C_7	38.68(3)	33.99(6)	32.69(7)	32.33(4)	
	C_8	39.59(6)	39.28(6)	39.08(4)	38.72(6)	
4	C_1	41.26(1)	41.09(2)	40.80(7)	39.48(8)	
	C_2	32.80(2)	30.35(8)	29.17(1)	26.37(3)	
	C_3	49.42(2)	49.24(7)	49.00(9)	48.40(2)	
	C_5	55.08(7)	55.2(1)	54.90(2)	53.36(1)	
	C_6					
	C_7	39.12(7)	40.2(1)	34.85(1)	34.76(2)	
	C_8	37.0(1)	37.20(2)	36.17(2)	35.7(1)	

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

Table 2 Logarithms of the Stepwise Basicity Constants of 6-fluorospermine (1), 6,6- difluorospermine (2), 6-flurospermidine (3), 6,6-difluorospermidine (4), and spermine (5) determined at 40 °C in D_2O[a]

Compound	log K_1	log K_2	log K_3	log K_4	Number of points
1	10.9 (1)	9.91 (7)	8.27 (4)	6.90 (4)	166
2	10.9 (1)	10.0 (1)	7.99 (4)	5.39 (3)	189
3	10.69 (5)	9.2 (1)	7.19 (3)		91
4	10.4 (2)	9.95 (6)	5.48 (2)		114
5[b]	11.20 (2)	10.30 (4)	9.05 (4)	8.18 (2)	200

[a]Values in parentheses are estimated standard deviations on the last significant figure
[b]From ref. [30]

Table 3 Thermodynamic functions for the stepwise protonation of spermine (5), 6-fluorospermine (1), 6,6-difluorospermine (2), spermidine (6), 6-flurospermidine (3) and 6,6-difluorospermidine (4) calculated at 25 °C in H_2O[a]

Compound	Step	log K	$-\Delta G°$ kJ mol^{-1}	$-\Delta H°$ kJ mol^{-1}	$\Delta S°$ J K^{-1} mol^{-1}
5[b]	1	10.98(7)	62.7(4)	49.2(2)	45(2)
	2	10.09(8)	57.6(4)	51.7(2)	20(2)
	3	8.83(8)	50.4(4)	49.8(2)	2(2)
	4	7.94(7)	45.3(4)	47.3(2)	–7(2)
1	1	10.7(2)	61.1(9)	50.2(7)	36(5)
	2	9.7(1)	55.4(6)	51.2(5)	14(4)
	3	8.04(8)	45.9(5)	47.9(5)	–7(3)
	4	6.62(8)	37.8(5)	41.5(4)	–13(3)
2	1	10.7(1)	60.8(8)	50.2(6)	36(5)
	2	9.8(2)	55.7(9)	51.7(5)	13(5)
	3	7.74(8)	44.2(4)	45.1(3)	–3(3)
	4	5.11(7)	29.2(4)	39.8(4)	–36(3)
6	1	10.89[c]	62.2[c]	52.4(2)	32.9(5)
	2	9.81[c]	56.0[c]	54.0(1)	6.7(6)
	3	8.34[c]	47.6[c]	48.2(1)	–2.0(5)
3	1	10.49 (9)	59.9(8)	51.7(4)	28(3)
	2	9.0(2)	51.6(9)	53.5(3)	–6(4)
	3	6.91(8)	39.4(4)	41.5(3)	–7(2)
4	1	10.2(2)	58.1(9)	50.1(7)	27(5)
	2	9.76(9)	55.7(5)	52.6(5)	10(3)
	3	5.16(7)	29.5(4)	36.7(4)	–24(3)

[a]Values in parentheses are estimated standard deviations on the last significant figure
[b]From ref. [30]
[c]Values at 298 K and I = 0.1 mol dm^{-3} (KNO_3) taken from ref. [54].

the fact that we have re-determined the protonation enthalpies used to adjust the values to 25 °C, whereas before we used the enthalpies published by Palmer et al. [41]. The basicity constants for 3 and 4 have been published by Baillon et al. [39] from potentiometric data obtained at 25°C in the medium 0.1 M KNO_3. Their values, log K_1 = 10.40, log K_2 = 9.55, log K_3 = 7.18, for (3), and log K_1 = 10.34, log K_2 = 9.29, log K_3 = 5.70, for (4) are in satisfactory agreement with the values obtained in the present work.

Distribution diagrams of the various protonated species of the polyamines 1-4 were calculated using the program HYSS [42] and are shown in Fig. 3. No single species is present at any pH with the exception that in 6,6-difluorospermidine (4) H_2L is the predominant species in the pH-range 5–8.

The standard protonation enthalpies for spermine (5) and spermidine (6) obtained in this work are compared with published values in Table 4. In general the agreement is within three standard deviations except for the enthalpy for the first protonation step. We find that the $-\Delta H°$ value for the first protonation is less than that for the addition of the second proton in both these cases and also with the fluorinated polyamines (Table 4).

