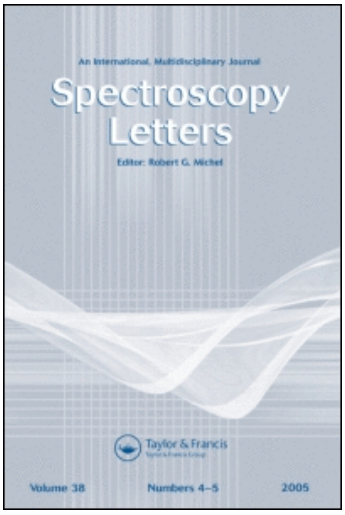

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STUDY OF THE PROTONATION/DEPROTONATION SEQUENCE OF TWO POLYAMINES: BIS-[(2S)-2-PYRROLIDINYLMETHYL] ETHYLENEDIAMINE AND SPERMIDINE BY ^{1}H AND ^{13}C NUCLEAR MAGNETIC RESONANCE

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STUDY OF THE PROTONATION/DEPROTONATION SEQUENCE OF TWO POLYAMINES: BIS-[(2S)-2-PYRROLIDINYLMETHYL] ETHYLENEDIAMINE AND SPERMIDINE BY ^{1}H AND ^{13}C NUCLEAR MAGNETIC RESONANCE

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ABSTRACT

In this paper we describe the study of protonation/deprotonation of two polyamines: bis-[(2S)-2-pyrrolidinylmethyl]ethylenediamine (tetra) and spermidine (Spd). A new synthetic route was established for the synthesis of tetra, which structure was confirmed by IR, elemental analyzes, ^{1}H-NMR, ^{13}C-NMR(Pendant) and 2D-NMR (COSY, ^{13}C-^{1}H HETCOR and HMQC) spectra. The protonation/deprotonation sequence studies of tetra and Spd were determined by potentiometric and NMR methods. For the NMR studies, the tetra and Spd samples were dissolved in D_2O and the pD adusted with NaOD. The protonation/deprotonation sequences of tetra and Spd were determined by means of the values and the variations of the hydrogen atom and ^{13}C NMR chemical shifts as a function of hydrogen atom pD. The variation of δ^{1}H with pD clearly showed that the first protonation of tetra occurs at the pyrrolidine nitrogen atoms and the second protonation occurs at the ethylenediamine nitrogen atom. The analysis of the ^{13}C-NMR spectra confirmed the results obtained by ^{1}H-NMR, as a greater chemical shift variation was observed for C-6 (5.6 ppm), as compared to C-8 (1.8 ppm). In the study with Spd, the greater chemical shift variation was observed for C-2 (6.75 ppm) and C-5 (4.95 ppm), indicating that the deprotonation occurs first at the secondary nitrogen atoms and the second and third deprotonation steps occur at the primary nitrogen atoms.

Key Words: *bis*-[(2S)-2-Pyrrolidinylmethyl]ethylenediamine; Synthesis; Spermidine; Protonation/deprotonation; ^{1}H and ^{13}C NMR

INTRODUCTION

Putrescine (Put, $NH_2(CH_2)_4NH_2$), spermidine (Spd, $NH_2(CH_2)_3NH(CH_2)_4NH_2$) and spermine (Spm, $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$) are polyamines that are found in most living organisms and play an essential role in several biochemical processes.[1] Many alkaloids have been found to be built from polyamines. For example, molecules such as pithecolobine and palustrin contain substituted diamino butane residues, whereas lunarine

contain a diacyl spermidine that accounts for all the nitrogen atoms of the molecule.[2] The peptidic antibiotic edeine A, obtained from *Bacillus brevis*, has a spermidine residue at one of its ends.[2] Graser and Hartmann,[3] have recently discovered that, in root cultures of *Senecio vulgaris L*, spermidine is an essential biosynthetic precursor of pyrrolizidine alkaloids. It provides the aminobutyl group, which is transferred to putrescine, yielding homospermidine, the specific building block of the necine base of pyrrolidizine alkaloids. The enzymatic formation of spermidine performed by S-adenosylmethionine decarboxylase and spermidine synthase, have been studied in relation to the unique role of this polyamine as an alkaloid precursor.[3]

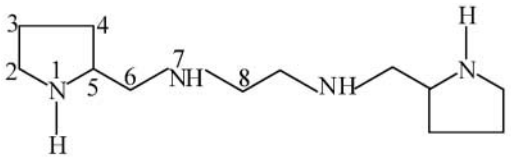
Because of the sustained increase in polyamine biosynthesis in preneoplasic and neoplastic tissues, a great deal of attention has been given to polyamine biosynthesis as a target in antineoplasic strategies. Initial work focused on the design and synthesis of compounds, which would inhibit L-ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC)[4] and continued by developing several powerful inhibitors. Unfortunately, attempts to completely deplete cellular polyamine content with these inhibitors have not been entirely successful and a major reason for this is that cell growth ceases on exposure to such inhibitors and the residual spermine is not degraded. Therefore, the cellular spermine content is not greatly reduced and it has been proposed that the presence of this residual spermine maintains cellular viability.[5]

Recent studies[5] have demonstrated that some bis(alkyl)polyamine derivatives can be used to deplete cellular polyamine levels. Although these compounds are not direct inhibitors of the polyamine biosynthetic enzymes, it seems that they act by depleting polyamines by down-regulating the biosynthesis of the key enzymes ODC and AdoMetDC.

Bergeron and coworkers[6] have synthesized and evaluated the antiproliferative proprieties of a series of analogues and homologues of the naturally occurring tetraamine spermine.

