Neuroactive Polyamine Wasp Toxins: Nuclear Magnetic Resonance Spectroscopic Analysis of the Protolytic Properties of Philanthotoxin-343

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Acid−base properties (pK_a values and proton distribution patterns) of philanthotoxin-343 (PhTX-343) were investigated by ^{1}H and ^{13}C NMR titration. Chemical shift data and the total ionization shifts were used to assign carbon atoms of the polyamine chain. Nonlinear analysis of the ^{13}C NMR titration curves gave four pK_a values (pK_1 8.5, pK_2 9.5, pK_3 10.4, pK_4 11.4) and the intrinsic chemical shifts of the non-, mono-, di-, tri-, and tetraprotonated forms. The changes of intrinsic chemical shifts enabled analysis of the deprotonation sequence of fully protonated PhTX-343. The results of analysis of the ^{13}C NMR titration curves were supported by ^{1}H NMR data obtained from two-dimensional ^{1}H,^{13}C chemical shift correlation experiments. Thus, the first deprotonation mainly takes place at the inner amino group. The phenol group is deprotonated in the second and third deprotonation steps. The preferential deprotonation of the inner amino group is also apparent in the diprotonated form. The monoprotonated form carries a practically fully ionized phenol group and the proton shared between the three amino groups. This characteristic is in agreement with existing data on polyamines. At physiological pH, the tetraprotonated form of PhTX-343 predominates, but the proportion of the triprotonated form becomes significant at low ionic strength. The terminal, primary amino group, which has been shown to be essential for biological activity, remains practically fully protonated at biologically relevant pH values, and this charge is likely to participate in the receptor-binding event. Protonation of the central amino group does not appear to be necessary for biological activity.

Introduction

Venoms of spiders[1,2] and wasps[3] contain a number of structurally related polyamines with the general formula shown in Figure 1, that exhibit pronounced noncompetitive antagonist activity at ionotropic receptors for L-glutamate, acetylcholine, and serotonin. Such receptors are playing important roles in analyzing a variety of physiological and pathophysiological mechanisms in the central nervous system, making these polyamines valuable pharmacological tools and potentially useful pharmacological agents. Philanthotoxin-433 (PhTX-433; Figure 1), isolated from the parasitic wasp *Philanthus triangulum*, is a prototype of the most extensively investigated group of polyamine toxins. A large number of naturally occurring toxins and their structural analogues, including PhTX-343 (Figure 1), have been synthesized[3−5] and used to explore structure-activity relationships.[6−9]

The polyamine chain present in philanthotoxins is essential for their biological activity. Thus, shortening of the chain or acetylation of the primary amino group has been shown to reduce activity. In some cases, however, unchanged or even increased noncompetitive antagonist effects have been measured upon acylation of the terminal amino group with glycine, lysine, or arginine.[6,7] Increase of the lipophilicity of the R_1 and R_2 groups (Figure 1) results in increased potency.[6,7] According to the current model of action, the polyamine chain enters the channel, whereas the lipophilic moiety is anchored to the outer part of the receptor complex.

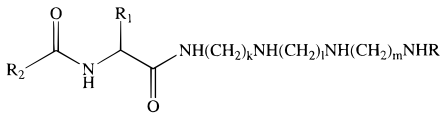

Figure 1. General structure of wasp and spider toxins. Philanthotoxin-433 (PhTX-433): R = H, R_1 = 4-hydroxybenzyl, R_2 = propyl; k = 4, l = m = 3. Philanthotoxin-343 (PhTX-343): R = H, R_1 = 4-hydroxybenzyl, R_2 = propyl; k = m = 3, l = 4.

This model is consistent with recent photoaffinity labeling studies using nicotinic acetylcholine receptors[10] and with the observed changes in activity upon alteration of the basic amino groups in the polyamine chain.[6,7] Thus, the interior of the channel complex is likely to interact with the protonated nitrogen sites. The acid−base properties and proton distribution patterns are, therefore, of considerable interest in connection with the modeling of the action of philanthotoxins. Because of the sensitivity of NMR chemical shifts to the ionization state of acidic and basic groups, NMR spectroscopy is a useful tool to investigate protolytic properties of polyamines. In this work, we report the results of such studies with PhTX-343.