The macro-protonation constants of spermine and spermidine decrease with fluoro substitution and decrease

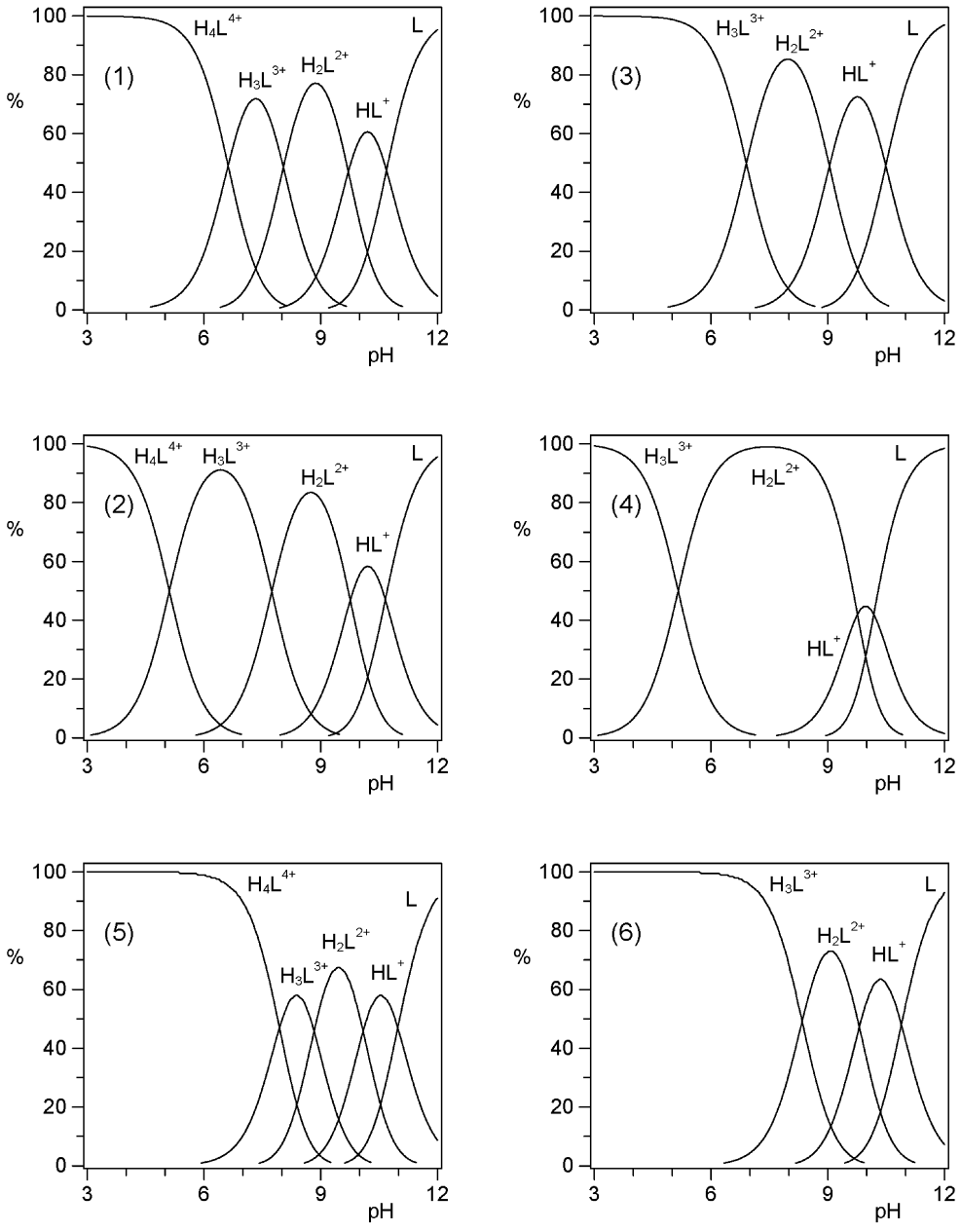


Fig. 3 Calculated species distribution plots for the fluorinated polyamines: 6-fluorospermine (1), 6,6-difluorospermine (2), 6-fluorospermidine (3), 6,6-difluorospermidine (4), spermine (5) and spermidine (6)

again as a second fluoro-substituent is introduced, as shown in Fig. 4. This reduction in the pK_a values is a direct result of the high electronegativity of fluorine: electron density is pulled towards the fluorine atoms via the methylene groups from the nearest nitrogen atom(s), so reducing the nitrogen atom's basicity. The effect is presumably more marked the closer the nitrogen atom, which undergoes protonation, is to a fluorine substituent.

The values of the thermodynamic functions, $\Delta G°$, $\Delta H°$ and $\Delta S°$, for each protonation step of the polyamines 1-6 are given in Table 3. The trends are illustrated in Fig. 5: the values both of $-\Delta G°$ and of $\Delta S°$ decrease as more protons are added in all cases. However the standard enthalpy change, $-\Delta H°$, is largest, by a small amount, for the second protonation step. This trend has been reported for other open-chain polyamines [43].

