Norbadione A: Kinetics and Thermodynamics of Cesium Uptake in Aqueous and Alcoholic Media

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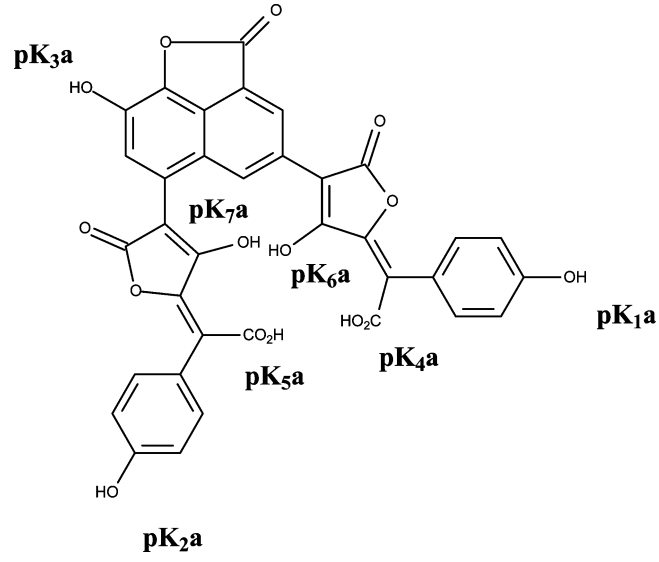
Received: April 2, 2010; Revised Manuscript Received: August 24, 2010

Norbadione A (NbA) is a mushroom pigment, which is assumed to be involved in ^{137}Cs accumulation all over Europe during the Chernobyl nuclear accident. NbA bears seven acid−base functional groups, among which are two enolic and two carboxylic acid moieties. This work deals with complex formation of Cs^+ and NbA in ethanol, ethanol/water (9:1) (M1), and water with, when required, the support of two Cs^+ ionophore probes, calix[4]arene-bis(crown-6-ether)dioxycoumarine (A1) and its tetrasuslfonated form (A2). In ethanol, two Cs^+ complexes are formed, with the affinity constants $K_{1EtOH} = (1.1 \pm 0.25) \times 10^5$ and $K_{2EtOH} = (2.1 \pm 0.4) \times 10^3$. In M1, a single Cs^+ complex occurs when only the enols are deprotonated, whereas a bicomplex is formed when both enols and carboxylic acids are deprotonated: $K_{1M1} = (1.5 \pm 0.3) \times 10^5$ and $K_{2M1} = (4 \pm 2) \times 10^3$. These data are confirmed by stopped-flow and T-jump kinetics. In ethanol, a fast Cs^+ exchange occurs between NbA and A1: direct rate constant, $k_1 = (3.1 \pm 0.1) \times 10^7\ M^{-1}\ s^{-1}$; reverse rate constant $k_{-1} = (2.8 \pm 1) \times 10^5\ M^{-1}\ s^{-1}$; and Cs^+ exchange constant, $K_{1Exchange} = (9 \pm 4) \times 10^{-3}$. In M1, the quenching of A2 fluorescence by NbA is used to determine the kinetics of complex formation with Cs^+: $k_2 = (1.8 \pm 0.4) \times 10^9\ M^{-1}\ s^{-1}$; $k_{-2} = (1.80 \pm 0.15) \times 10^4\ s^{-1}$; and $K_{1M1} = (1.5 \pm 0.5) \times 10^5$. The affinity of NbA for Cs^+ is probably the result of the particular structure in which the two pulvinic acid arms adopt a conformation that forms two complexation sites composed of the two enolates and/or the two carboxylates. This renders the efficiency in Cs^+ uptake comparable to that of some calixarenes or crown ethers.