In physiological medium, polyamines are found as the protonated cation form. Due to their protonated $-NH_3^+$ groups, polyamines interact with negatively charged fragments of amino acids, proteins, fatty acids, phospholipids, nucleotides and nucleic acids.[1,7,8] Both the charge and the structure of the protonated amines influence conformational stability of nucleic acids.[1,9]

For the reasons above mentioned it is very important to study the protonation/deprotonation sequence of these polyamine in order to understand their binding to biological molecules. We chose spermidine (Spd) for our study as well as another polyamine: bis-[(2S)-2-pyrrolidinylmethyl]ethylenediamine (tetra).



Bis-[(2S)-2-pyrrolidinylmethyl]ethylenediamine - tetra

Spermidine - Spd

Figure 1. Structures of tetra and Spd.

The interest on the synthesis and study of tetra is due to its relation to compounds such as, N,N′ -bis(4-piperidinylmethyl)-1-4-diaminobutane [PIP(4,4,4)], N,N′ -bis[2-(4-piperidinyl)ethyl]-1-4-diaminobutane [PIP(5,4,5)], N,N′ -bis(4-pyridylmethyl)-1,4-diaminobutane [PYR(4,4,4) and N,N″ -bis[2-(4-pyridyl)ethyl]-1,4-diaminobutane, which are derived from piperidine and pyridine and have antineoplasic properties.[6]

Tetra has already been synthesized by Kitagawa et al.[10] and Jun et al.[11] Both groups investigated the stereoselectivity of this ligand to form nickel (II) and Co(III) ion complexes, respectively.

The present paper describes a new synthesis of tetra and reports an investigation of the protonation/deprotonation sequence of tetra and Spd, Fig. 1, performed by the use of potentiometric and nuclear magnetic resonance methods.

EXPERIMENTAL SECTION

General

N-tert-butoxycarbonil-L-proline (Boc-L-proline), isobutylchloroformate, ethylendiamine, lithium aluminum hydride and Spd were purchased from Aldrich Chemical Co. All others chemicals used were analytical grade.

Spermidine hydrochloride (Spd.3HCL) was produced by the reaction of concentrated hydrochloric acid with Spd in absolute ethanol. The white solid obtained was filtered under suction filtration and washed with absolute

ethanol several times. The residual ethanol was removed under high vacuum. The white solid was dried over P_4O_{10}. The identity of this hydrochloride was confirmed by ^{1}H-NMR, ^{13}C-NMR and elemental analysis, and comparison with literature data.

Synthesis of Tetra (3)

Tetra was synthesized as described in Fig. 2.

bis-(N-t-Butoxycarbonyl-L-prolyl)Ethylenediamine (1)

To a solution of 21.7 g (100 mmol) of N-t-butoxycarbonyl-L-proline (2) in 250 mL of anhydrous tetrahydrofuran (THF) at $-10°C$ 14.1 mL (100 mmol) of distilled triethylamine was added. After 5 min of reaction, 13.1 mL (100 mmol) of isobutyl chloroformate were added and the resulting white suspension was stirred for 5 min. After cooling the mixture to $-30°C$, 3.37 mL (50.5 mmol) of distilled ethylenediamine in 10 mL of anhydrous THF were added. The temperature was maintained below $-20°C$ for 40 min

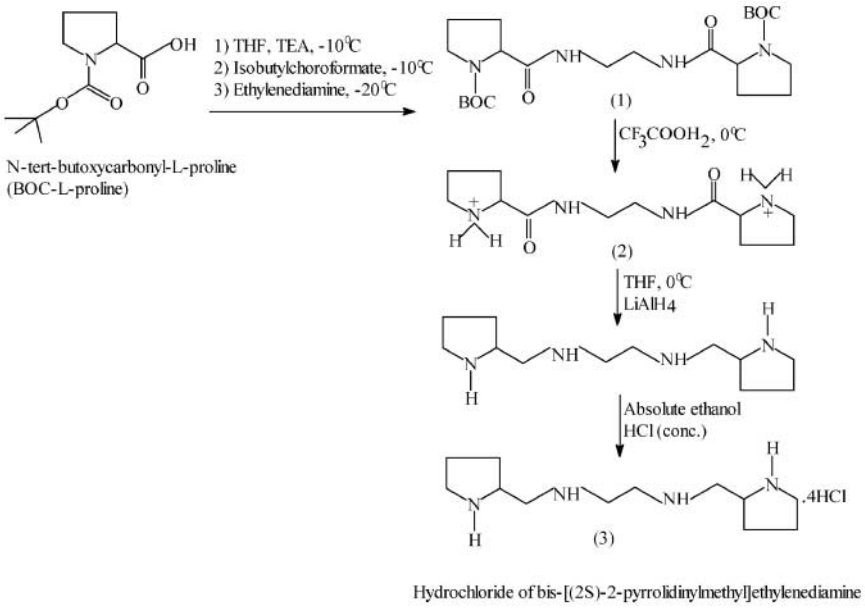


Figure 2. Scheme of the synthesis of tetra.

