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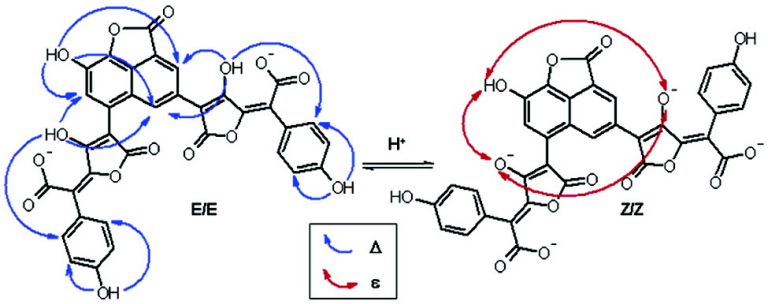

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Inframolecular Protonation Process of Norbadione A: Influence of the Ionic Environment and Stereochemical Consequences

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Abstract: The microscopic protonation mechanism, at an inframolecular level, of norbadione A, a pigment extracted from mushrooms and known to complex cesium cations, was determined by using ^{1}H NMR titrations and the cluster expansion method. This study revealed a pH dependent Z to E isomer switch that occurs in both pulvinic moieties. As a consequence, norbadione A can exist in solution in four stereomeric forms (E−E, E−Z, Z−E, and Z−Z), which can be of interest in the development of molecular-level devices. In the presence of 0.15 M NaCl, the calculated microconstants showed an unusual apparent cooperativity between the enol groups, which results from the release of the sodium cations upon protonation of norbadione A.

Introduction

Norbadione A is a pulvinic acid derivative first extracted from the brown pileus of the bay boletus (*Xerocomus badius* (Fr.) Kühn. Ex Gilg.)[1] and more recently from *Pisolithus arhizus* (Pers. Rauschert)[2] where its concentration may reach 10% of the dry weight of this mushroom. The structure of norbadione A has been characterized by Steglich's group about twenty years ago[1]. Investigations of radionuclide quantities present in edible mushrooms after the reactor accident at Chernobyl indicated that this pigment is able to capture cesium 137[3] and is therefore mainly responsible for the high level of radioactivity detected. Thus, the ability of norbadione A to selectively complex cesium cations opened the way for many promising applications. Among them, the separation of ^{137}Cs from the waste of nuclear power stations or its removal from contaminated soils. The latter application is particularly of interest as currently there is no efficient means for cleaning up ^{137}Cs after an accidental release into the environment.[4] Moreover, although by complexation of ^{137}Cs, norbadione A cannot prevent the radionuclide's radiative effects, remarkably, this ligand is able to neutralize the generated reactive oxygenated species due to very strong antioxidant activity.[5;6]

Two recent independent complexation studies of cesium cations by norbadione A[7;8], which mainly employed electrospray mass spectroscopic data, diverge in the complexed species that occur as well as in their stability constants. Both studies, however agree that the complexation of this cation is uncommon and is intimately linked to the protonation mechanism of the molecule.

Normally, only specialists appreciate that NMR titrations represent a most powerful tool to address protonation behavior of individual functional groups within complex molecules. Nevertheless, this approach has provided unique information in order to clarify the microscopic ionization mechanisms for a wide variety of molecules, such as, proteins,[9] dendrimers,[10] or

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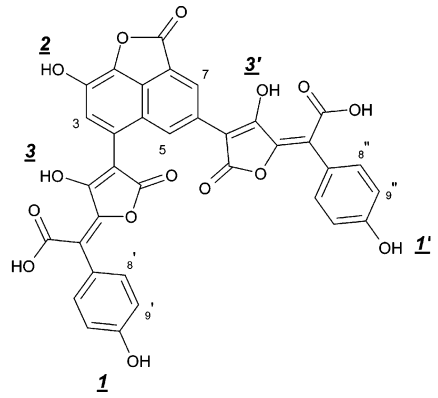
inositol-phosphates.[11] This technique follows the chemical shift of a nucleus, such as ^{31}P, ^{15}N, ^{13}C, or ^{1}H, as a function of pH, and relates this quantity to the degree of protonation of the ionizable group in question. We refer to this type of information as inframolecular, meaning that one considers the physicochemical properties at the level of a single functional group.[11] Such analysis requires, however, the full resolution of microscopic protonation equilibria, a task, which has been accomplished for very few molecules carrying more than three functional groups. Indeed, in the latter case 12 microconstants must be considered, 32 microconstants for a tetrafunctional compound, and more generally $N2^{N-1}$ microconstants for systems bearing N ionizable groups. However, recent cluster expansion techniques[12] circumvent this problem, and microscopic equilibria can be resolved based on NMR titration data for molecules with many ionizable sites. In addition to the determination of the microprotonation schemes, the titration curves provide further insights into the complex interplay of the intramolecular interactions, which ultimately govern the molecular conformation. This aspect, which is important for the complexation studies, becomes essential for the prediction of the antioxidant behavior of a given compound.[13]

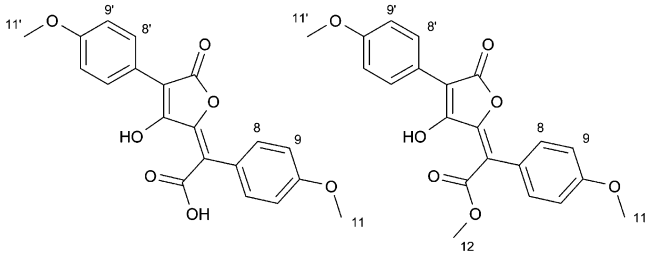
On the basis of potentiometric, spectrophotometric, and ^{1}H NMR titrations, in this paper we determine the protonation mechanism of norbadione A (**1**) at an inframolecular level. To ascertain the protonation sequence of norbadione A, we additionally examined two pulvinic acid analogues exhibiting methoxylated functional groups, where only two (**2**) or one (**3**) ionizable site(s) remain (see structures). We demonstrate that the protonation of norbadione A has most interesting stereochemical consequences, which in turn may even induce a remarkable cooperative effect between the ionizable sites. These results have important implications on the binding of alkali metal ions to norbadione A. Moreover, we shall see that analyzing NMR titrations with cluster expansion techniques represents a powerful tool to unravel protonation and complexation mechanisms in complex molecules.

Experimental Section

Materials. The dipotassium salt of Norbadione A (1) was obtained by extraction from Pisolithus arhizus according to reference 2. The two pulvinic derivatives (1 and 3) were synthesized according to the synthetic routes described in ref 6 and used without any further purification. The fully protonated form of norbadione A was obtained by lyophilizing a sample of potassium salt after passing it through a column containing a cationic IR120 Amberlite exchange resin with water as eluent. The purity was confirmed by IR, NMR and mass spectroscopy. The base used as titration reactant was tetramethylammonium hydroxide (Merck).