Introduction

Norbadione A (NbA) is a naphtholactone (Scheme 1) related to a family of mushroom pigments that includes the pulvinic acids.[1,2] NbA is found in the brown pilius of the bay boletus and at high concentrations (25% of the dry weight) in *Pisolithus arrhizus*.[1−4] During the Chernobyl disaster, a cloud of several radioactive isotopes, including ^{137}Cs and ^{131}I, was released into the environment. The half-life of ^{131}I is 8 days, whereas that of ^{137}Cs is 30 years. A substantial part of the ^{137}Cs was complexed by NbA and accumulated in the edible and appreciated bay boletus mushroom.[1−3] This led to contaminations of game meat and people all over Europe.[5] In the face of such a major health problem and the promise that NbA may constitute a ^{137}Cs decontaminant, several studies were undertaken at both the experimental and theoretical levels. Some showed that NbA possesses interesting antioxidant and antiradiation capabilities, whereas others dealt with Cs^+ uptake in aquo-alcoholic media, as well as with the role of the protodissociations of the pulvinic acid moieties in complex formation and the role of intramolecular hydrogen bonding.[6−12] NbA bears seven functional groups all of which undergo acid−base dissociation reactions. However, there is a disagreement in the attribution of the protodissociation constants to the different acid base centers of

SCHEME 1: Norbadione A (NbA)



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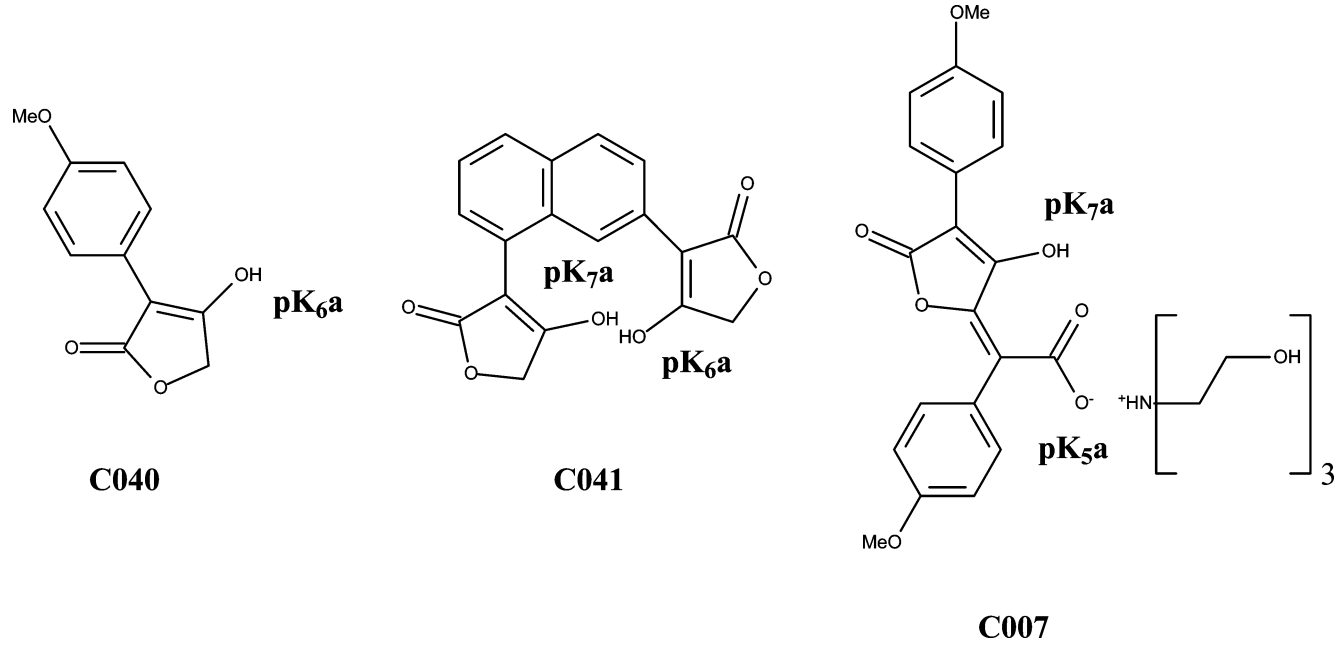
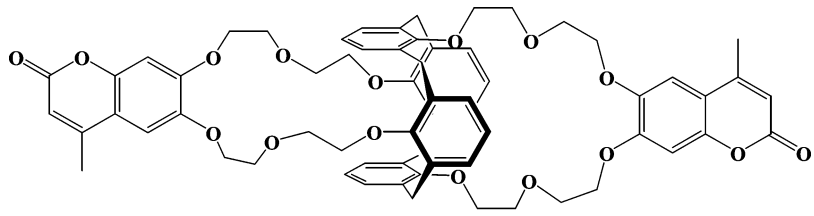
10.1021/jp1060232
Published on Web 09/10/2010