and then the mixture was allowed to warm to room temperature and was maintained in this temperature for 14 h. The solids formed (principally triethylamine hydrochloride) were removed by filtration and washed with ethyl ether (100 mL). The ether extracts and the filtrate were mixed and the volatile solvent was removed by means of a rotatory evaporator. Most of the residual isobutyl alcohol was removed under high vacuum. The residual material was dissolved in 300 mL CH_2Cl_2 and washed with 1 M H_3PO_4(2×40 mL) followed by saturated aqueous $NaHCO_3$ (75 mL). The CH_2Cl_2 solution was concentrated in a rotatory evaporator to yield 19 g of a white solid. Crystallization from ethyl acetate gave 17 g (74%) of diamide (1) as white, fluffy crystals, mp 168–170°C, suitable for further transformations. The spectral analysis confirmed its structure: IR (KBr pellets) 3283, 3079, 1653, 1541, 1385, 1364, 1162 cm^{-1}, ^{1}HNMR (200 MHz, $CDCl_3$) δ 1.43 (s, 18H), 1.75–2.18 (br m, 8H), 3.41 (br m, 8H), 4.17 (br m, 2H), 6.6–7.2 (br m, 2H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 24.70, 28.57, 29.20, 31.39, 39.79, 47.28, 60.46, 61.30, 80.41, 173.56; Elemental analysis, Calcd. for $C_{22}H_{38}N_4O_6$: C, 58.12%; H, 8.42%; N,12.32%. Found: C, 58.26%; H, 8.38%; N, 12.23%.

bis-[(2S)-2-Pyrrolidinylmethyl]Ethylenediamine-tetra (3)

To 17 g (37.4 mmol) of the synthezised diamide (1) in a 250 mL flask cooled in an ice/H_2O bath was added 20 mL (260 mmol) of trifluoroacetic acid. A strong bubbling was observed and after 20 min all of the solid had dissolved. The ice/H_2O bath was removed and the stirring was continued for 30 min Most of the trifluoroacetic acid was removed by means of a rotatory evaporator and the residual acid was removed under high vacuum to yield 19.7 g of a white powder. This material was added in portions over a 30 min period to a rapidly stirring suspension of 21.3 g (562 mmol) of $LiAlH_4$ in 250 mL of dry THF at 0°C. The mixture was allowed to warm to room temperature over 30 min and, after this time, it was heated under reflux for 24 h. After cooling to 0°C, 21.3 mL of saturated NH_4Cl, 21.3 mL of 15% NaOH and 64 mL of saturated NH_4Cl were added in succession. The solids were removed by vacuum filtration and thoroughly washed with warm (60°C) THF. The filtrate and the washings were mixed and the solvent was removed by means of rotatory evaporator. The reaction residue was dissolved in CH_2Cl_2 and dried over anydrous K_2CO_3. Suction filtration followed by removal of the solvent under vacuum afforded 9.5 g of a yellow oil. This oil was dissolved in absolute ethanol and treated with concentrated hydrochloric acid. Most of the alcohol was removed under vacuum to give a yellow oil, which was dissolved in absolute ethanol and stored in a

refrigerator for 2 days. The white precipitated were collected and washed with absolute ethanol several times. The residual ethanol was removed under high vacuum. The white solid was dried over P_4O_{10}, affording pure 3 in 65% yield (9.0 g). $[\alpha]^{D}_{28}$+0.02 (c 1g/100 mL of H_2O).

METHODS

Potentiometric titrations were carried out under dry nitrogen in order to obtain the dissociation constants of both polyamines Spd and tetra. Although the Spd constants are already known, we determined them to have the data for the two compounds at the same conditions. All solutions were prepared with bi-distilled, deionized and CO_2 free water. KNO_3 (Merck) was used as supporting electrolyte in all potentiometric measurements. The 10^{-3} mol L^{-1} Spd and tetra solutions were titrated with carbonate-free 0.1000 mol L^{-1} KOH solutions prepared from Titrisol ampoules (Merck).

The pH measurements and titrations were made in a Methrom digital pH-meter, using a combined glass electrode calibrated with appropriate buffers with an ionic strength of approximately 0.1 mol dm^{-3} (25 $\pm$ 0.1°C, $\mu = 0.1$ mol dm^{-3}). The titrimetric data were obtained using a E386 Multihand semiautomatic microburette, a B375 Micronal potentiometer and a combined glass electrode. During the titrations the cell was coupled to a double wall vessel where controlled temperature water was circulating to maintain the temperature constant.

The dissociation constants were calculated using HYPERQUAD.[12] The species distribution curves were drawn with the microcomputer program SPE,[13] under the experimental conditions used.

Spectral Measurements

The infrared spectra of the solid samples were recorded using KBr pellets on a Magma IR760-Nicolet IR spectrometer. The ^{1}H-NMR, ^{13}C-NMR(Pendant), COSY, ^{13}C-^{1}H-HMQC, ^{13}C-^{1}H-HETCOR and ^{13}C-^{1}H-HETCOR-LR spectra were determined in a Varian Unity-300 (300 MHz) and a Burker NMR (200 MHz) spectrometers. The solvent used for the diamide was $CDCl_3$ containing 0.03% v/v tetramethylsilane (TMS) and the solvent for tetra and Spd hydrochlorides was D_2O with the pD adjusted with NaOD when necessary.

Elemental analyses of the solids samples were carried out using a EA 1110 CHNS-O-CE instrument.

RESULTS AND DISCUSSION

In this paper we describe a new methodology for the synthesis of tetra, which has some advantages over the previously described methods,[10,11] such as good reproducibility, easiness of purification and shorter reaction times.

All the synthetic work done here is an improvement of previous work developed by Hansen, M. M,[14] R.S.C. Lopes and C.C Lopes.[15]

The tetra structure was explained by infra red spectroscopy, ^{1}H-NMR, ^{13}C-NMR (Pendant) spectra and elemental analysis.