Table 4 Standard enthalpy change, $-\Delta H°$ / kJ mol^{-1}, for the stepwise protonation of spermine and spermidine in H_2O at 25 °C

Compound	Step	This work[a]	Ref. [41][b]	Ref [54][c]	Ref [27][d]
Spermine	1	49.2(2)	55.0		
	2	51.7(2)	52.0		
	3	49.8(2)	52.0		
	4	47.3(2)	47.0		
Spermidine	1	52.4(2)		56.7(7)	56.0(1)
	2	54.0(1)		53.5(4)	53.4 (2)
	3	48.2(1)		48.9(5)	48.9(2)

[a] I = 0.15 mol dm^{-3} NaCl
[b] I = 0.1 mol dm^{-3} KNO_3
[c] I = 0.1 mol dm^{-3} KNO_3
[d] I = 0.1 mol dm^{-3} NaCl

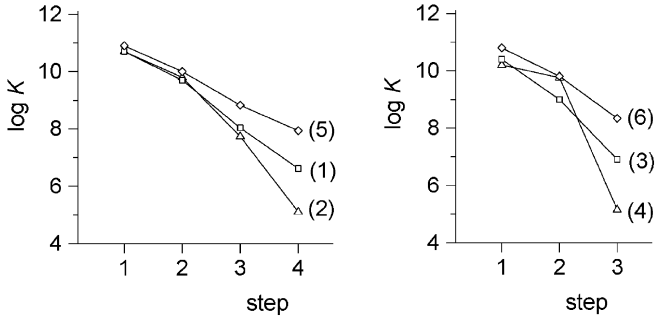


Fig. 4 Stepwise protonation constants: 6-fluorospermine (1), 6,6-difluorospermine (2), 6-fluorospermidine (3), 6,6-difluorospermidine (4), spermine (5) and spermidine (6)

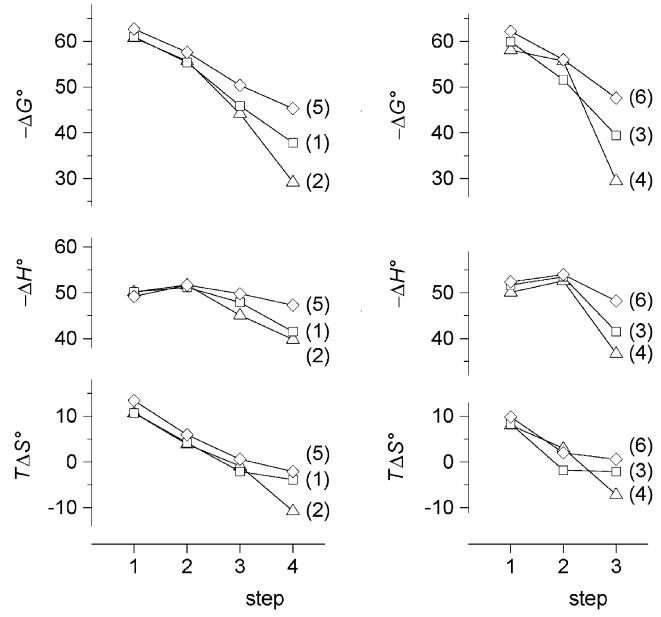


Fig. 5 Thermodynamic functions (kJ mol^{-1}) for the stepwise protonation of: 6-fluorospermin(1), 6,6-difluorospermine (2), 6-fluorospermidine (3), 6,6-difluorospermidine (4), spermine (5) and spermidine (6)

The protonation sequence of linear polyamines is still today a matter for controversy. Some authors suggest that the primary (terminal) amine groups are the first to be protonated and that the secondary amine groups (internal) accept protons only after the terminal groups are protonated [34, 39]. Other authors have suggested that protons are in rapid exchange between various acceptor sites resulting in an equilibrium mixture of tautomers [31, 33, 44, 45]. We have previously suggested that the first protonation stage involves both primary and secondary amino groups [43], but when a second proton is added electrostatic repulsion causes any protons on secondary amino groups to migrate towards terminal positions. This proposal is based on the facts that (*i*) in aqueous solution secondary amino groups are more basic than primary amino groups (have higher pK_a values) and so have a higher affinity for protons and (*ii*) the protonation of a primary amino group is more exothermic than the protonation of a secondary or tertiary amino group. These facts provide the basis for a rational explanation of the trends previously found in the stepwise protonation enthalpies.

The same trend has been found for all the polyamines investigated in this study, namely, $-\Delta H^\circ_1 < -\Delta H^\circ_2$. Therefore the same protonation sequence can be invoked also for these polyamines.

As can be seen in Table 3, the decrease in basicity with increasing substitution of hydrogen by fluorine is determined by both enthalpy and entropy factors. For example, the values of $-\Delta H^\circ$ for compounds 5, 1 and 2 are about the same for the first and second protonation steps, decrease a little for the third step and decrease markedly for the fourth step. A similar sequence is to be seen for compounds 6, 3 and 4. The values of ΔS° are also markedly less favourable for the last step compared to the first. Thus, both enthalpy and entropy become less favourable as a result of fluoro–substitution.