Data Analysis

Since PhTX-343 contains four acid−base centers (three amino groups and one phenol group), the deprotonation equilibria of the fully protonated form can be described as follows:

$$XH_4 \rightleftarrows XH_3 + H^+ \quad (1)$$

$$XH_3 \rightleftarrows XH_2 + H^+ \quad (2)$$

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$$XH_2 \rightleftarrows XH + H^+ \quad (3)$$

$$XH \rightleftarrows X + H^+ \quad (4)$$

where XH_n (n = 4, 3, 2, 1, or 0) designates tetra-, tri-, di-, mono-, or nonprotonated PhTX-343, respectively. The corresponding equilibrium constants $[XH_n][H^+]/[XH_{n+1}]$ for reactions 1, 2, 3, and 4 are K_1, K_2, K_3, and K_4, respectively. Chemical shift of ith nucleus (^{13}C or ^1H) at any pH value can be described as a sum:

$$\delta_i = \sum_{n=0}^{4} \delta_i^n x_n \quad (5)$$

where δ_i is the observed chemical shift of the ith site, δ_i^0, δ_i^1, δ_i^2, δ_i^3, and δ_i^4 are the chemical shifts of the ith site in non-, mono-, di-, tri-, and tetraprotonated species, and x_n is the mole fraction of n protonated species present. It can be shown that:

$$x_0 = K_1K_2K_3K_4/F$$

$$x_1 = K_1K_2K_3[H^+]/F$$

$$x_2 = K_1K_2[H^+]^2/F$$

$$x_3 = K_1[H^+]^3/F$$

$$x_4 = [H^+]^4/F$$

where $F = [H^+]^4 + K_1[H^+]^3 + K_1K_2[H^+]^2 + K_1K_2K_3[H^+] + K_1K_2K_3K_4$.

The chemical shift of the ith site at any pH defined by eq 5 can be expressed, therefore, in terms of equilibrium constants as:

$$\delta_i = (\delta_i^0K_1K_2K_3K_4 + \delta_i^1K_1K_2K_3[H^+] + \delta_i^2K_1K_2[H^+]^2 + \delta_i^3K_1[H^+]^3 + \delta_i^4[H^+]^4)/([H^+]^4 + K_1[H^+]^3 + K_1K_2[H^+]^2 + K_1K_2K_3[H^+] + K_1K_2K_3K_4) \quad (6)$$

This equation describes the NMR titration curve, and equilibrium constants as well as δ_i^n parameters can be obtained by fitting the equation to experimental data.[11,12]

Provided that the precise, individual effects of full protonation of individual basic sites on chemical shifts of individual nuclei are known, the chemical shift changes observed experimentally upon protonation can be used to calculate residence times of protons at various basic sites. Thus:

$$\Delta\delta_i = \sum_j c_{ij}f_j \quad (7)$$

where $\Delta\delta_i$ is the observed protonation shift for a given protonation step, c_{ij} is the protonation shift constant for the ith nucleus for total protonation of the jth site, and f_j is the fraction of time the jth site is protonated.[13,14]

Results

Assignment of ^{13}C NMR Resonances. NMR spectra of PhTX-343 and spermine (Chart 1) were investigated as a function of ionization state of the molecules in D_2O solution at 25 °C and 1 M ionic strength. The ^{13}C NMR titration curves for PhTX-343 are shown in