The IR spectrum of tetra, Fig. 3, showed the expected bands for this compound. It is relevant a band at 2766 cm^{-1} which appears when more than two adjacent -CH are in *trans* position relatively to the electron pair of the nitrogen atom. The main vibration modes are listed in Table 1 and the results of the elemental analysis can be seen in Table 2.

Figure 4 (a and b) shows the ^{1}H-NMR and ^{13}C-NMR spectra of tetra, respectively. In the ^{1}H-NMR spectrum it can be observed that the hydrogen atoms (H-5, H-6, H-8 and H-2) bounded to carbon atoms which are closer of the nitrogen atoms have greater chemical shifts than the hydrogen atoms

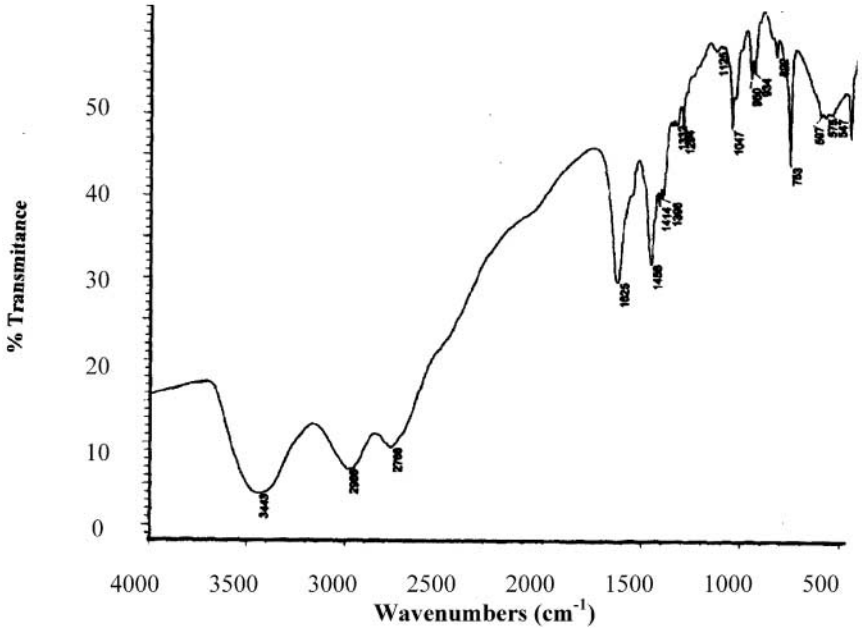


Figure 3. Infrared spectrum of tetra.

Table 1. Main Attribution of Tetra (cm^{-1})

ν(NH)	ν(CH2)	ν(CH)	δ(N–H)
3443	2986	2766	1625

Table 2. Elemental Analysis Data of Tetra

Found (Calculated)		
C%	H%	N%
38.48(38.72)	8.94(8.27)	14.44(15.05)

bound to carbon atoms which lie further away from the nitrogen atoms (H-4a, H-4b and H-3). The conclusive chemical shift assignments for all the hydrogen and carbon atoms in tetra were possible using ^{1}H-NMR, ^{13}C-NMR (Pendant) and 2D-RMN (COSY and ^{13}C-^{1}H-HMQC) data. These data can be seen in Table 3.

Dissociation Equilibria

The dissociation constants of Spd and tetra were determined potentiometrically by titrating 0.1 mmol dm^{-3} of each polyamine with a standard KOH solution, under the experimental conditions, $T = 25 \pm 0.1°C$ and $\mu = 0.1$ mol dm^{-3} (KNO_3).

The deprotonation equilibria of the polyamines: Spd and tetra are as follows:

Spd

$$H_3Spd^{3+} \Leftrightarrow H_2Spd^{2+} + H^+$$
$$H_2Spd^{2+} \Leftrightarrow HSpd^{+} + H^+$$
$$HSpd^{+} \Leftrightarrow Spd^{0} + H^+$$

tetra

$$H_4tetra^{4+} \Leftrightarrow H_3tetra^{3+} + H^+$$
$$H_3tetra^{3+} \Leftrightarrow H_2tetra^{2+} + H^+$$
$$H_2tetra^{2+} \Leftrightarrow Htetra^{+} + H^+$$
$$Htetra^{+} \Leftrightarrow tetra^{0} + H^+$$