Hague et al [46, 47] have shown that the ^{13}C chemical shifts of linear aliphatic polyamines, where the nitrogen atoms are separated by two or three methylene groups, can all be expressed in terms of a 2-parameter empirical equation:

$$\delta_{calc} = \pi_1(\text{or } \pi_1^+) + \pi_2(\text{or } \pi_2^+) \qquad (6)$$

It is assumed that the chemical shift, δ, of a ^{13}C nucleus is a linear combination of two terms which depend on the nature of the nearest amino groups. Both terms depend on (*i*) the nature of the adjacent amino groups, be they primary or secondary (*ii*) the electrical charge, π^+ for a protonated group, π for a non-protonated group (*iii*) the position of the carbon atom relative to the nitrogen atom (α, β or γ).<001><fn><001> Both Hague's and our measurements were made in a solvent which mainly consisted of D_2O. For the sake of simplicity deuteriated species such as DL^+ and D_2L^{2+} may, in what follows, be represented as HL^+ and H_2L^{2+} and may be referred to as protonated species. Likewise basicity constants involving deuterium may be described as protonation constants and deuteriated amino groups may be described as protonated groups. Hague's values are shown in Table 5.

Applying Hague's formula to spermine, the chemical shifts of the carbon atoms both in the free polyamine and in its fully protonated form (reported in Table 1) were found to be systematically lower than the observed values by 1.67 ±0.1 ppm. This difference is mainly due to the fact that Hague used dioxane as internal standard, with shifts

Table 5 Individual ^{13}C amine shift parameters for primary and secondary amino groups / ppm[a]

	α-Position	β-Position	γ-Position
NH_2	26.1	18.0	13.4
NH_3^+	24.5	13.0	13.2
NH	34.0	14.9	13.8
NH_2^+	32.3	11.9	13.2

[a]From ref. [55]. The estimated errors are generally ± 0.1 ppm

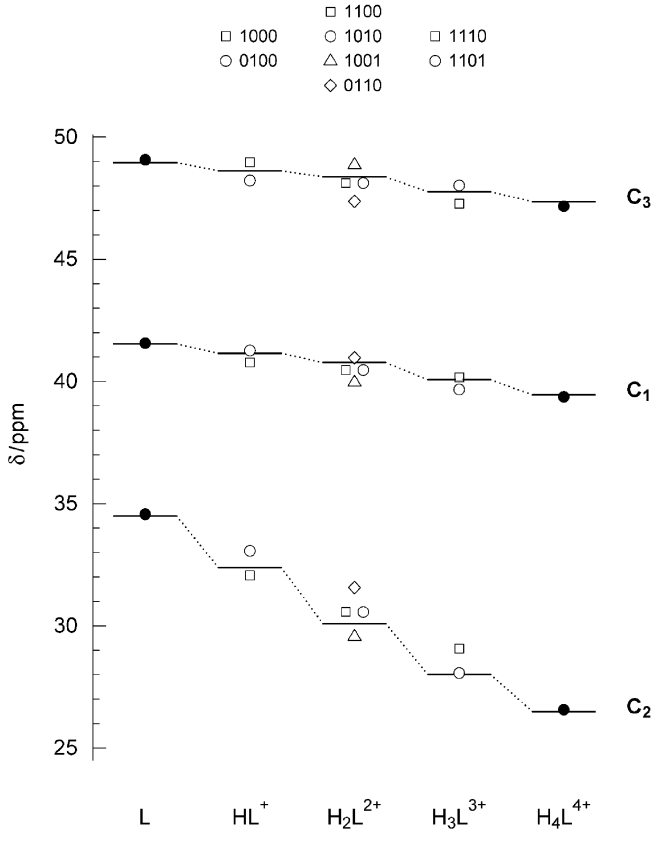


Fig. 6 ^{13}C NMR chemical shifts (δ, ppm) for the carbon atoms in the fragment $N[CH_2]_3N$ of spermine (5). The chemical shifts of the individual species H_iL^{n+} (i = 0–4) obtained in the least squares refinement of the protonation constants are given by *horizontal lines*. *Solid circles* represent the values appropriate to unprotonated and fully protonated species. The *hollow symbols* represent the values δ_{calc} evaluated by Eq. 7 for the microstates in which protonation has occurred at the nitrogens identified by 1 for a protonated atom and 0 for an un-protonated atom

corrected by 67.71 ppm to TMS, whilst we used as internal standard TSP which has a shift of 1.7 ppm with respect to TMS. Also, a different temperature was used.

$$\delta_{calc} = \pi_1\left(\text{or } \pi_1^+\right) + \pi_2\left(\text{or } \pi_2^+\right) + 1.67 \tag{7}$$

Eq 7, which includes a correction for the internal standard, was applied to the variously protonated species of all the polyamines investigated, including the micro-species. The values calculated by this method are compared with the macro-constants obtained with HypNMR2000 in Fig. 6. Information concerning the protonation sequence can be obtained from this comparison.