(1) norbadione A



(2) **(3)**

Structures

Potentiometric, UV−Visible and NMR Spectrometric Titrations. Potentiometric and NMR experiments were carried out in a CD_3OD:D_2O-80:20 (by weight) medium chosen for solubility purposes. The experiments were performed in two steps in which 0.50 mL of the same initial solution of the studied compounds of about 2×10^{-3} M was successively subjected to potentiometric and ^{1}H NMR titrations. The processing of the pH measurements and chemical shifts allowed the macroscopic protonation constants to be determined by using the programs Hyperquad[14] and HypNMR.[15] The ^{1}H NMR titrations were performed at 300.08 MHz on a Bruker DPX-300 FT-NMR spectrometer. Spectra were acquired with water presaturation over a spectral width of 12 ppm using a 3 s relaxation delay and a $\pi/2$ pulse. 12 K data points were sampled with a corresponding 1.14 s acquisition time. The spectra had a digital resolution of 0.30 Hz per point. The temperature in both cases was controlled at 25 ± 0.5 °C. The proton resonances of compounds **2** and **3** were assigned by performing proton−proton (2D-COSY) and carbon-proton 2D correlation experiments. All the NMR titrations were at least duplicated. For further experimental details, see ref 11a. UV−Visible spectra of compounds **1**, **2**, and **3** were recorded with a SHIMADZU UV-2401 PC spectrometer. The experiments were performed in two steps in which 0.50 mL of the same initial solution of the studied compounds of about 1×10^{-4} M, in a CH_3OH:H_2O-80:20 (by weight), Et_4NClO_4 0.1M medium, was successively subjected to potentiometric and UV−Visible titrations. Macroscopic protonation constants were determined by using the Hyperquad[14] program.

For the potentiometric measurements, it should be noted that the glass electrode was calibrated in a concentration scale and the measurements done in CD_3OD:D_2O-80:20 (by weight) when associated to the NMR titrations and in CH_3OH:H_2O-80:20 when preceding the UV−Visible titrations. In this work, for simplicity pH will stand for the cologarithm of the concentration of D^+ in the former medium and the cologarithm of the concentration of H^+ in the latter medium.

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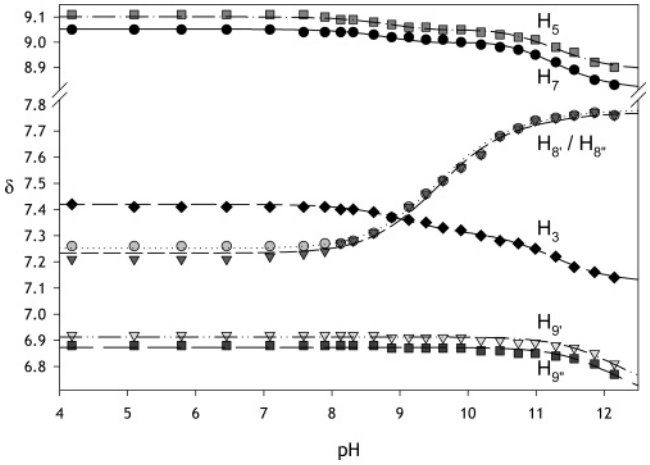

Figure 1. Chemical shifts δ from ^{1}H NMR titrations for norbadione A (**1**) as a function of pH at 25 °C (CD_3OD:D_2O-80:20). The least-squares fit is shown in solid line. The protons are labeled according to the structure.

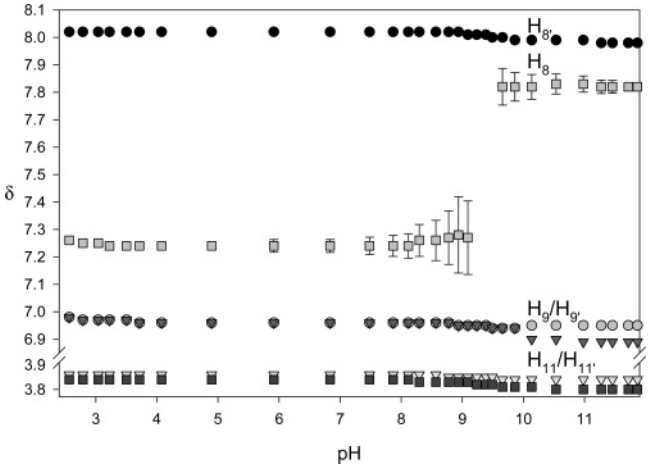

Figure 2. Chemical shifts δ from ^{1}H NMR titrations for the pulvinic derivative **2** as a function of pH at 25 °C (CD_3OD:D_2O-80:20). The protons are labeled according to the structure. The bar corresponds to the half-width of the resonance.

Results and Discussion

Protonation Sequence of Norbadione A. Norbadione A carries seven ionizable functional groups, namely, two carboxylic acids, two enols, two phenols, and one naphtholactonic alcohol. The potentiometric titrations clearly show that two of these groups exhibit close to strong acid characteristics, and that the logarithm of the protonation constants for the remaining five groups are equal or higher than 9.0 in conditions where no cations interfere. This observation prevents an initial assignment of macroscopic protonation constants to a specific functional group, since a given protonation step undoubtedly involves several groups. However, before the microscopic protonation process can be derived, an approximate knowledge of the acid−base character of each functional group is necessary. This information can be obtained by following the nuclei whose chemical shifts change as a result of the protonation of nearby basic groups, although the aromatic character of norbadione A renders this task less straightforward than for nonaromatic compounds.

A plot of chemical shift versus pH for norbadione A is shown in Figure 1. The titration curves will be discussed in terms of protonation, starting at high pH and then proceeding to lower pH values. Thus, it can be observed that the curves for the three naphthalenic protons (H_3, H_5, and H_7) parallel, undergoing a first marked downfield shift until pH 11.0, which later on softens until pH 8.0. The curves for phenolic $H_{9'}$ and $H_{9''}$ protons remain constant over nearly all the pH range with a first slight shift to lower fields (until pH 11.5). These curves can be interpreted based on the fact that the capture of a proton from a basic site leads to an electron density decrease and thus, via a through-bond effect, to a shift of the proton resonances to lower fields. Thus, the protonation of the two phenolates, is responsible for the initial downfield shift mentioned for $H_{9'}$ and $H_{9''}$, indicating that the logarithm of their protonation constants should be higher than 11.5, as expected for a phenol moiety. A same downfield shift occurs from pH 12.0 to 11.0 for the naphthalenic ring protons, which must be caused by the protonation of the β-naphtholate. Indeed, changes in the electron density near this site can be easily transmitted through the π system to more distant places so that H_5 and H_7 are affected in addition to H_3. Thus, the logarithm of the protonation constant of this group should be close to 11.0, which is in agreement with the literature.[7]

In contrary to the previously discussed protons, $H_{8'}$ and $H_{8''}$ are far more sensitive to the protonation of norbadione A as they undergo an upfield chemical shift change of ca. 0.6 ppm when titrating pH 11.0 to 8.0. This behavior is the so-called "wrongway shift", and has been observed in nucleotides[16] and inositol-phosphates[11d;11e], typically arising from conformational effects and occurring when a highly negatively charged group approaches a hydrogen atom. This effect, which is electrostatic in origin, operates through the field and affects the chemical shift of the hydrogen atoms in the neighborhood of the negative charge. However, taking into account the extent of the chemical shift variation, it is likely that anisotropic ring current effects also largely contribute to this effect.[17] Thus, the upfield shift of $H_{8'}$ and $H_{8''}$ can be attributed to the protonation of the carboxylates as well as to the enolates. We favor the latter attribution for two reasons. First, the expected log K of approximately 10 would be very high for a carboxylate group. Indeed, as shown by Rosés et al.,[18] the pK_a of a carboxylic acid in 80:20-MeOH:H_2O increases only by about 2 orders of magnitude with regard to water. Further, the downfield shift observed for H_3, H_5, and H_7 from pH 11 to 8 must be the result of the protonation of the closer enolate group rather than of the more distant carboxylate group.