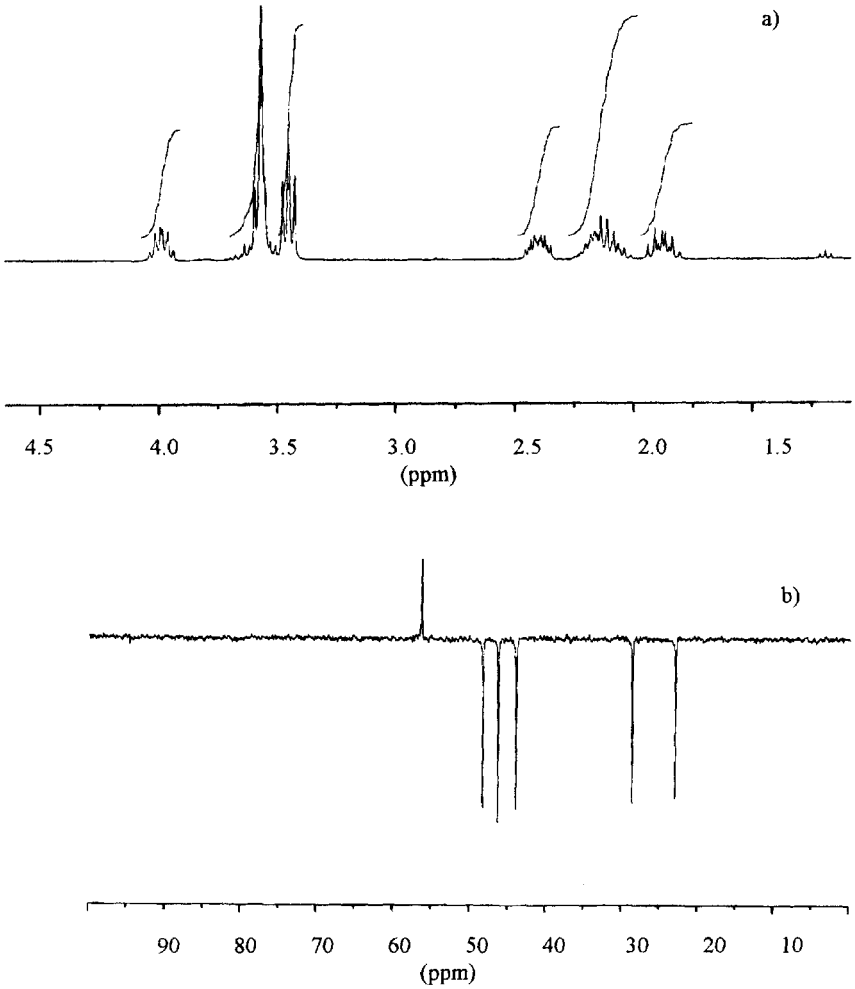


Figure 4. a) ^{1}H - NMR spectrum of tetra; b) ^{13}C - NMR (pendant) of tetra.

with the corresponding equilibrium constants, K_1, K_2 and K_3 for Spd, and K_1, K_2, K_3 and K_4 for tetra.

The HYPERQUAD program was used to determine the global formation constants (logβ), corresponding the following equilibria:

Table 3. Chemical Shifts (δ/ppm) in ^{1}H-NMR (200 MHz) and ^{13}C-NMR(Pendant) (50 MHz) of Tetra

C-2/H-2	C-3/H-3	C-4/H-4a/H-4b	C-5/H-5	C-6/H-6	C-8/H-8
46.3/3.44 (dd*, 2H)	22.9/2.12 (m, 2H)	28.6/2.40/1.88 (m, 1H) / (m, 1H)	56.3/4.0 (m, 1H)	48.3/3.6 (2^0, 2H)	43.9/3.6 (s, 2H)

$$H^+ + PA^0 \Leftrightarrow HPA^+ \quad \beta_1^H \tag{1}$$

$$2H^+ + PA^0 \Leftrightarrow H_2PA^{2+} \quad \beta_2^H \tag{2}$$

$$nH^+ + PA^0 \Leftrightarrow H_nPA^{n+} \quad \beta_n^H \tag{3}$$

The protonation constants (log β) of Spd and tetra defined by Eq. (3) are presented in Table 4.

With these results it was possible to obtain the species distribution curves shown in, Figs. 5 and 6. It can been seen in the group of Spd (Fig. 5) that H_3Spd^{3+} is the main species until approximately pH 7. AtpH 8–9 the species H_2Spd^{2+} predominates and at higher pH the species $HSpd^{1+}$ is the main one. In the group of tetra (Fig. 6), the species H_4tetra^{4+} predominates only at very low pH (≈ 3). The dissociated species H_3tetra^{3+}, H_2tetra^{2+} and $Htetra^{1+}$ are the main species at pH 5, 8 and higher than 10, respectively.

Tetra and Spd Deprotonation Study

To carry out the NMR spectra, the pD values of each polyamine was previously chosen according to the distribution of species in function of pH from the above determination. The analysis of ^{1}H-NMR,

Table 4. Overall Formation Constant, (logβ), of Spd and Tetra

Species	Logβ (25°C, $\mu = 0.1$ mol dm^{-3}, KNO_3)
H_3Spd^{3+}	29.89 ± 0.01 (29.49)[16]
H_2Spd^{2+}	21.41 ± 0.01 (21.03)[16]
$HSpd^{+}$	11.39 ± 0.01 (11.61)[16]
H_4tetra^{4+}	29.95 ± 0.01
H_3tetra^{3+}	26.64 ± 0.01
H_2tetra^{2+}	20.15 ± 0.01
$Htetra^{+}$	10.54 ± 0.01

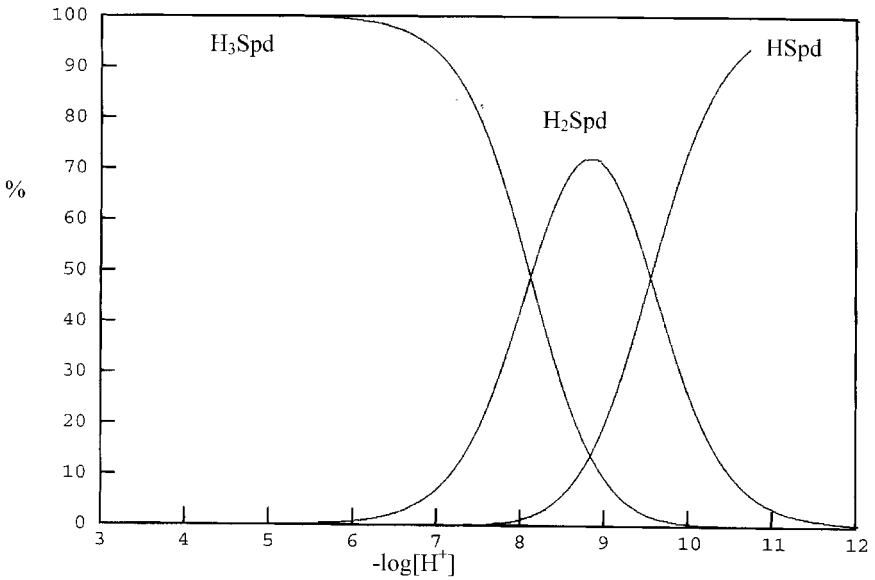


Figure 5. Species distribution in the spd system.