Chart 1

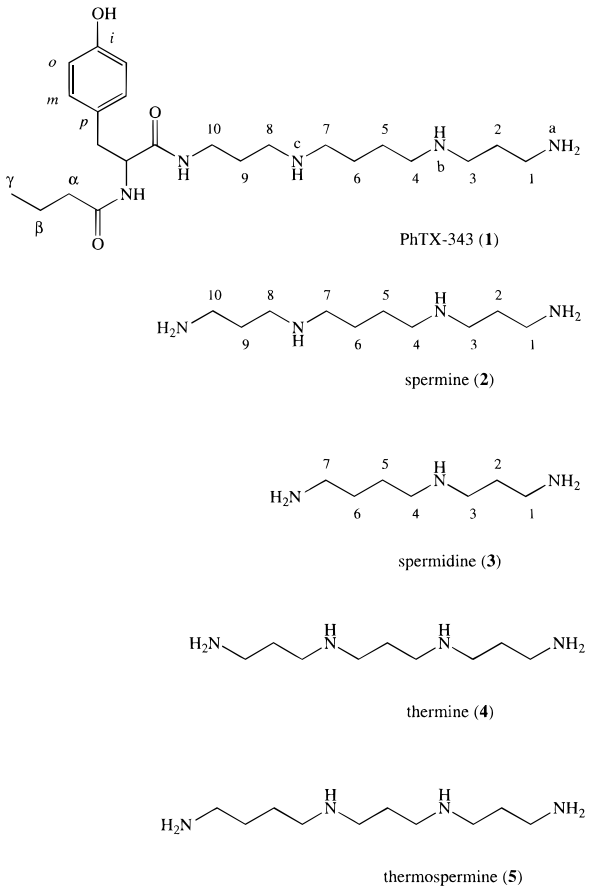

Figure 2. Assignment of carbon resonances into classes was carried out by comparison with the resonances of model polyamines. Thus, the available data for spermine and spermidine[14−17] suggest that the resonances of the polyamine chain of PhTX-343 should be found in three regions, around 30 ppm (methylene groups separated from nitrogen by at least one carbon on each side, i.e., C2, C5, C6, and C9), around 40 ppm (methylene groups next to a primary amino group, i.e., C1), and around 50 ppm (methylene groups adjacent to a secondary amino group, i.e., C3, C4, C7, and C8). Since acylation of primary amines has only a minor effect on the chemical shift of the α-carbon,[18,19] the chemical shift of C10 was expected to be similar to that of C1. Assignment of individual carbons within these three groups could be achieved by considering changes of chemical shift values upon full protonation of all nitrogens. Thus, protonation of aliphatic amines leads to the upfield shift of attached carbons as far as five bonds away, the shifts being largest for β-carbons.[20,21] The β-carbon shifts in primary amines are larger than those observed in secondary amines. Consequently, C1 and C10 could be readily distinguished since the former, affected by one α- and one γ-nitrogen, is expected to be shifted more upon protonation than the latter, which is only affected by one γ-nitrogen.

In the group of resonances with δ around 25−35 ppm, the carbon experiencing the largest ionization shift must be C2 (two β-effects), whereas the δ values of C5 and C6 are expected to be closely similar in fully protonated as well as fully deprotonated PhTX-343. Each of these two carbons is affected by one β- and one γ-nitrogen, and their ionization shift is expected to be somewhat

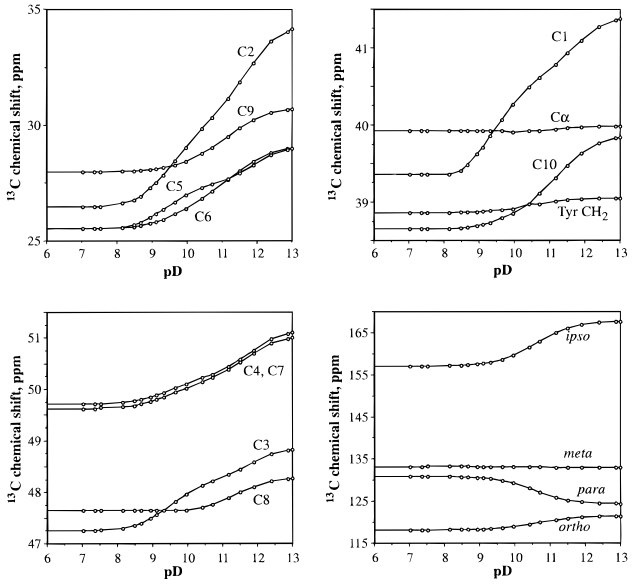

Figure 2. ^{13}C NMR titration curves for the polyamine chain and aromatic carbons in 22 mM PhTX-343 solution (D_2O, 25 °C, ionic strength 1 M). pD is the negative logarithm of the deuterium ion activity.

larger than that of C9, experiencing only a β-nitrogen. For the four carbons with δ around 50 ppm, the ionization shift of C8, affected by one protolytic center in the α-position, is expected to be smaller than the ionization shift of C3, C4, and C7, each being affected by one α- and one γ- or δ-nitrogen. As in the case of C5 and C6, the limiting δ values of C4 and C7 are expected to be very similar. This yielded the assignment of all carbons of the polyamine chain of PhTX-343 except for the two pairs in the central tetramethylene unit. The assignment of C5 and C6 will be discussed below.