To ascertain this protonation sequence of norbadione A, two pulvinic acid derivatives have been used, where the number of ionizable functional groups was reduced. Figure 2 shows the ^{1}H NMR titration curves of compound **2**, which keeps free both the carboxyl and the enol groups. These curves demonstrate that upon protonation of **2**, $H_{8'}$ and $H_{9'}$ are slightly shifted to lower fields while simultaneously H_8, the equivalent of $H_{8'}$ or $H_{8''}$ for norbadione A, is upfield shifted by 0.6 ppm. Remarkably, the H_8 signal broadens until pH 9.0, and then even

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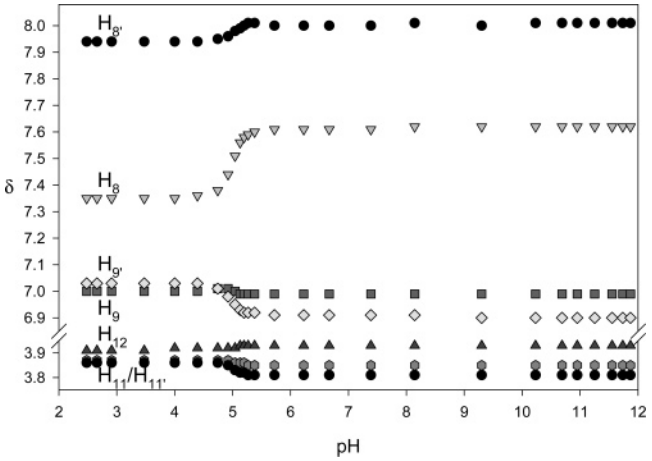

Figure 3. Chemical shifts δ from ^{1}H NMR titrations for the pulvinic derivative **3** as a function of pH at 25 °C (CD_3OD:D_2O-80:20). The protons are labeled according to the structure.

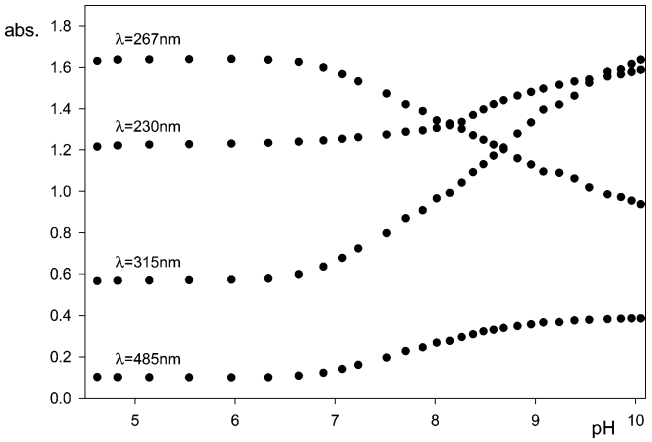

Figure 4. Absorbances of the four main absorption maxima versus pH for norbadione A.

disappears for three points of the titration curve, before becoming again well resolved for pH < 8.0. Figure 3 displays the ^{1}H NMR titration curves for compound **3**, which only carries the enol group. At first glance, these curves appear different from the previous ones, since the upfield shift of H_8 occurs at pH 5.0, which is 4.5 pH units lower than before. In addition, at pH 5.0, the signals of all the hydrogen atoms appear affected by the protonation of the enolate. This behavior can be explained by considering that for compound **3** only one negative charge leads to weak intermolecular repulsive forces, allowing thus the molecules to stack. More importantly, these results indicate that protonation of the enolate causes an upfield shift for proton H_8 for compounds **2** and **3**, and therefore of protons $H_{8'}$ and $H_{8''}$ for norbadione A. On the basis of NMR titration data, we thus conclude that norbadione A's functional groups decrease in basicity in the following order, namely, phenolates, naphtholate, enolates, and carboxylates.

This protonation sequence can be further confirmed by examining the UV−Visible spectra for the three compounds under study. Plots of the four main absorption maxima versus pH for norbadione A are shown in Figure 4. The absorbance of the three maxima at 230, 315, and 485 nm decreases from pH 10.0 to 7.0, whereas the one at 267 nm increases. For compound **2** two absorption maxima remain at 257 and 313 nm, while for

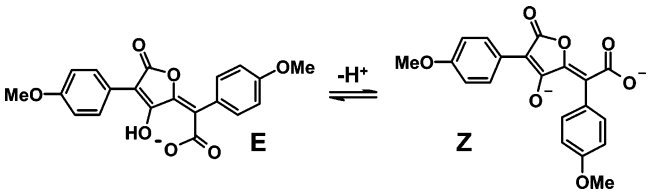

Figure 5. Z to E isomerization for the pulvinic derivative **2**.

compound **3** there is no longer an isobestic point since the absorption at 257 nm slightly varies in the opposite direction, and only the main peak at 313 nm subsists (see Supporting Information). Thus, it can be concluded for norbadione A that the absorbance decrease at 230 nm is due to the protonation of the naphtholate group and this at 315 nm to the protonation of the enolates. By considering now the pH at which the absorption transitions occur, it can be seen that the resulting protonation sequence is the same as this provided by the NMR titrations. Thus, we have proven by two independent techniques that the basicity of functional groups of norbadione A decreases in the order: phenolates, naphtholate, enolates, and carboxylates. The latter sequence differs from the one reported recently,[7] where the enols are considered being the most acidic groups. However, this difference becomes less critical if one considers the fact that intramolecular resonance-assisted O−H···O hydrogen bonding and π-delocalization confer to the seven-membered ring, including the enolate and carboxylate groups, a pseudo-aromatic character, which leads to the partial sharing of the proton by both groups. This hydrogen bond is further confirmed by the existence of the isomer switch, the 260 nm absorbance band in the UV−Visible spectra (see Supporting Information) and the quantum mechanical calculations.

Further examination of the NMR titration curves allows us to draw important conclusions concerning an unexpected pH dependent isomerization. This isomerization is evidenced by the large upfield shift and the significant broadening of the H_8 protons for norbadione A and compound **2**. Indeed, such broadening is typical for switching between two conformers or isomers that have an interconversion lifetime comparable to the NMR time scale. Our interpretation of this phenomenon is a Z to E isomer switch as shown in Figure 5. Such isomerization reactions are also well-known in Z−E isomer mixtures of pulvinic acid analogues, which easily self-isomerize to an exclusive isomer in ambient light.[19] In the case of norbadione A and of compound **2**, the E isomer of the pulvinic moiety is favored by a hydrogen bond between the carboxyl oxygen and the enol hydrogen as long as the enol group remains protonated. The E to Z isomerization is further triggered by the deprotonation of the enol allowing for the minimization of the electrostatic repulsion between both anionic groups. In addition, this mechanism implies a low energy barrier to rotation due to conjugation by resonance. The reason for the large upfield shifts now becomes obvious; it is due to the release of the interaction between the observed proton and the negatively charged enolate as the pulvinic moieties switch from a Z to E configuration. Such an isomerization is remarkable by its pH dependency and full reversibility; the back-titration namely yields the initial situation.