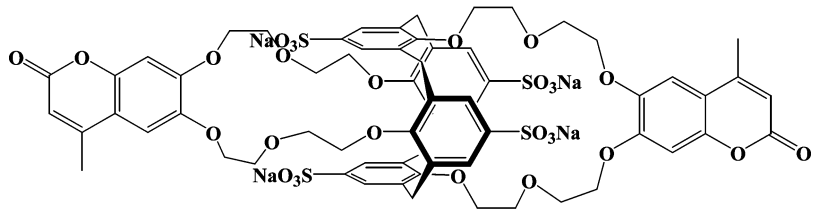
SCHEME 2: C040, C041, and C007



SCHEME 3: A1 and A2



A1



A2

the molecule.[7,8] Moreover, NbA exists in four different configurations, with conformational changes triggered by the protodissociations.[8]

On the other hand, despite its possible interest in radioprotection and in ^{137}Cs decontamination,[10,11,13] NbA also constitutes a major challenge for coordination chemistry. Indeed, Cs^+ ligands are mainly macrocyclic, such as crown ethers and calixarenes, whereas NbA acts as a chelator capable of binding two Cs^+.[14−18] However, the reports dealing with this process are controversial and relate affinity constants ranging from 10^2 to 10^7 in the methanol/water (8:2) mixture.[7,8]

The aim of this study is not to explore the mechanism of ^{137}Cs accumulation in mushrooms but to examine in aqueous and ethanolic media the protodissociations and Cs^+ complex formation with NbA and three of its constitutive pulvinic and/or tetronic acid elements (Scheme 2).[19] This will allow us to compare our results to those already available in media different from ours (8:2 CH_3OH/H_2O)[6−9] and in particular, to investigate the coordination chemistry of a new family of Cs^+ ligands. One of the major problems encountered with NbA is the difficulty in detecting the Cs^+ complex by spectrophotometric techniques. We, therefore, analyzed, in water, ethanol/water, and absolute ethanol, the kinetic and thermodynamic behavior of NbA and the three substructures in the presence of Cs^+, as well as in the presence or absence of fluorescent calix[4]arene-bis(crown-6-ether)-dioxycoumarin or the same molecule tetrasulfonated (Scheme 3, A1 and A2).[14,20,21] These highly Cs^+ selective molecules were used as probes to analyze either Cs^+ complex formation between Cs^+ and NbA or its exchange between the latter and A1 or A2. These two approaches allowed us to propose here a mechanism for Cs^+ uptake by NbA. The role of proton transfers in such mechanisms was also analyzed. This study is based on the use of the methods and techniques of chemical relaxation associated with absorption and emission spectroscopy.[22−24]

Experimental Section

The acidic form of NbA was extracted from *Pisolithus arrhizus*,[4] and the NbA substructures (Scheme 2) A1 and A2 were synthesized and purified according to published procedures.[2,12,13,19−21,25] All other products were of the purest possible grade (Sigma, Merck, Acros, or Aldrich). Ethanol was Merck spectroscopy grade, and water was demineralized and doubly distilled.

Stock Solutions. The ethanol-to-water ratio is given in volume. The solubilities of NbA and A1 in water and of A2 in ethanol are very poor. Therefore, NbA and A1 were first dissolved in pure ethanol [(2−5) × 10^{-4} M] and then, respectively, diluted [(0.5−500) × 10^{-6} M] and [(1−5) × 10^{-6} M] in the final media. As for A2, it was first dissolved in pure water (2 × 10^{-4} M) and then diluted [(2−4) × 10^{-7} M] in the final media.