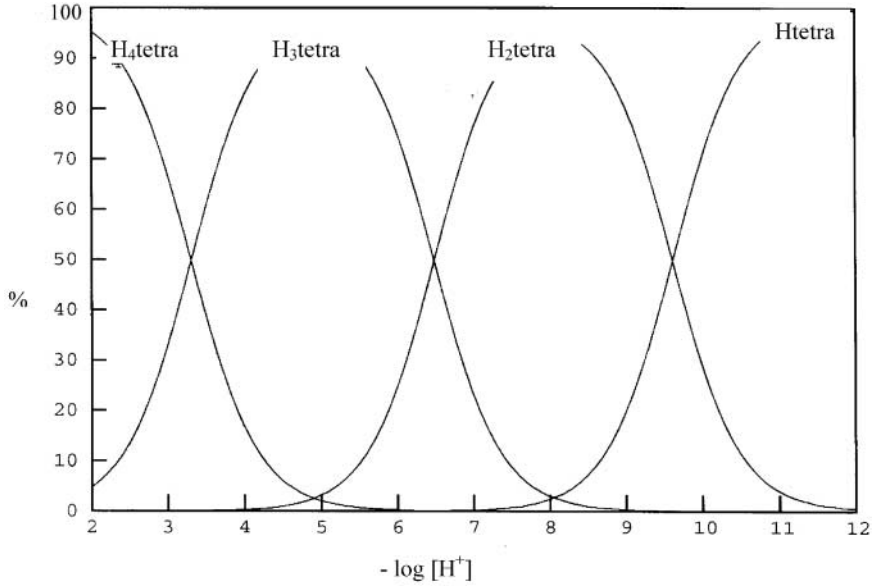


Figure 6. Species distribution in the tetra system.

^{13}C-NMR(Pendant) and 2D-NMR (COSY, ^{13}C-^{1}H-HMQC, ^{13}C-^{1}H-HETCOR and ^{13}C-^{1}H-HETCOR-LR) spectra allowed the determinate the protonation/deprotonation sequences of tetra and Spd with good precision. The sequences were determined using the values and the variations of the chemical shifts of the hydrogen and carbon atoms as a function of pD. The deprotonation of the various basic sites affects the hydrogen and carbon atoms chemical shift, which are in the neighborhood of these sites.

The chemical shifts of the various atoms were monitored as a function of pD. The resulting data are shown in Table 5.

The ^{1}H-NMR spectra of tetra in pD values: 2, 5, 8 and 12, Fig. 7, show changes in the chemical shifts as a function of pD. At the inicial pD (2), the hydrogen atoms H-6 and H-8 have closer chemical shifts, while, at pD 5 and 8 it can be seen that they are further apart. Using the COSY spectra at pD = 8, the chemical shifts due to these two hydrogen atoms are well evidenced to hydrogen atoms H-6 and H-8.

Table 5. Chemical Shifts (δ/ppm) of ^{1}H-NMR (200 MHz) and ^{13}C-NMR (50 MHz) in Function of pD of Tetra and Spd

Tetra				
pD	2	5	8	12
C-5/H-5	56.3/4.0	56.5/3.9	59.5/3.8	57.7/3.58
C-6/H-6	48.3/3.6	48.3/3.26	49.9/3.06	53.9/2.82
C-2/H-2	46.3/3.44	46.2/3.4	47.7/3.34	48.2/3.2
C-8/H-8	43.9/3.6	44.2/3.18	45.8/2.96	45.7/2.82
C-4/H-4a	28.6/2.40	28.5/2.30	28.4/2.26	29.9/2.14
C-3/H-3	22.9/2.12	22.9/2.08	23.5/2.06	24.9/1.98
H-4b	1.88	1.80	1.76	1.62
Spd				
pD	6	9	12	
C-4	47.14	47.45	48.32	
C-3	44.62	45.20	46.16	
C-7	38.85	39.15	40.32	
C-1	36.62	37.37	38.64	
C-2	23.99	25.65	30.74	
C-5	23.87	24.52	28.82	
C-6	22.86	23.91	25.66	