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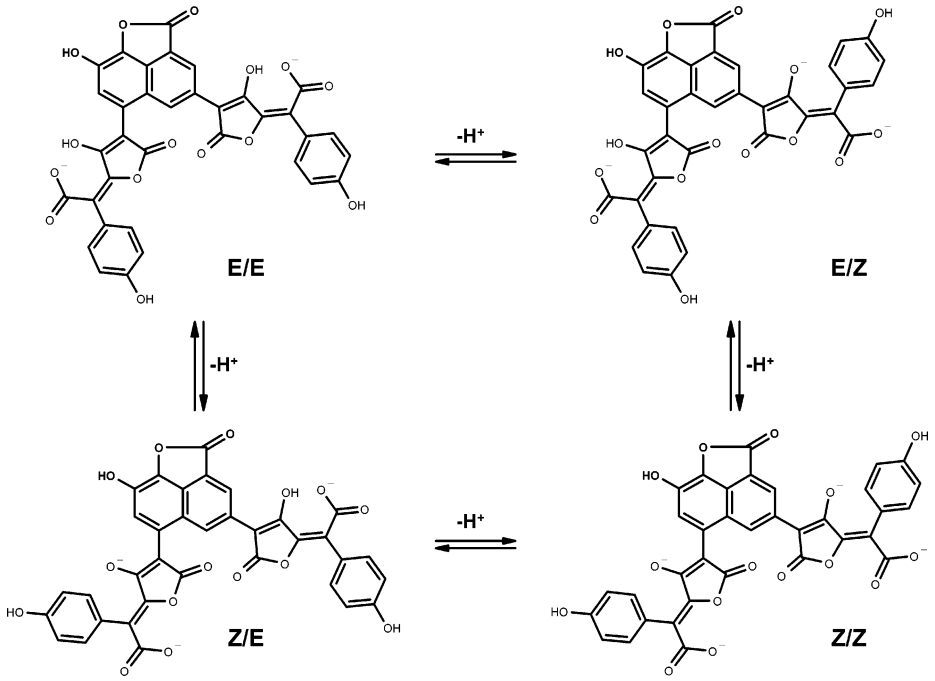

Figure 6. Four stereomeric possible forms for norbadione A (**1**).

Table 1. Logarithms of the Macro Protonation Constants for the Studied Compounds. Pair Interactions and Populations of Isomers for norbadione A

		norbadione A (**1**)								
		without salt			Et_4NClO_4 0.1M			NaCl 0.15M		
macroconstants	$\log K_1$	12.49 (>11)			11.63 (>11)			11.49 (>10)		
	$\log K_2$	11.93 (>11)			11.07 (>11)			10.90 (>10)		
	$\log K_3$	11.17			10.20			9.36		
		11.05 ± 0.06			*10.04 ± 0.02*			*9.30 ± 0.06*		
	$\log K_4$	9.79			9.07			6.94		
		9.34 ± 0.10			*8.88 ± 0.05*			*7.20 ± 0.08*		
	$\log K_5$	8.96			8.11			6.90		
		8.97 ± 0.09			*8.07 ± 0.05*			*7.06 ± 0.07*		
pair interactions	ϵ_{23}	0.86			0.82			0.95		
	$\epsilon_{23'}$	0.86			0.82			0.95		
	$\epsilon_{33'}$	0.00			0.00			−0.83		
isomers (%)		Z/Z	E/E	E/Z−Z/E	Z/Z	E/E	E/Z−Z/E	Z/Z	E/E	E/Z−Z/E
	$n = 5$	0	100	0	0	100	0	0	100	0
	$n = 4$	0	32	68	0	47	53	0	28	72
	$n = 3$	79	<1	21	65	<1	34	98	<1	2
	$n = 2$	95	<1	4	95	<1	5	100	<1	<1
	$n = 1$	99	0	1	99	0	1	100	0	<1
	$n = 0$	100	0	0	100	0	0	100	0	0
macroconstants		pulvinic acid **2**						pulvinic acid **3**		
	[a]$\log K_1$	9.09 ± 0.12						5.04 ± 0.03		
	[a]$\log K_2$	<4						/		
		calculations by Hyperquad from UV measurements in $MeOH/H_2O$ 80/20								
	[b]$\log K_1$	*8.46* ± 0.01						*4.68* ± 0.07		
	[b]$\log K_2$	<4						/		

Macroconstants calculated by the cluster expansion method (roman) and determined by potentiometry (*italics*). For compounds **2** and **3**, values [a] were obtained from NMR titrations without any salt and values [b] by UV−visible spectrometry in 0.1 MEt_4NClO_4 ($MeOH/H_2O$ 80/20). The uncertainties are estimates of the standard deviation as calculated by HypNMR[15] and Hyperquad[14] for the macroconstants. The error on the macroconstants determined by the cluster expansion method is ±0.05.

To confirm the existence of this isomerization reaction, titration experiments were repeated at −15 °C for compound **2**, which shows the largest broadening of the H_8 protons. At this temperature, the rate of interconversion should be sufficiently slow to enable the observation of the isomer resonances. The corresponding titration curves (see Supporting Information) show indeed two resonances at 7.84 and 7.24 ppm for the H_8 protons, whose relative intensities vary largely. The signal at 7.84 ppm, assigned to the Z isomer is present in the entire 8.5 to 12.5 pH range. In contrary, the signal at 7.24 ppm attributed to the E isomer vanishes progressively as soon as the enol deprotonates. [The effect of the protonation state on the Z/E