The assignment of the remaining carbon atoms of PhTX-343 presented no difficulty. The chemical shifts of the butyryl moiety and the aliphatic side chain of tyrosine were largely unaffected by the ionization state of the molecule, as expected. Thus, the chemical shift differences [δ(base) − δ(acid)] observed over the whole pH range were 0.05−0.08 ppm for the propyl group carbons, 0.21−0.24 ppm for the CH_2-CH part of tyrosine, and −0.13 to −0.18 ppm for the carbonyl groups. The assignment of the aliphatic carbons was confirmed by COSY, NOESY, and ^1H,^{13}C chemical shift correlation experiments, which unequivocally identified respective interproton and proton−carbon spin systems, even in cases of overlapping ^1H resonances. In the aromatic ring, the carbon bearing the hydroxy group and the carbons *ortho* to the hydroxy group experienced large downfield shifts upon ionization, whereas the *para* carbon was shifted strongly upfield. The *meta* carbons were practically unaffected by the ionization (shift of −0.27 ppm). The directions of the ionization shifts and their magnitudes are in agreement with published data for simple phenols.[22]

Acidity Constants and Proton Distribution Patterns from ^{13}C NMR Data. Nonlinear fitting of the NMR titration curve eq 6 to the experimental data (Figure 2) yielded four macroscopic pK_a values: pK_1 = 9.05 ± 0.14, pK_2 = 10.01 ± 0.19, pK_3 = 11.01 ± 0.16, and pK_4 = 11.97 ± 0.11, as well as the corresponding δ_i^n values (Table 1). The values obtained in the D_2O solution must be corrected for solvent isotope effect in order to obtain values in H_2O solution using the well-known relationship p$K_a(D_2O)$ = p$K_a(H_2O)$ + ΔpK_a.[23] There is a large body of data showing that for primary and secondary amines ΔpK_a = 0.59 ± 0.07.[24] This gave the corrected pK_a values of PhTX-343, believed to be accurate within 0.1−0.2 unit; they are shown in Table 2. The distribution curves for PhTX-343 corresponding to these pK_a values are shown in Figure 3.

The δ_i^n values were used to derive information about proton distribution patterns in mono-, di-, and triprotonated PhTX-343. Chemical shifts of β-carbons (C2, C5, C6, and C9) were used for the estimations concerning the polyamine chain, since their deprotonation shifts are large and can be only marginally influenced by long-range effects. The chemical shifts of C9 and C10 are expected to be influenced significantly only by the protonation state of N_c (Chart 1), and hence the total deprotonation shifts experienced by these carbons were assumed to represent pure β- and γ-effects valid for N_c as well as N_b. Similarly, the chemical shifts of the aromatic carbons were assumed to be influenced only by the ionization state of the phenol group. The β-effect of the primary amino group was derived from statistical analysis of a number of primary amines.[20] Solving simultaneous eqs 7 gave the fractional protonation data shown in Table 3.

^1H NMR Data. In order to assess the usefulness of ^1H NMR data to support conclusions obtained on the basis of the ^{13}C NMR spectra, chemical shifts of all protons in the polyamine side chain were measured for each pD value (Figure 2) by means of two-dimensional ^1H,^{13}C chemical shift correlation experiments (Figure 4). Since the experiments separate ^1H NMR shift according to the already assigned ^{13}C NMR shifts, ^1H NMR titration curves could be obtained (not shown) in spite of severely overlapping signals in ordinary proton spectra. The protons of the polyamine chain exhibited upfield shifts upon titration of protonated amino groups.[26,27] The largest ionization shifts were observed for the secondary α-amino groups, but γ-effects were also apparent (0.11 ppm for H10). A good qualitative agreement between the ^1H and ^{13}C NMR data was observed. For example, the titration curves of H5 and H6 were closely similar except in the region around pD 10, as for C5 and C6 (Figure 2). The late titration of N_c was apparent from the titration curves of H8 and H9. However, because the ionization shifts of ^1H are generally small (0.1−0.6 ppm) and consequently the titration curves less well defined, no quantitative analysis of the data was attempted.

NOESY spectra of PhTX-343 recorded at several pH values showed no cross-peaks corresponding to interactions between distant parts of the molecule. The coupling constants in the tyrosine side chain (both ^3J ≈ 8 Hz) exhibited no systematic variations with pH.

Discussion

Unambiguous assignment of all diagnostic ^{13}C NMR signals of PhTX-343 could be obtained by considering ionization shifts expected for the individual resonances. The NMR titration curves were obtained at a relatively high salt concentration in order to maintain a constant ionic strength during the experiment. Subsequently, the titration curves could be analyzed in terms of