In Fig. 6 the ^{13}C chemical shifts of C_1, C_2 and C_3 in the species H_iL^{i+} (i = 0–4), obtained by refinement with HypNMR2000, are shown as horizontal lines; the values calculated by means of the modified Hague's formula, Eq. 7, are shown as filled circles for i = 0 and 4, and as open symbols for i = 1–3. Each micro-state is represented by a four-digit binary string in which 0 or 1 are used to specify whether a nitrogen atom is (1) or is not (0) protonated. Thus, 1000 represents the micro-species $H_3N^+(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$ and 0110 represents the micro-species $H_2N(CH_2)_3NH_2^+(CH_2)_4NH_2^+(CH_2)_3NH_2$. (N.B. Spermine is symmetric about the central C-C bond, so 1010 and 0101 are one and the same micro-species).

The result is very satisfactory. There is excellent agreement for L and H_4L^{4+}, where only one micro-state is possible, between "observed values" (δ, i.e. values derived from observed spectra by means of HypNMR2000) and values calculated from Eq. 7. For the partially protonated species δ is seen to be between the values obtained for the micro-species. In fact δ is a concentration-weighted average of the shifts of the micro-species. Thus, the nearer the shift of a micro-species is to δ, the greater the fractional population of that micro-species should be. Unfortunately the errors on δ_{calc} of ca. ±0.2 ppm are too large to allow reliable quantitative conclusions to be reached, that is, calculated micro-constants are subject to excessive error. However, it is clear from Fig. 6 that when spermine is partially protonated all possible tautomers are present in the equilibrium mixture. In particular it is clear that the monoprotonated species is a mixture of 1000 and 0100, that is, both the terminal primary amino group and the secondary amino groups are involved in the first protonation step.

A similar treatment has been applied to the fluorinated polyamines. When one or two fluorine atoms are present at position 6 in spermine C_1 and C_{12} become non equivalent, as do the pairs C_2, C_{11} and C_3, C_{10}. The spectra therefore are more complicated, but the overall situation is similar. Fig. 7 is illustrative of the results obtained with the fluorinated polyamines.

Recently, Borkovec and Koper have published "A cluster expansion method for the complete resolution of microscopic ionisation equilibria from NMR titrations" [48]. The mathematical basis for this method can be briefly summarised as follows. It is assumed that there is a series of micro-protonation reactions of the type:

$$A\{s\} + H^+ \rightarrow A\{s'\} \tag{8}$$

between two generic micro-states, s and s' of a base containing N sites capable of accepting a proton. Each micro-state is specified by an N-digit binary string of 0 and 1, as above. On protonation of the ith site the corresponding binary digit changes from 0 to 1. The micro-constant for this protonation reaction, log$K$, is expressed as a series expansion in terms of so-called *cluster parameters*, as shown:

$$\log K = p\hat{K}_i - \sum_j^N \varepsilon_{ij} s_j - \frac{1}{2}\sum_{j,k}^N \lambda_{ijk} s_j s_k - \ldots \tag{9}$$

where $p\hat{K}_i$ is the micro-constant for protonation of the site when all other sites are deprotonated and $\varepsilon_{ij}$and λ_{ijk} are parameters for interaction with 2 or 3 neighbouring sites. If the series is limited to primary interactions Eq. 9 becomes the simpler:

$$\log\ K = p\hat{K}_i - \sum_j^N \varepsilon_{ij} s_j \tag{10}$$

in which the coefficients represent the decrease due to the presence of a proton on site j. The influence of protonation on chemical shift is given by:

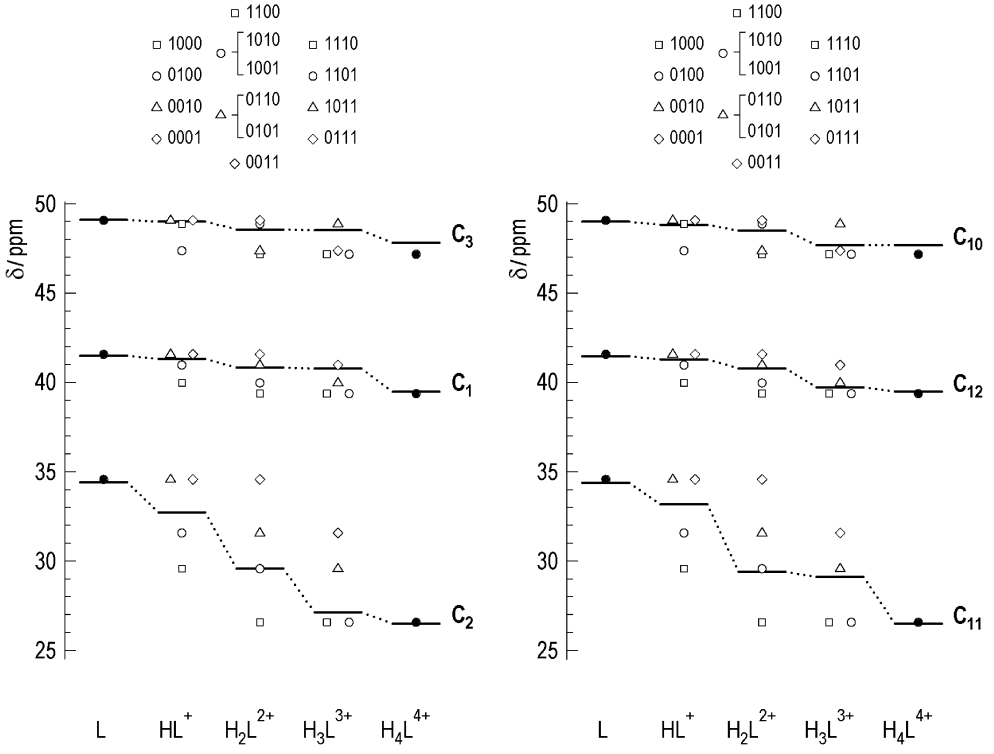


Fig. 7 ^{13}C NMR chemical shifts (δ, ppm) for the carbon atoms in the two fragments $N[CH_2]_3N$ of 6-fluorospermine (1). Symbols as in Fig. 6

$$\delta_l = \delta_l^{(0)} + \sum_m \Delta_{lm}\theta_m \qquad (11)$$

in which the terms $\delta_l^{(0)}$ and Δ_{lm} are called *chemical shift parameters*: $\delta_l^{(0)}$ is the value for the fully deprotonated base, Δ_{lm} is the change caused by protonation of the site m and θ_m is the degree of protonation of that site.

With this model, the observed chemical shifts are expressed as a function of four types of parameter, as shown:

$$\delta = f\left(\mathrm{p}\hat{K}, \varepsilon, \delta^{(0)}, \Delta\right) \qquad (12)$$

These parameters were optimised by non-liner least-squares refinement, using Powell's conjugate directions method [49], of the sum of squared residuals between observed and calculated chemical shifts.

Applying this procedure to spermine, there are 16 parameters, which comprise two $\mathrm{p}\hat{K}$ values, one for the primary (terminal) amino group and one for the secondary (bridging) amino group, two ε values, one for the interaction between primary and secondary amino groups and the other for interaction between the two secondary amino groups, five $\delta_l^{(0)}$ values, one for each of the non-equivalent ^{13}C nuclei, seven Δ values, three of which relate to interactions with protonated primary amino groups in α, β and γ positions and the other four relating to the secondary amino groups in the α, β, γ and δ positions. The number of experimental chemical shift values is 100, so that the system is sufficiently over-determined to give statistically valid results. Refined parameters are shown in Fig. 8 and Table 6.

The agreement between observed and calculated chemical shifts is excellent with a root mean square residual of 0.05 ppm. The calculated errors on the parameters are small enough for the values to be well-defined. The calculated residuals are scattered more or less randomly. Furthermore, the macro-constants, obtained by adding together the apposite calculated micro-constants, log K_1=11.07, log K_2=10.42, log K_3=8.99 and log K_4= 8.30 are in good agreement with the values calculated with HypNMR (11.20, 10.30, 9.05 and 8.18) [30]. It is most interesting that the micro-basicity constant for the secondary amino group, $\mathrm{p}\hat{K}^{(\mathrm{II})}$, is only slightly higher than that of the primary amino group. The value of ε' is larger than the value of ε''. It is expected that the nearer the groups are to each other the stronger the interaction will be. The values of the cluster parameters obtained in this work can be compared with the values published for 1,4,7,10,13-pentaazatridecane (tetren, $H_2N(CH_2)_2NH(CH_2)_2NH(CH_2)_2NH(CH_2)_2NH_2$) [48]: $\mathrm{p}\hat{K}$ = 10.05 for the primary amino groups and 9.58 for the secondary amino groups. These values are lower than the values for spermine in line with the fact that the CH_2CH_2 units have a smaller inductive effect than the $CH_2CH_2CH_2$ unit in spermine. The value of ε, 2.49, is larger than that of spermine, as expected as the amino groups are closer together.

The calculation also provides chemical shifts for each nucleus in each micro-species, the micro-constants for protonation of all the non-protonated sites in each micro-species and the percentage of each micro-species present in the macro-species. The values are shown in Fig. 9. HL^+ is a mixture of approximately equal amounts of the micro-species 1000 and 0100. Thus, both primary and secondary amino groups are involved, to an approximately equal extent, in the first protonation step. This is in line with our