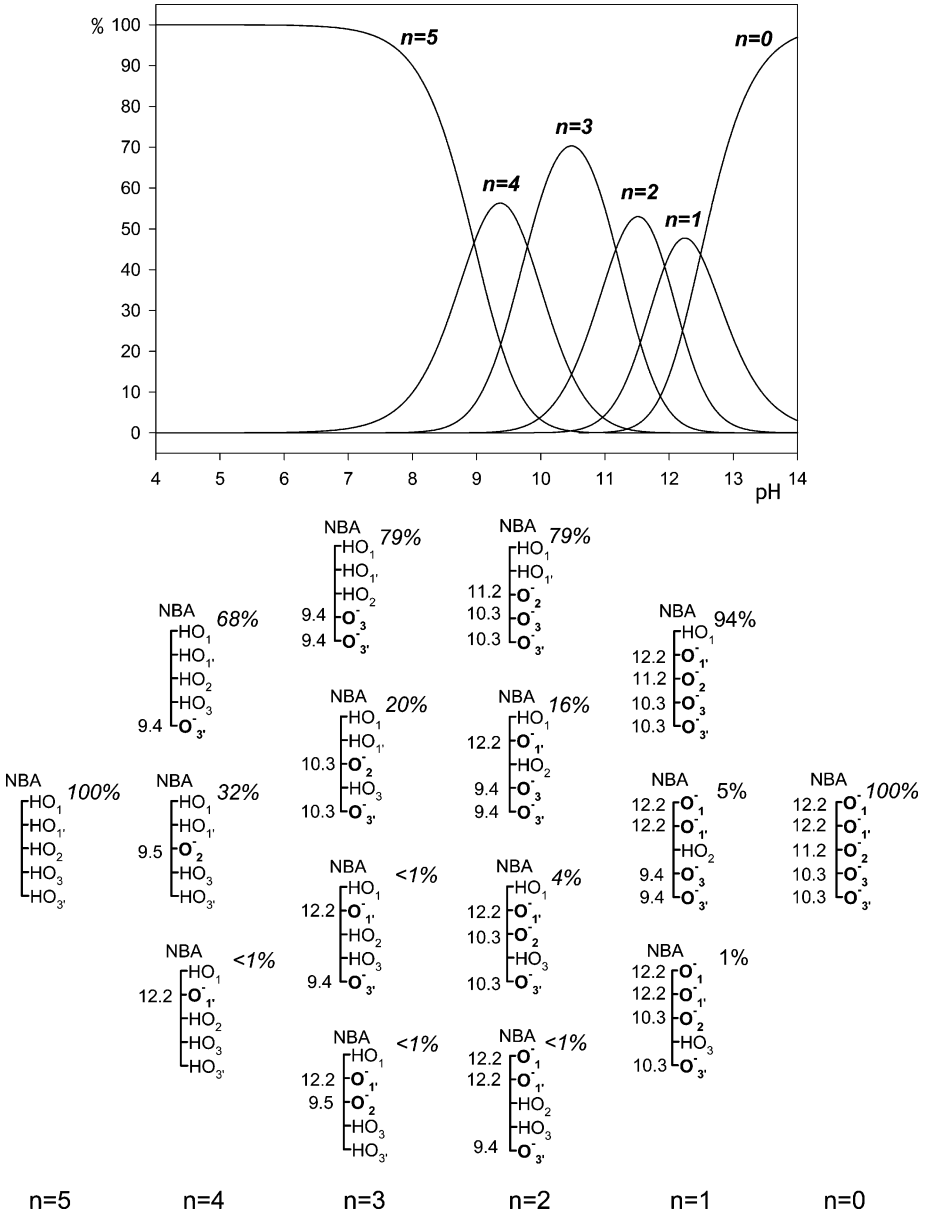

Figure 7. Relative concentrations of the macrospecies and micro conditional probabilities for norbadione A (**1**) determined without supporting-electrolyte at 25 °C in $CD_3OD:D_2O$-80:20. The calculated microconstants at each protonation step are associated to their functional group labeled as the protonation sites in the structure of norbadione A (**1**). The error on the microconstants is ±0.1.

configuration was confirmed by quantum mechanical calculations (HF /6-31G** level) on the $\mathbf{2}^-$ and $\mathbf{2}^{2-}$ species, optimized in their E versus Z forms. $\mathbf{2}^-$(E) is found to be much more stable than $\mathbf{2}^-$(Z) (by 30.3 kcal/mol), due to a strong internal hydrogen bond between the coplanar enol and carboxylate groups (with $O_{enol}\cdots H-O_{CO2}$ distances of 1.56 and 0.97 Å). Deprotonation of $\mathbf{2}^-$ to form $\mathbf{2}^{2-}$ leads to a small preference for the Z isomer (by 1.5 kcal/mol). In these two isomers, the carboxylate group positions perpendicularly to the enolate fragment, thus minimizing repulsions between the oxygen atoms. The calculations further support the stabilization of the E form of **3** by internal hydrogen bonding (with $O-H_{enol}\cdots O_{CO2}$ distances of 0.96 and 1.66 Å). R. Diss and G. Wipff, private communication.].

The E to Z switch for each two pulvinic moiety leads therefore to four stereomeric forms of norbadione A, namely, E/E, E/Z, Z/E, and Z/Z, all of which being in pH dependent equilibrium (Figure 6). Thus, defining the protonation process at the inframolecular level will assist to understand why an isomer is preferred at a given pH.

Quantitative Macro and Micro Protonation Process of Norbadione A. As previously discussed, even though norbadione A carries seven protonable groups, only five of them are able to be protonated for pHs ranging from 12.0 to 3.0. Thus, stepwise macroscopic protonation can be defined by a stepwise constant $K_{y,}$ characterizing the equilibrium

$$H_{y-1}L^{(8-y)-} + H^+ \overset{K_y}{\Leftrightarrow} H_yL^{(7-y)-} \qquad (1)$$

These macroconstants can be determined from potentiometric and ^1H NMR titration curves by analyzing the pH or chemical shift data with the Hyperquad[14] or HypNMR[15] programs. However, these constants contain no a priori information concerning the ionization of individual sites.

When each ionizable site is considered individually, microscopic protonation equilibria must be discussed. However, the

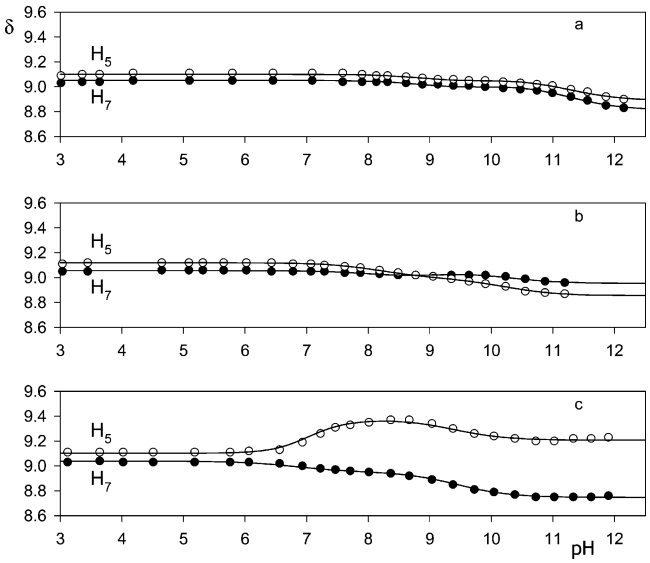

Figure 8. Chemical shifts δ from ^{1}H NMR titrations for norbadione A (**1**) as a function of pH for the H_5 and H_7 protons at 25 °C. a) without supporting-electrolyte, b) in 0.1 M Et_4NClO_4, c) in 0.15 M NaCl. The least-squares fit is shown in solid line.

resolution of microscopic protonation equilibria remained a substantial problem for complex molecules until the "cluster expansion method" became available.[12] Its mathematical basis can be summarized as follows. The entire microprotonation scheme for a *N*-functional base, is made up with a series of microequilibria of the type

$$A\{s_i\} + H^+ \leftrightarrows A\{s_i'\} \qquad (2)$$

Protonation microstates can be specified with a two-valued state variable s_i for each individual site i ($i = 1, 2, \ldots N$), such that $s_i = 1$ if the site is protonated and $s_i = 0$ if the site is deprotonated. Thus, a given protonated microstate is defined by the set of state variables s_1, s_2, ... s_N. If now a particular site j among all the sites i is being protonated, then in eq 2, $s_j = 0$ and $s_j' = 1$, whereas $s_i = s_i'$ otherwise. The microconstant for this protonation equilibrium, $\log k_{s_i}$, can be expressed as an expansion in terms of cluster parameters according to eq 3

$$\log k_{s_i} = \log k_i - \sum_j^N \epsilon_{ij} s_j \qquad (3)$$

where $\log k_i$ is the first microprotonation constant for site i of the fully deprotonated species and ϵ_{ij} the parameter for interaction with 2 neighboring sites. The pair interaction ϵ_{ij} represents the change in the microscopic protonation constant of site i when site j is being protonated.

Moreover, as well established in NMR studies, the protonation of a nearby group leads to a change in the observed chemical shift of a nucleus l, and is given by

$$\delta_l = \delta_l^{(0)} + \sum_{m=1}^N \Delta_{lm}\theta_m \qquad (4)$$

where $\delta_l^{(0)}$ is the chemical shift of l for the deprotonated base, Δ_{lm} is the change due to the protonation of the site m and θ_m, the averaged degree of protonation of that site. We refer to $\delta_l^{(0)}$ and Δ_{lm} as *chemical shift parameters*.

Therefore, each chemical shift measured as a function of pH can be expressed as a function of the *cluster parameters* and *chemical shift parameters.* These parameters can then be obtained by a nonlinear least-squares procedure, provided that the quality of the available experimental chemical shifts is pertinent, and their number sufficient to keep the system overdetermined. More details of these procedures are provided elsewhere.[12]

For the treatment of the microprotonation process of norbadione A, the phenol groups will be labeled sites *1* and *1′*, the naphthol group, site *2*, and the enol groups sites *3* and *3′*. The carboxylic acids will not be considered, since for the present concentration conditions they behave as strong acids. Even with this simplification, we must still consider five functional groups for norbadione A. This leads to 15 cluster parameters, whose determination is highly uncertain. Fortunately, however, the number of these parameters can be significantly reduced based on structural considerations. First, we observe that the titration curves of sites 1 and 1′ as well as sites 3 and 3′ cannot be really distinguished, and therefore the two phenols and the two enols display the same intrinsic basicity, leading to $k_1 = k_{1'}$ and to $k_3 = k_{3'}$. In addition, due to the substantial distance between the ionizable groups, we can reasonably assume that there are no interactions between both phenols, as well as between the phenols and the enol or naphthol groups.