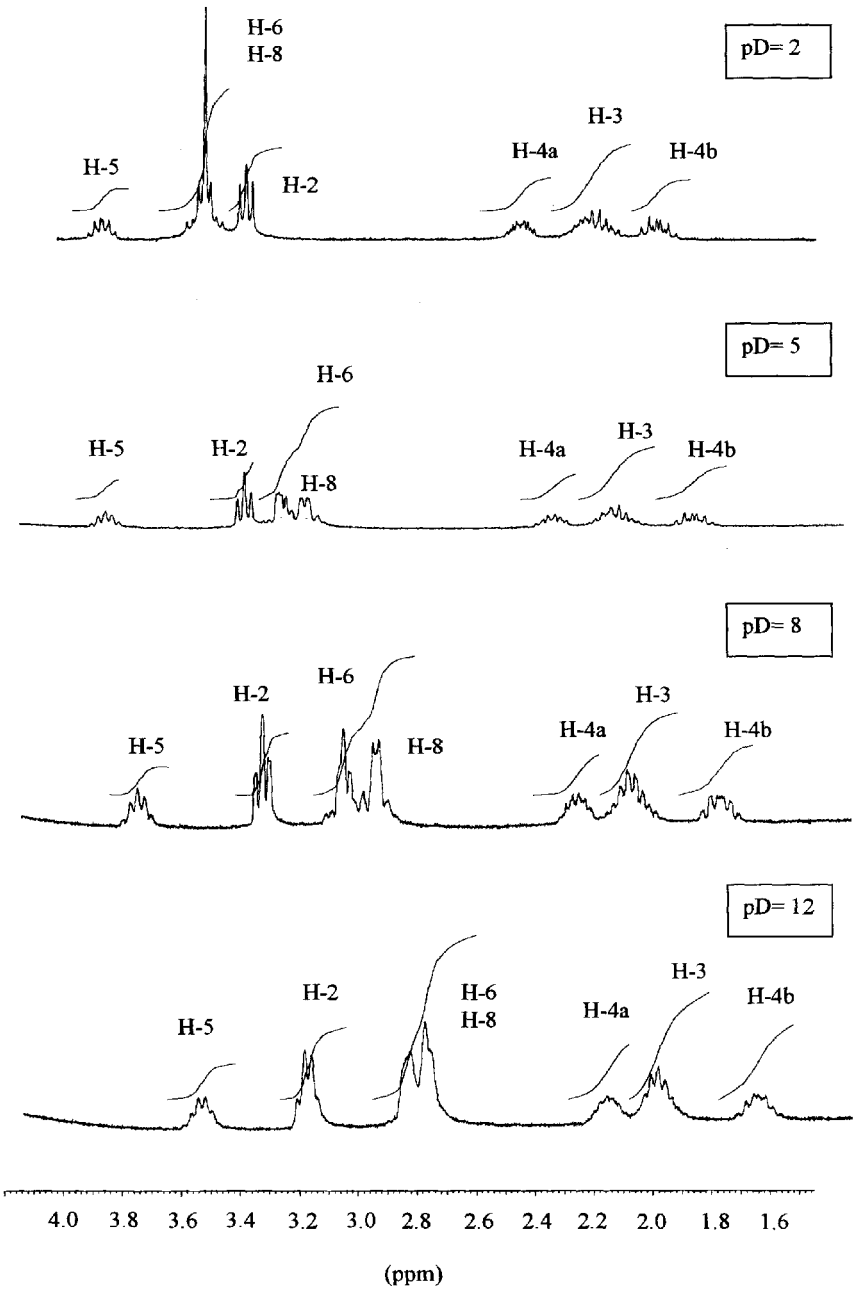


Figure 7. ^{1}H NMR spectrum of tetra in various pD values.

The variation of the values of δ for ^{1}H with pD neatly shows that the first stage of the protonation of tetra occurs at the nitrogen atoms of pyrrolidine and the second stage occurs at the nitrogen atoms of the ethylenediamine moiety. Clearly, the deprotonation sequence is exactly the reverse. This is evidenced by the greater δ ^{1}H variation for the hydrogen atoms which are closer to the ethylenediamine moiety. For, e.g., the greater chemical shift variations are observed for H-8 and H-6 (0.8 ppm) and also for the H-5 (0.4 ppm). It could also be seen that H-3 has the smallest chemical shift variation inside the pD range studied (2 to 12), with almost no modification between pD 2 and 8, thus indicating that the pyrrolidinic nitrogen atom might be completely protonated over all this range.

The ^{13}C-NMR spectra, Fig. 8, confirms the results obtained by ^{1}H-NMR. It was observed that the variation of $\delta^{13}C$ for C-6 (5.6 ppm), was much greater than the variation for C-8 (1.8 ppm). This probably occurs because of conformational changes due to the pD variations affect more intensely this carbon atom (C-6), since it lies near the two protonation sites of the molecule.

Concerning the study of Spd, the ^{13}C-NMR(Pendant), ^{13}C-^{1}H-HMQC, ^{13}C-^{1}H-HETCOR and ^{13}C-^{1}H-HETCOR-LR analyses clearly defined the protonation/deprotonation sequence. Fig. 9 shows the ^{13}C-NMR(Pendant) of Spd at different pD values.

Kimberly and Goldstein,[17] in a similar study showed that the chemical shift of C-6 is greater than that for C-5 in spermidine. In our work we found the opposite behavior. The chemical shift of C-5 was greater than that of C-6. Through the ^{13}C-^{1}H-HETCOR-LR analysis it was possible to refer the signals at 23.87 and 22.86 ppm to C-5 and C-6 respectively. The values for the ^{13}C NMR chemical shifts of Spd at different pD values are shown in Table 5.

On the basis of NMR studies, Kimberly and Goldstein[17] showed that the first stage of Spd protonation occurs at the primary (Na) and secondary (Nb) nitrogen atoms. In this work, the variation of δ ^{13}C with pD shows that the deprotonation first occurs at the secondary nitrogen atom (Nb). This conclusion arise because of the greater variations of δ ^{13}C observed for C-2 (6.75 ppm) and C-5 (4.95 ppm), inside the studied pD range (6 to 12). The second and third stages of deprotonation occur at the primary nitrogen atoms, (Na and Nc), being the protonation sequence the inverse.