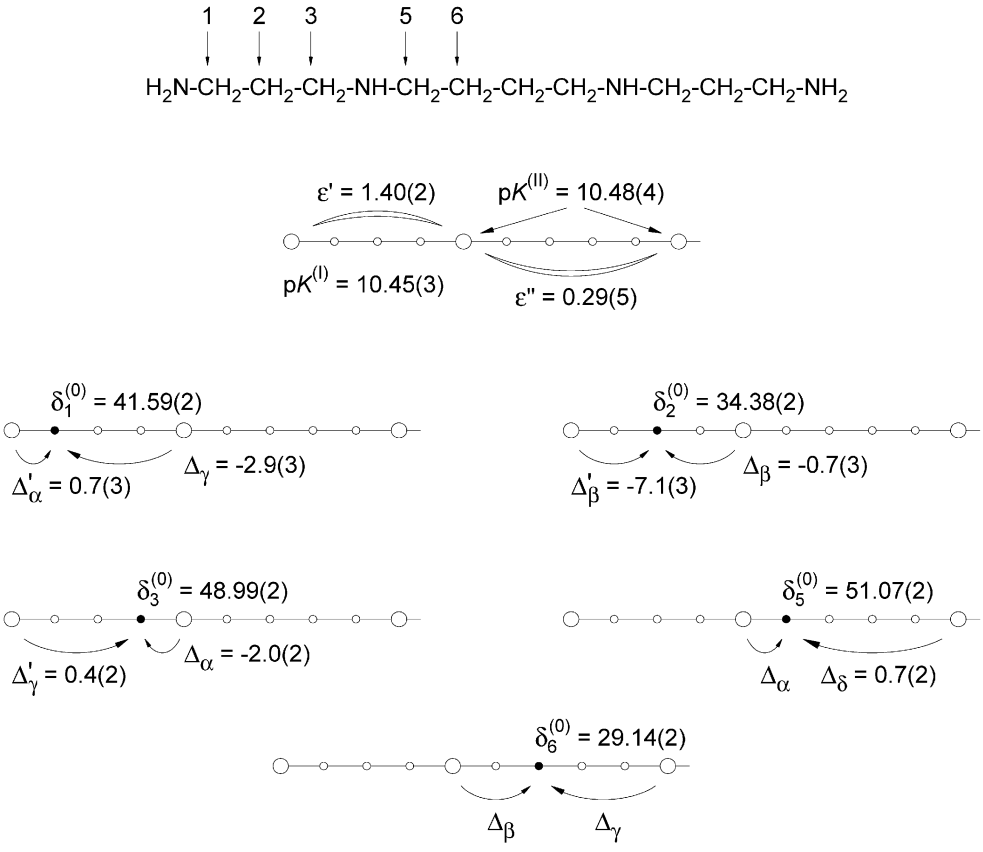


Fig. 8 Cluster and chemical shift parameters refined for spermine (5)

previous proposals for the protonation sequence [43]. H_2L^{2+} comprises 1010 and 1001 as the major species. 0110, in which the two secondary amino groups are protonated is a minor component. The micro-species 1100, in which a primary and adjacent secondary amino group are protonated, makes virtually no contribution to the ensemble of micro-states. This supports our previous suggestion that when two sites are protonated electrostatic repulsion will push the protons as far apart as is reasonable [43]. In this case, there are eight atoms in the chain between the protonated sites in the 1010 tautomer and this appears to be sufficient for this species to be present at about the same level as 1001, in which the protons are indeed as far apart as possible. For LH_3^{3+}, the micro-species 1101 is the major contributor as in 1110 the protonated amino groups are nearer to each other overall.

Compounds 1-4 were also subjected to cluster analysis and selected results of the calculations are shown in Table 6. It can be seen that the precision of the parameters for the fluorinated spermine derivatives is lower than for spermine itself (5) due to a lower S/N ratio in the data and/or the increased number of parameters needed for the non-centrosymmetric molecules. What is clear, however, is the reduction in the basicity, $p\hat{K}^{II}$, of the secondary amino groups which are near to the carbon carrying the fluorine substituents. All the other parameters are more or less constant.

The spatial distribution of protonated sites in the polyamines has an important bearing on the mechanism of activation of PKC. With spermine, the species distribution diagram, Fig. 3, shows that H_4L^{4+} is the principal species present at physiological pH (about 7); there is a very small amount of H_3L^{3+} and negligible amounts of the less protonated species. Indirect studies of the interaction of spermine with biological membranes and artificial membranes (liposomes) [50, 51, 52, 53], have led to the suggestion that the inhibition of PKC is mainly due to the fact that the 4 protonated amino groups interact with the negatively charged phosphate and carboxylate groups on PS in such a way that the spermine takes up a position flat on the membrane surface and so hinders the penetration of the lipid bilayer by the protein which is need for its activation. It was also suggested that the interactions between posi-

Table 6 Selected cluster parameters for 6-fluorospermine (1), 6,6-difluorospermine (2), 6-flurospermidine (3) and 6,6-difluorospermidine (4) and spermine (5)[a]