Initial calculations indicated that $\epsilon_{33'}$, the interaction parameter between both enols, is close to zero. Therefore, we retain as possible interactions only those between the enols and the naphthol group, which are likely to occur through the π system. Thus, 5 cluster parameters remain, namely the microscopic protonation constants $k_1 = k_{1'}$, k_2 and $k_3 = k_{3'}$ in addition to the interaction parameters ϵ_{23} and $\epsilon_{23'}$. The subscripts denote the individual group position on norbadione A labeled as indicated above.

Since experimental observations showed that the presence of salts markedly affects the protonation process of norbadione A, the titrations were first carried out without supporting-electrolyte. In the calculations performed on the curves of Figure 1, it was assumed that protons H_3, H_5, and H_7 responded to the protonation of the naphthol, the group of the H_8 protons to the enols and those of the H_9 protons to the phenols. While the fit is satisfactory, it could be improved considering that protons H_3, H_5, and H_7 respond to the protonation of the enols slightly. Moreover, since ϵ_{23} and $\epsilon_{23'}$ were found very close, they were kept equal in the further calculations. The fact that these two interactions must be similar can be also inferred from structural considerations.

The calculated macroconstants, pair interaction parameters and isomer populations are listed in Table 1. Note that the calculated macroconstants agree well with independent determinations by potentiometry. The different microstates are shown in Figure 7 with the respective microconstants and the conditional microstate probabilities. The summation of these probabilities for a given protonation step are displayed in the same figure (see Supporting Information for the Δ_{lm} chemical shift parameters).

The protonation mechanism of norbadione A in the absence of any supporting electrolyte can now be discussed in detail. By decreasing pH, a first phenolate, which has a microconstant

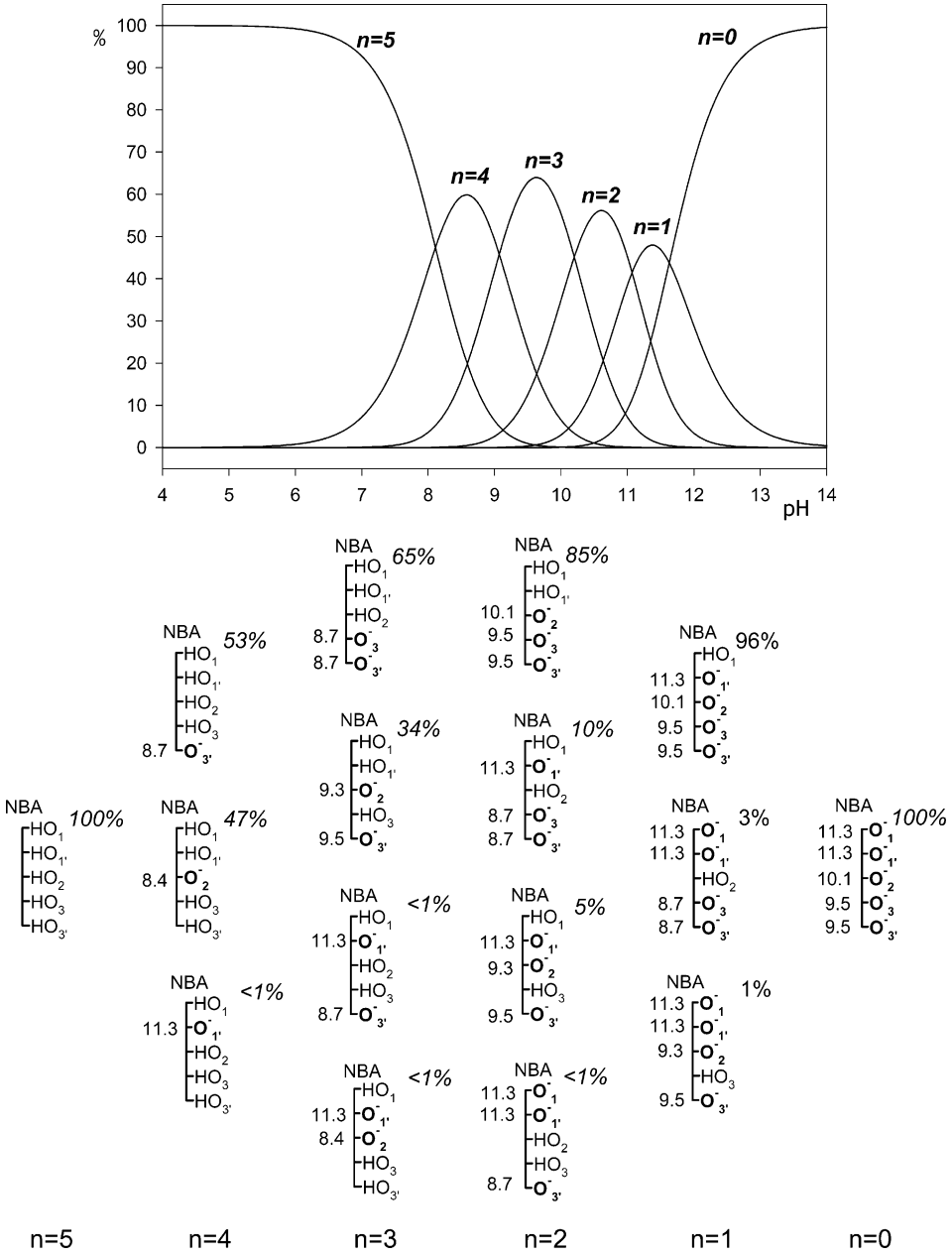

Figure 9. Relative concentrations of the macrospecies and micro conditional probabilities for norbadione A (**1**) determined at 25 °C in 0.1 M Et_4NClO_4 (CD_3OD:D_2O-80:20). The calculated microconstants at each protonation step are associated to their functional group labeled as the protonation sites in the structure of norbadione A (**1**). The error on the microconstants is ±0.1.

of 12.2, protonates almost exclusively (94%). The most abundant microstate species with two protons corresponds to the bisphenol species (79%), along with a phenol−naphthol species (16%) and the minor phenol−enol species (4%). The next step mainly involves the protonation of the naphthol group with a microconstant of 11.2, leading to the dominating microstate with three protons involving the bisphenol−naphthol form (79%), and to a lesser extend through protonation of the first enol group with a microconstant of 10.3 to the bisphenol−enol form (20%). Finally, the species with four protons is dominated by the bisphenol−naphthol−enol form (68%), which is generated by the protonation of the enolate of microconstant 9.4, and the minor bisphenol−bisenol form (32%).

From this discussion, we can quantify the proposed protonation sequence for the major microspecies: the phenolates have a microconstant of 12.2, the naphtholate 11.2, and the enolates 9.4. The latter value is well comparable to the protonation constant of the enol group for compound **2**, $\log K_1 = 9.09$. This similarity in values indicates the validity of the microscopic picture proposed.