CONCLUSIONS

Since it is necessary that the polyamine be protonated for its interaction with the negative regions of the biomolecules, it was important to verify

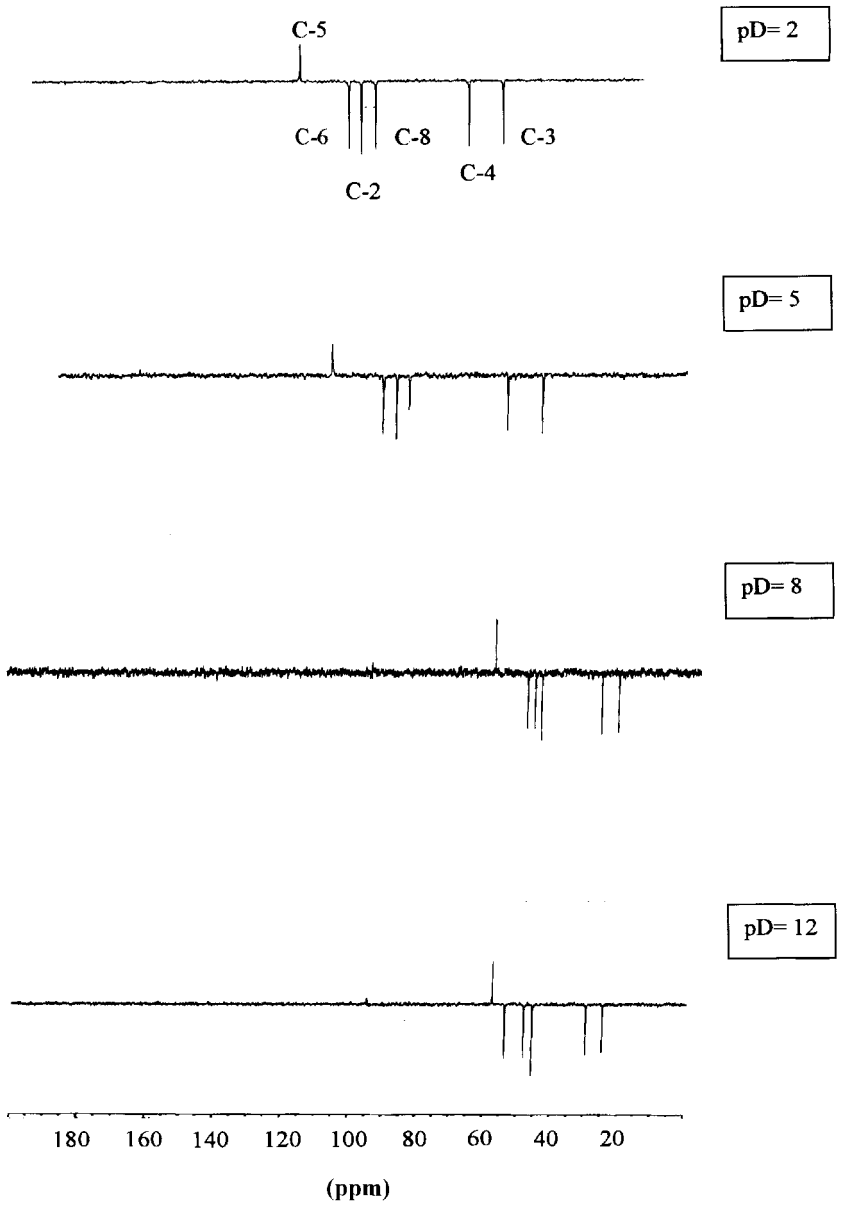


Figure 8. ^{13}C NMR spectrum of tetra in various pD values.

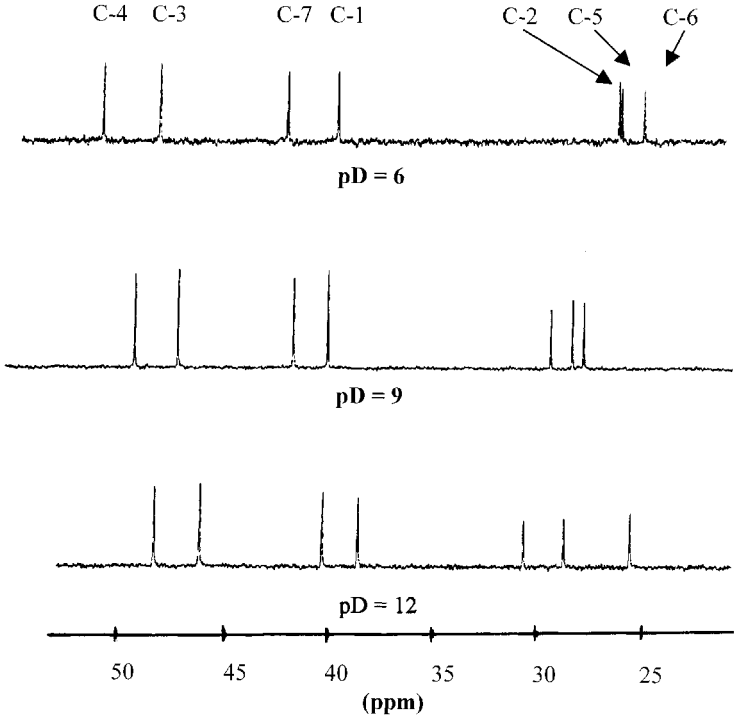


Figure 9. ^{13}C NMR spectrum of Spd in various pDs.

that both polyamines are protonated at physiological pH. Spd is completely protonated while tetra is only partially protonated. The NMR analysis allowed for the precise determination of the protonation/deprotonation sequence of tetra and Spd.

These results add an important information valuable for the undestanding of the formation equilibria for metal complexes at various pH values. The protonation/deprotonation sequence of polyamines, specially spermidine, a biological amine, is also important for the understanding of its biological mechanism action.

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