	$p\hat{K}^{I}$	$p\hat{K}^{II}$	$p\hat{K}^{II}$	$p\hat{K}^{I}$	ε'	ε''	σ (ppm)[b]
1	10.07(7)	8.76(4)	9.76(3)	10.15(6)	1.4(1)	0.5(2)	0.07
2	10.38(5)	7.30(5)	9.6(2)	10.5(2)	1.3(3)	0.5(3)	0.11
3	10.3(1)	8.44(8)	10.02(8)		0.8(2)	0.2(3)	0.15
4	10.5(1)	6.8(1)	9.8(2)		1.0(3)	0.2(4)	0.20
5	10.45(1)	10.48(2)			1.40(1)	0.29(5)	0.05

[a]Values in parentheses are standard deviations on the last significant figure

[b]Root mean square deviation

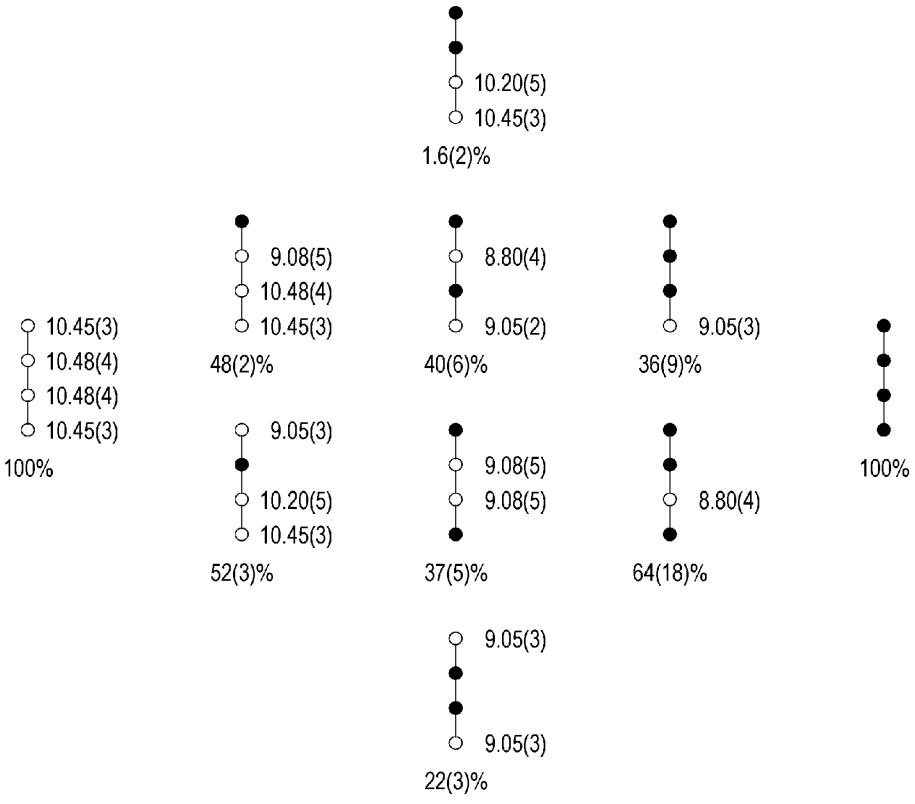


Fig. 9 Micro-constants and conditional probabilities for all the distinguishable micro-states of spermine (5), calculated using the parameters shown in Fig. 8. In each micro-state protonated amino groups are represented by *filled circles* and unprotonated ones by *empty circles*

tively and negatively charged sites on H_4L^{4+} and PS respectively cause the PS to form clusters which are not good receptors for PKC.

With compound 1 the speciation diagrams, Fig. 3, show that H_4L^{4+} is not the predominant species at pH ca. 7, having a concentration of less than 30% of the total ligand present. For compound 2 the concentration of H_4L^{4+} is negligible at pH 7. At pH 7 the principal species for both 1 and 2 is H_3L^{3+} (70%–80% formation). Now, this species can exist in 4 tautomers, 1011, 1101, 1110 and 0111. The analysis described above indicated that in the fluoro-substituted polyamines the secondary amino group nearest to the electron-withdrawing substituent is less basic, so protonation of the primary amino groups is more favourable than in spermine. This means that the microstate 1011 is likely to be the predominant one. In this case there are only two adjacent sites which are protonated as opposed to all four in spermine. Consequently the complexes formed by the fluorinated spermine species with PS are likely to be different from those formed by spermine itself and so the effect on the activation of PKC is likely to be different.

Similar considerations apply to spermidine and its fluorinated derivatives. At physiological pH the main species with spermidine is H_3L^{3+}, but for compound 3 there are roughly equal concentrations of H_3L^{3+} and H_2L^{2+} at the same pH and compound 4 is practically 100% H_2L^{2+} under the same conditions. The consequences in terms of the distribution of charge along the molecular backbone of spermidine are therefore quite similar to the consequences of fluorination on spermine.

Work is in progress to study the polyamine/lipid interaction directly by NMR spectroscopy. The objectives of this work are to ascertain (*i*) the molecular arrangement of spermine and its fluoro-derivatives on the surface of the lipid vesicles that contain PS and (*ii*) the stoichiometry of the spermine-PS and fluoro-spermine-PS complexes. These investigations should provide detailed information at the molecular level regarding the mechanism of PKC activation.

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