Table 1. Intrinsic ^{13}C NMR Chemical Shift Values of Various Protonated Forms of PhTX-343[a]

carbon	δ_i^0	δ_i^1	δ_i^2	δ_i^3	δ_i^4	$\delta_i^0 - \delta_i^4$	**2**[b]	**3**[c]
1	41.43	40.85	40.62	40.00	39.27	2.16	2.20	2.04
2	34.40	31.53	30.11	28.14	26.43	7.97	7.90	7.78
3	48.87	48.38	48.23	47.76	47.25	1.62	1.63	1.52
4 or 7	51.05	50.46	50.16	49.90	49.63	1.42	1.45	1.26
6	29.10	28.06	26.86	25.96	25.51	3.59	3.62	3.19
5	29.12	27.65	27.47	26.63	25.43	3.69		3.65
7 or 4	51.15	50.48	50.24	50.00	49.71	1.44		1.58
8	48.29	48.00	47.65	47.65	47.65	0.64		
9	30.74	29.95	28.70	28.16	27.98	2.76		
10	39.87	39.51	38.94	38.75	38.62	1.25		
ipso	167.64	166.98	161.86	157.45	157.12	10.52		
ortho	121.40	121.22	119.65	118.29	118.19	3.21		
para	124.39	124.72	127.86	130.64	130.88	-6.49		

[a] 22 mM solution in D_2O, 25 °C, ionic strength 1 M. [b] Total ionization shift in spermine [δ(pD 13.37) − δ(pD 1.91), 40 mM solution in D_2O, 25 °C, ionic strength 1 M]. [c] Total ionization shift in spermidine [$\delta_i^0 - \delta_i^3$ from ref 14 (51 mM solution in H_2O, 31 °C)].

Table 2. Macroscopic pK_a Values of PhTX-343 and Related Polyamines

compd	pK_1	pK_2	pK_3	pK_4
PhTX-343 (**1**)[a]	8.5	9.5	10.4	11.4
spermine (**2**)	8.69[b]	9.31[b]	10.65[b]	10.80[b]
	7.91[c]	8.68[c]	10.21[c]	10.56[c]
spermidine (**3**)	8.93[b]	10.30[b]	10.86[b]	
	8.15[c]	9.74[c]	10.24[c]	
thermine (**4**)[d]	7.36	8.81	10.09	10.82
thermospermine (**5**)[e]	7.59	9.24	10.49	11.14

[a] This work, 25 °C, ionic strength 1 M. [b] Potentiometric titration data at 25 °C, ionic strength 1 M (ref 25). [c] Potentiometric titration data at 25 °C, ionic strength 0.1 M (ref 25; the values reported in refs 14−16 are very similar). [d] Potentiometric titration data at 27 °C, ionic strength 0.1 M (ref 15). [e] Potentiometric titration data at 27 °C, ionic strength 0.1 M (ref 15).

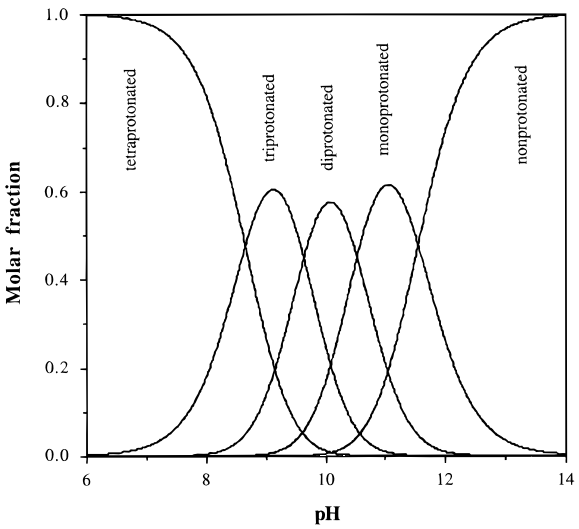

Figure 3. Species distribution diagram for PhTX-343.

Table 3. Proton Distribution Patterns for PhTX-343 at Various Stages of Protonation[a]

	protonation, %			
protonation stage	N_a	N_b	N_c	OH
monoprotonated	0.35	0.34	0.29	0.06
diprotonated	0.66	0.23	0.74	0.54
triprotonated	0.89	0.47	0.93	0.97

[a] The values of c_{ij} used for calculation: $c_{2b} = c_{5b} = c_{6c} = c_{9c} = 2.74$, $c_{2a} = 5.56$, $c_{5c} = c_{6b} = 1.25$, $c_{ortho,d} = 3.21$, $c_{ipso,d} = 10.52$, $c_{para,d} = -6.49$, and remaining $c_{ij} = 0$.

successive deprotonation constants (pK_a values) and fractional deprotonation shifts. The procedure used was essentially the same as employed in earlier studies of amino acids and polyamines.[11−15] The pK_a values