This discussion shows clearly that a single protonation step in norbadione A involves equilibria between several microspecies. A macrospecies can be dominated by a single microspecies in special situations, and in that case a macroconstant often coincides with the microconstant of the proton transferred[12;20]. In contrast to previous suggestions, however, a macroconstant cannot be attributed to a unique binding site in norbadione A.[7]

Let us now connect the microscopic protonation mechanism to the Z to E isomer switch. In general, each microspecies can comprise several conformers or isomers,[21] and the microconstants and interaction parameters represent averages of the

corresponding parameters for the individual conformations or configurations. In the case of norbadione A, the ionization of the enol groups triggers the E to Z isomer switch, and thus an individual diastereoisomer can be associated to an individual microstate. Indeed, two protonated enols impose the E/E form, whereas the entire deprotonation of these groups sets the Z/Z form. The intermediate E/Z or Z/E forms result from the ionization of the enol of one pulvinic moiety only. On the basis of these considerations, we can thus estimate the percentages of the diastereoisomers at each protonation step (see Table 1). The obtained microconstants can be directly assigned to the different isomers, since each microstate is dominated by a single configuration. Therefore, since the putative complexation properties toward alkali metal ions of these diastereoisomers are expected to be very different, the knowledge of their pH dependency appears to be fundamental in further complexation studies.

Salt Effects on the Micro Protonation Process of Norbadione A. To keep the ionic strength of the solution constant, we have initially employed tetraethylammonium perchlorate (Et_4NClO_4) at 0.1 M, whose large cation and anion are usually presumed to have a negligible influence on protonation constants. However, titration experiments resulted in significant differences due to the presence of this supporting-electrolyte (see Supporting Information). Except for H_7, all of the curves keep the same shape but are shifted by 0.6 log unit to lower pH values, when compared to titration curves without salt present. Moreover, H_5 differentiates from H_7 (Figure 8b) indicating that Et_4NClO_4 exerts a distinct contribution on the enol and naphthol groups. The least-squares fit performed on the titration curves leads again to $k_3 = k_{3'}$, and $\epsilon_{33'} = 0$ indicating weak interaction between both enol groups.

The microscopic protonation mechanism is similar as in absence of salt. However, the calculated microscopic constants (Figure 9) are about 0.8 log unit lower for the phenols and the enols and even 1.1 log units lower for the naphthol group compared to the titration without salt. This decrease clearly favors the Z/E−E/Z stereoisomer in step three and the E/E isomer in step four (Table 1). The macroscopic protonation constants exhibit the same trend as the microscopic ones and are commensurate with those published by Garaudee et al..[7] The decrease in basicity of all the functional groups could be the result of the interaction between norbadione A and the Et_4N^+ cations, but more likely is due to the changes in the structure of the solvent, which is in a more ordered state in the presence of hydrophobic groups. It has indeed been shown that hydrophobic hydration of tetra-alkylammonium cations leads to water structuring.[22] It can therefore be expected that these cations may indirectly affect the solvation shell of norbadione A and interfere with the complex network of hydrogen bonds, which contributes to determine the acid−base character of the functional groups.

Due to the complexation properties of norbadione A toward cesium as well as for its antioxidant properties, the use of this compound as therapeutic agent was proposed[6;8]. In biological media, norbadione A will be surrounded by many ionic species among them alkali cations present in the hundred millimolar concentration range. To investigate the influence of the ionic environment on the microprotonation process of norbadione A, NMR titration experiments were carried out in 0.15 M NaCl. The resulting curves, displayed in Figure 10, show that the protons are selectively affected by the sodium cations. Thus, in the presence of Na^+, the curves of the $H_{9'}$ and $H_{9''}$ protons are almost unaltered, but those of $H_{8'}$ and $H_{8''}$ are shifted by approximately 3 orders of magnitude to lower pH values. This observation points toward a strong competition between these protons and the sodium cations, and can be interpreted as a sign of complexation. Moreover, the curves for H_5 and H_7 assume shapes never previously observed (Figure 8c). Upon protonation, H_5 first shifts downfield from pH 10.2 to 8.5, and then moves in the opposite direction until pH 6.5, whereas H_7 only shifts to lower fields for the same pH range. Figure 10 indicates a slight downfield shift for the H_8 protons upon protonation at high pH, which suggests that these protons also respond to the protonation of the naphthol groups.

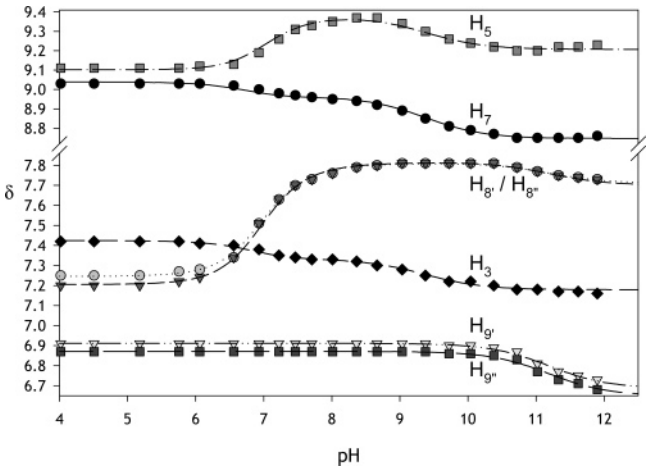

Figure 10. Chemical shifts δ from ^{1}H NMR titrations for norbadione A (**1**) as a function of pH at 25 °C in 0.15 M NaCl (CD_3OD:D_2O-80:20). The least-squares fit is shown in solid line. The protons are labeled according to the structure.

Table 1 lists the macroscopic protonation constants determined in the presence of sodium cations, which show, with regard to those obtained in both previous media, widely different values of log K_3 and log K_4, and for log K_4 and log K_5 values that are unusually close. The large decrease observed on log K_3, log K_4, and log K_5 with respect to the other media, indicates a very strong influence of Na^+ on the protonation mechanism of norbadione A, which exerts particularly on the enol groups. The proximity of log K_4 and log K_5 clearly indicates a cooperative effect, which is most unusual for protons. Recall that for two noninteracting sites, such as the two enols when the other site are protonated, log K_5 − log K_4 should amount 0.6, and a smaller value can be only caused by a cooperative effect. These constants lead to a species with four bound protons that is here not very prominent suggesting a sudden transition between the species with three and five protons (Figure 11).

The origin of this cooperative effect can be traced by analyzing the NMR titration data and the corresponding microscopic equilibria. To obtain a satisfactory description of the slight downfield shift for the H_8 protons (see Figure 10), it is necessary to include additional $\Delta_{H8'-1}$ and $\Delta_{H8''-1}$ chemical shift parameters in the calculations (see Supporting Information). Moreover, the fit immediately yields a strongly negative