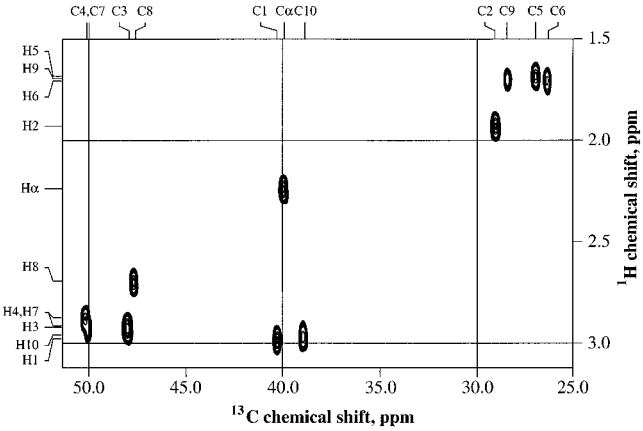

Figure 4. Example of a $^1H,^{13}C$ chemical shift correlation spectrum of PhTX-343 showing resolution of polyamine chain resonances (22 mM solution in D_2O, pD 9.95, 25 °C, ionic strength 1 M).

obtained are compared with those of model compounds[14−16,25] in Table 2.

The pK_1 and pK_2 values of PhTX-343 are closely similar to those of spermine at the same ionic strength, as might be expected. The pK_4 value is considerably higher than in spermine (Table 2). Since the last deprotonation step in PhTX-343 involves removal of a proton from a neutral molecule to give the negatively charged species (see below), the observed decrease of acidity relative to spermine can be readily rationalized.

The observed total ionization shifts in PhTX-343 are in good agreement with those for spermine and spermidine (Table 1).[14] From the point of view of biological action, it is interesting to determine whether deprotonation of fully protonated PhTX-343, present in an acidic solution, occurs preferentially from one of the four possible sites. The structures of particular, partially protonated forms of PhTX-343 can be derived from fractional deprotonation shifts ($\delta_i^n - \delta_i^{n-1}$) (Table 1). For example, the intrinsic chemical shift changes observed for the aromatic carbons in each deprotonation step represent a direct measure of the extent of change in the ionization state of the phenol group. As seen in Figure 5, the aromatic carbons change their chemical shifts practically exclusively during the second and third deprotonation steps. The behavior of the three carbons (*ipso*, *ortho*, and *para* relative to the phenol group) is fully consistent, which supports the validity of the analysis. Thus, triprotonated PhTX-343 contains an almost fully un-ionized and monoprotonated PhTX-343

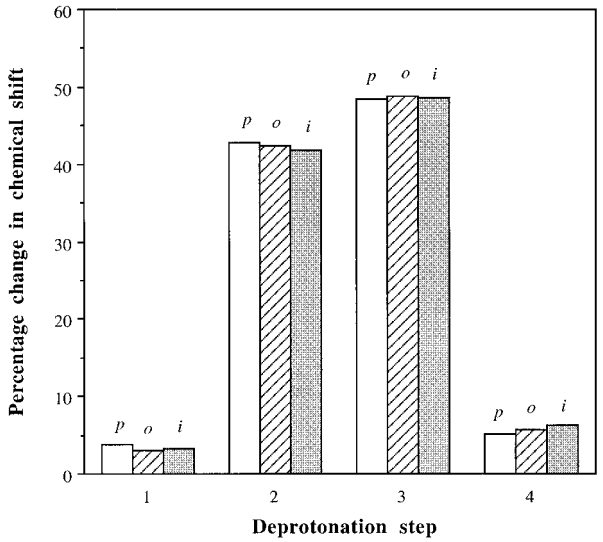

Figure 5. Fractional changes of ^{13}C chemical shifts of carbons *para*, *ortho*, and *ipso* to the phenol group during stepwise deprotonation of fully protonated PhTX-343.

an almost fully ionized phenol group. In the diprotonated PhTX-343, the phenolic group is 50% ionized.

Similarly, the data in Table 1 show that for carbons C8, C9, and C10 very little chemical shift change (0−10% of the total) is observed in the first deprotonation step. Since the chemical shift of these carbons can be assumed to be affected only by the protonation state of N_c, the first deprotonation does not take place at this site. In other words, the triprotonated PhTX-343 is practically fully protonated on N_c. Also in this case there is a high degree of internal agreement between the ^{13}C resonances involved.