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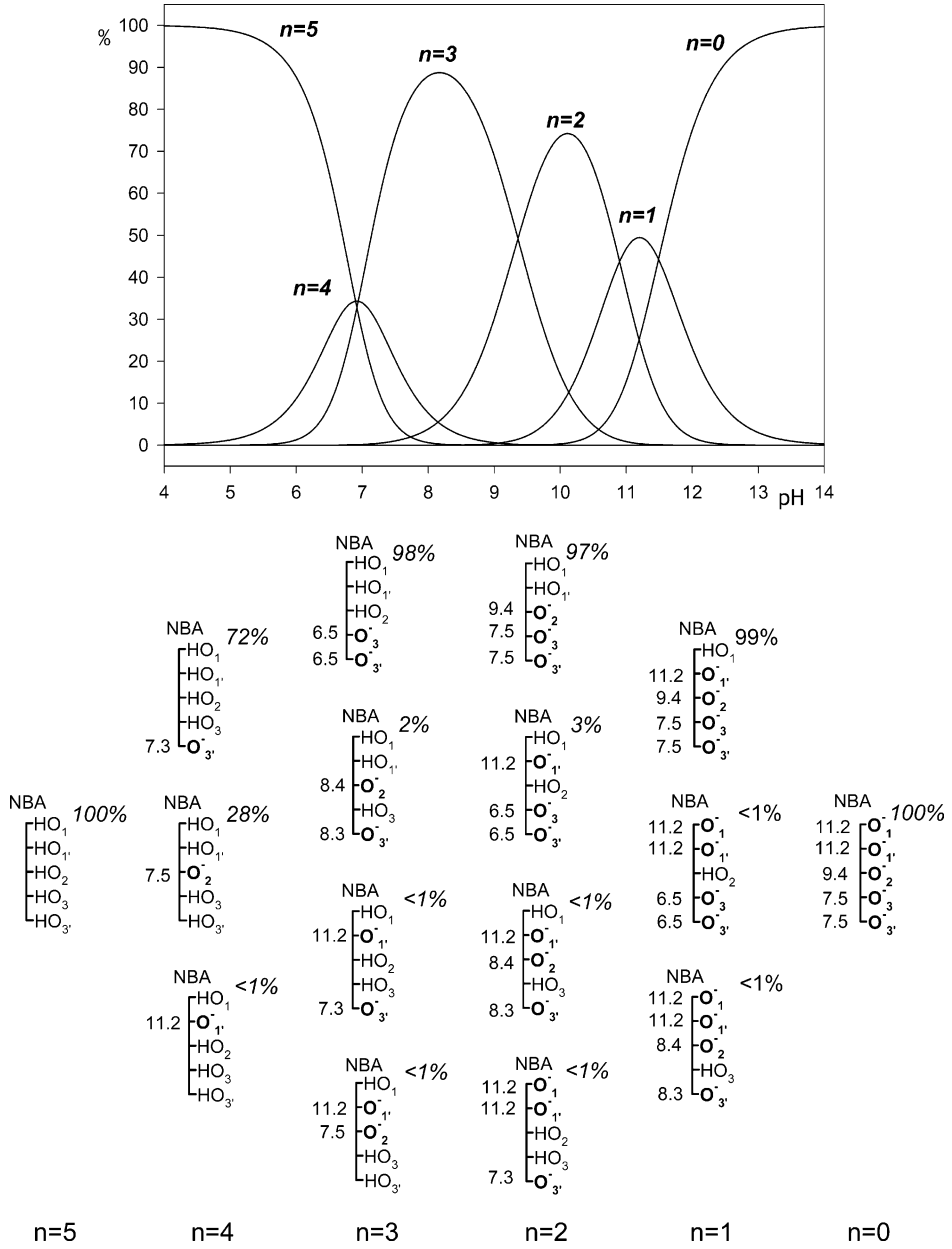

Figure 11. Relative concentrations of the macrospecies and micro conditional probabilities for norbadione A (**1**) determined at 25 °C in 0.15 M NaCl (CD_3OD:D_2O-80:20). The calculated microconstants at each protonation step are associated to their functional group labeled as the protonation sites in the structure of norbadione A (**1**). The error on the microconstants is ±0.1.

(cooperative) value for the interaction between the enol groups $\epsilon_{33'} = -0.83$ in the presence of sodium cations. This trend is in marked contrast to the previous data in the absence of sodium cations, and setting this parameter to zero leads to a poor fit. This model derived on the basis of the NMR data yields very similar macroconstants log K_4 and log K_5, thus confirming the cooperative nature of the protonation process.

The resulting protonation microconstants of the enols involving the main species of steps 3 and 4 show an unusual increase from 6.5 to 7.3 (Figure 11). This trend, which is related to the negative value of the interaction $\epsilon_{33'} = -0.83$, indicates that the protonation of one enol group strongly promotes the protonation of the second. Figure 11 further illustrates that the relative percentages of the microspecies for a given protonation step substantially differ from previous ones. The first three steps correspond almost exclusively to the successive protonation of the phenols followed by the naphthol, and the fourth and fifth steps to the protonation of mainly the enols. By taking into account this protonation pathway, we propose that the sodium cations strongly interact with norbadione A in a Z/Z form where both enol groups are required to be deprotonated. In addition, the sodium cations are most likely to constrain norbadione A in a conformation, where the two enolates are oriented inward, namely being positioned oppositely to each other. This proposal can be further confirmed by the observation that the H_5 proton undergoes a highfield shift from pH 8.5 to 6.5. At pH 8.5, the enolate of site 3 faces site 3′ due to its mutual interaction with the Na^+ cations, leading to a small distance between site 3 enolate and H_5, that deshields the latter for the same reasons as described above for H_8. With decrease in pH, one enolate protonates and releases the sodium cations. This release simultaneously provides a larger conformational freedom to the molecule, increasing the distance between site 3 and H_5, which leads to the observed highfield shift.

From the NMR and potentiometric titration data we thus conclude that protonation of one enolate triggers the release of the sodium cations and, via a conformational change, renders the second enolate more favorable to protonation conditions. Thus, the first proton acts as an allosteric effector, which leads to an apparent cooperativity between both enols. Such cooperative effects are well-known for binding of metals and ligands to proteins,[23] but are most remarkable for protons interacting with a molecule of a rather modest molecular mass.

Conclusions

Full resolution of microscopic protonation equilibria of polyprotic molecules can be achieved with NMR titrations and data analysis by cluster expansion methods. On the basis of ^{1}H NMR, potentiometric, and spectroscopic titrations for norbadione A and related pulvinic acid derivatives, we do not only obtain a detailed microscopic picture of the protonation process, but we can also demonstrate that the protonation is accompanied by an isomerization of the molecule. We have shown that the basicity decreases in the following order, namely, phenolates, naphtholate, enolates, and carboxylates. Due to the existence of several prevalent microspecies, however, macroscopic constants cannot be assigned to individual ionizable groups.

Inspection of the microprotonation process allows a preferred diastereoisomer to be associated to an individual microstate. Two protonated enols impose the E/E form, whereas the entire deprotonation of these groups defines the Z/Z form. The intermediate E/Z or Z/E forms result from the ionization of the enol of only one pulvinic moiety. The pH dependent interconversion of these diastereoisomers is most remarkable from the point of view that great interest is currently devoted to the design of molecules that exist in different forms able to be interconverted by an external stimulus. Such molecules may lead to molecular-level switching devices of high practical and fundamental interests. Norbadione A may appear as the first candidate of pulvinic acid derivatives for applications of information processing at the molecular level. We have further shown that protonation of norbadione A is strongly influenced by sodium cations, which interact with the enol groups, thus favoring the Z/Z isomer in a conformation, where both enolates are placed opposite each other. This interaction leads to a cooperative effect in the protonation of two enolate groups, which contrasts the situation in the absence of Na^+, where both pulvinic moieties keep much conformational freedom. Further pH decrease leads to the protonation of one enolate, triggers the release of the sodium cations, and promotes the protonation of the second enolate in a mechanism where the basicity of this group is strongly raised with regard to the first one.

This observation is fascinating, as it demonstrates the existence of an allosteric effect in norbadione A. We suspect that a similar allosteric effect is most important in the understanding of the complexation of cesium by norbadione A under physiological conditions.

Supporting Information Available: UV−Vis titrations, 1H-NMR spectra, chemical shift parameters, and titration curves for the substances in this manuscript. This material is available free of charge via the Internet at http://pubs.acs.org.

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