Since triprotonated PhTX-343 has an approximately fully protonated nitrogen N_c and an un-ionized phenol group, deprotonation of the tetraprotonated species must involve N_a or N_b, or both. Which site is actually deprotonated can be decided by consideration of the titration behavior of C2, C5, and C6 (Figure 2 and Table 1).

As pointed out above, the assignment of the resonances of PhTX-343 on the basis of total ionization shifts could not distinguish between C5 and C6. However, although the two carbons have nearly identical limiting chemical shift values, they show distinctly different pH profiles, especially during titration of the tetraprotonated form. The chemical shift of C6 is expected to be affected mainly by the protonation state of N_c and the chemical shift of C5 by the protonation state of N_b because the β-effects are much larger than the γ-effects. Since nitrogen N_c is not deprotonated during the first deprotonation step (according to the behavior of C8, C9, and C10), the nonidentity of the titration curves of C5 and C6 must reflect the deprotonation of N_b and at the same time provides the basis for assignment of C5 and C6. Specifically, the chemical shift of C5 changes by 33% of the total ionization shift during the first deprotonation, whereas the chemical shift of C6 changes only by 13%, being in this respect similar to C9, which shifts by 10%. It should also be emphasized that the chemical shift change of C2 during the first deprotonation step (1.71 ppm) is not compatible with any significant deprotonation of N_a, since the deprotonation shift of the β-carbon in primary amines is 5.56 ppm.[14,20]

Although the assignment of C5 and C6 could be obtained by the analysis of titration curves, similar arguments cannot be applied to C4 and C7 because α- and γ-protonation shifts are very similar,[20,21] and hence the titration curves will be nearly identical irrespective of the deprotonation sequence (cf. Figure 2).

The results of calculation of proton distribution patterns according to eq 7 fully agree with the arguments presented above, although the fractional protonation values are generally overestimated by 4−9% (Table 3). The latter error is expected when long-range effects are not accounted for (protonation of a given site is overestimated if the observed chemical shift change is solely ascribed to the effect of the change at the nearest site), but the differences are small. The total ionization shifts in the polyamine chain of PhTX-343 are very similar to the corresponding shifts in spermine (Table 1), providing further evidence that no pronounced specific interactions take place. Moreover, the obtained proton distribution pattern is internally consistent and in agreement with the results previously observed for polyamines. Thus, the preference for deprotonation of inner nitrogen(s) was deduced from NMR data for spermine, spermidine, and other tri- and tetraamines.[14−17]

Upon further titration of the triprotonated species, further deprotonation of N_b and the phenol group is the main process (Table 3). In the monoprotonated form, the single proton is shared practically equally between the three nitrogen atoms, in agreement with the equal microscopic basicities expected for these sites.[25] In this respect, PhTX-343 is similar to spermine.[14]

The NMR titrations were in the present work carried out in D_2O in order to obtain ^1H NMR chemical shift data along with ^{13}C NMR shift values. ^1H NMR ionization shifts have previously been used in a titration study of spermidine,[25,26] and in fact, all 12 microscopic ionization constants were obtained by stepwise multiparameter fits. However, although all proton resonances of PhTX-343 could be assigned from two-dimensional experiments (Figure 4), the proton ionization shifts are small and hence the data do not have the precision necessary for an analysis by nonlinear fit. PhTX-343 has 26 microscopic ionization constants, and it is unrealistic to derive them from experimental titration curves. Besides giving general support to the conclusions obtained from the ^{13}C NMR data, the ^1H NMR spectra demonstrated that no specific interactions occur in the molecule during the titration, as shown by the absence of long-range NOEs, and that there is no systematic conformational changes of the tyrosine residue, as shown by the constancy of vicinal interproton couplings in the CH_2-CH group.

In conclusion, the main species present at different pH values in the solution of PhTX-343 (Figure 3) are as follows. (1) The monoprotonated form of PhTX-343 has an ionized phenol group and the proton shared between N_a, N_b, and N_c. (2) In the diprotonated form the two protons are shared between all basic sites, with the lowest occupancy of N_b. (3) In the triprotonated form, N_b is the least occupied site. The preferred protonation of the outer basic nitrogens N_a and N_c relative to inner nitrogen N_b can be readily explained in terms of electrostatic repulsion between the positively charged sites and follows the pattern previously established for polyamines.[14−17]

Since pK_a values become smaller as the ionic strength of the solution is decreased (Table 2), a solution of PhTX-343 at low salt concentration will contain a significant proportion of the triprotonated form. In a bioassay situation, the pK_1 of PhTX-343 may well fall below 8.0, where the content of the triprotonated form at pH 7.4 will exceed 20%, and its biological role cannot be excluded in advance. The observation that N_a remains highly protonated at all pH values relevant to the biological action is compatible with the significance of this charge in the channel-binding event and in agreement with the essential role of this amino group[6−9] for the biological activity of philanthotoxins. The nitrogen N_a is similar to N_c as far as the acid−base properties are concerned, and its charge is also likely to be involved in the binding process. The role of N_b is less clear. PhTX-433 is more potent than PhTX-343, and the former is expected to have pK_1 around 7.4 (cf. Table 2). The triprotonated form of PhTX-433 is, therefore, the predominant ionic species at physiological pH, and the preferred deprotonation of the inner protonated site (corresponding to N_b) is expected to be even more pronounced than in PhTX-343 because of the decreased distance between the charged sites. Thus, it does not appear that the charge at N_b is essential for the biological effects of philanthotoxins.

It should also be mentioned that substitution of PhTX-433 in the vicinity of the inner amino group results in a drastic increase of activity, which supports the possibility that this site is not involved in electrostatic interactions in the charged form. Interestingly, the analogue of PhTX-343 in which N_b and N_c have been replaced by methylene groups turned out to be highly active as determined electrophysiologically.[29,30] In conclusion, the basicity of the nitrogens is likely to contribute significantly to the differences in the biological effects of philanthotoxins and other polyamine toxins. The fact that the phenol group remains un-ionized at physiological pH is in agreement with the results of structure−activity studies, which strongly suggest that this part of the molecule takes part in lipophilic interaction only.[6−9]

Experimental Section

Chemicals. Spermine tetrahydrochloride was obtained from Sigma. PhTX-343 was obtained from Prof. P. N. R. Usherwood (University of Nottingham, England).[3] Solutions of PhTX-343 tris(trifluoroacetate) salt (10.3 mg) in D_2O (0.6 mL) contained 1 M KCl and were titrated with 0.5 M NaOH adjusted to ionic strength of 1 M with KCl. Acidity was measured directly in the NMR tubes, before and after acquiring the NMR spectrum, using a glass microelectrode. The measured values were converted to pD by adding 0.4.[28]

NMR Spectroscopy. NMR spectra were recorded with a Bruker AMX 400 WB spectrometer operating at 400.13 MHz for ^{1}H NMR and 100.62 MHz for ^{13}C NMR. Spectral widths and the number of data points were adjusted to obtain digital resolution in the frequency domain better than 0.3 or 0.8 Hz/data point for ^{1}H and ^{13}C NMR spectra, respectively. ^{13}C NMR spectra were obtained with WALTZ16 proton-decoupling scheme. Two-dimensional heteronuclear ^{1}H,^{13}C chemical shift correlation spectra were obtained with ^{13}C detection and proton decoupling in f_1. COSY spectra were obtained in the phase-sensitive mode. NOESY spectra were recorded in the phase-sensitive mode with mixing time of 600 ms. Water peak suppression in one- and two-dimensional NMR spectra was achieved by low-power presaturation. All spectra were recorded at 25.0 °C and standardized to internal sodium 3-(trimethylsilyl)propanesulfonate set to δ 0.0.

The ^{13}C NMR chemical shifts (δ, ppm) of PhTX-343 at pD 8.05 are as follows: 15.32 (Cγ), 21.56 (Cβ), 25.58 (C6), 25.58 (C5), 28.01 (C9), 26.63 (C2), 38.86 (C10), 38.65 (CH_2 in Tyr), 39.36 (C1), 39.92 (Cα), 47.30 (C3), 47.65 (C8), 49.66 and 49.74 (C4 and C7), 58.40 (CH in Tyr), 118.20 (*ortho*), 130.86 (*para*), 133.26 (*meta*), 157.15 (*ipso*), 176.50 and 179.85 (CO). The ^{1}H NMR chemical shifts (δ, ppm) of PhTX-343 at pD 8.05 are as follows (total ionization shifts in parentheses): H1 3.13 (0.50), H2 2.07 (0.54), H3 3.18 (0.60), H4 and H7 3.02 and 3.14 (0.42 and 0.59), H5 1.76 (0.33), H6 1.76 (0.33), H8 2.78 (0.36), H9 1.73 (0.18), H10 2.98 (0.11).

Data Analysis. The nonlinear curve fitting was carried out on a Macintosh Quadra 650 computer with Ultrafit v. 2.11 program from Biosoft, Cambridge, U.K., using Levenberg−Marquardt algorithm.

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