Spectrophotometric Measurements. Absorption measurements were performed at 25 ± 0.5 °C on a Cary 500 spectrophotometer equipped with a thermostated cell-carrier. Fluorimetric measurements were performed at 25 ± 0.5 °C on an Amino-Bowman series 2 luminescence spectrometer equipped with a thermostated cell carrier. The fluorescence intensity was corrected for the inner filter effect of the absorption of the calixarenes and NbA.[26,27]

pH Measurements. The pHs were measured at 25 ± 0.5 °C with a Jenco pH-meter. In M1 (ethanol/water 9:1) and EtOH, the pH values were corrected according to published procedures.[28]

Protodissociation and Affinity Constants Measurements. Protodissociations and affinity constants were determined spectrophotometrically by the use of the Global Analysis program SPECFIT 32.[29] SPECFIT is a multivariate data analysis program for data sets that are obtained from multiwavelength spetrophotometric measurements. The program utilizes a specially adapted version of the Levenberg−Marquardt method. This procedure returns optimized model parameters, their standard errors, and the predicted spectra of the unknown colored species.[29] For protodissociations, the spectra were measured at different pH values in water and M1. Neutralization and acidification were achieved with NaOH and HCl. The affinity constants were checked when possible by a variant of the Benesi and Hildebrand method.[30] Unless specified otherwise, they were measured in ethanol in the absence of alkali cations other than Cs^+. In M1, they were measured in media buffered with 5 × 10^{-4} M of MES (4-morpholinoethanesulfonic acid, sodium salt) or in its absence. The pH was adjusted to the vicinities of pH 7.00 or 9.30 with microinjection of HCl and NH_4OH.

Potentiometric Titration. Potentiometric titrations were performed in ethanol and M1 by adding microvolumes of cesium acetate in the presence of NbA (5 × 10^{-4} M) and in its absence (baseline). The potential was measured with a Jenco mV-meter.

T-Jump Kinetics. Experiments were run on a modified Joule effect Messanlagen and Studien absorption and fluorescence emission T-jump spectrophotometer. The apparatus was equipped with a 200 W Xe/Hg light source, a Jarrel Ash monochromator, and a thermostated cell holder maintained at 20 ± 1 °C.[14,24] The T-jump was achieved by discharging an 0.05 μF condenser charged at 10 kV, which gives an approximate temperature jump of about 5−6 °C.[14] The experiments were carried out in 90% EtOH with solutions containing 0.4 M NH_4Cl with 1 × 10^{-6} M ≤ c_0 ≤ 8.5 × 10^{-5} M, 2 × 10^{-7} M ≤ c_2 ≤ 8 × 10^{-7} M, and 4 × 10^{-7} M ≤ c_1 ≤ 8 × 10^{-7} M (with c_0 = analytical concentration of NbA, c_1 = analytical concentration of Cs^+, and c_2 = analytical concentration of calixarene). The experiments were performed at two different pH values (7.1 and 8.9). The pHs measured before and after the T-jump were identical, within the limits of uncertainty. They were adjusted by microinjections of HCl or NH_4OH.

Stopped-Flow Kinetics. Experiments were realized on a Hi-Tech Scientific SF61DX2 stopped-flow spectrophotometer equipped with a Xe/Hg light source and a thermostated bath at 25 ± 1 °C.[14,24] Solutions of NbA in water, M1 and EtOH (1 × 10^{-5} M ≤ c_0 ≤ 1 × 10^{-4} M) in the presence of Cs^+ (c_1 = 2 × 10^{-7} and 4 × 10^{-7} M) were mixed with solutions of A1 and A2 (2 × 10^{-8} M ≤ c_2 ≤ 5 × 10^{-6} M). A2 was used in M1 and water. As for A1, it was used in M1 and EtOH. All signals were accumulated at least 10 times.[14] The excitation wavelength was set to 365 nm, which is one of the emission peaks of the light source. Detection was set to λ_{em} ≥ 400 nm.[14]

Data Analysis. The kinetic data were analyzed by linear and nonlinear least-squares regressions, and all uncertainties were twice the standard deviations. All the observed kinetic processes were pure exponentials, and all experimental conditions were set so as to allow the use of the methods and techniques of chemical relaxation.[14,22−24,31]

Results

Our experiments were performed in a purely aqueous medium, in ethanol/water (9:1) mixture (M1) and in pure ethanol.

Thermodynamics. We began by analyzing the protodissociations of the acid−base centers involved in NbA (Scheme 1), in the absence or presence of Cs^+ along with those involved in the C040, C041, and C007 subtructures (Scheme 2).

Protodissociation Constants. The pK_as of the different acid−base functions in NbA were reported in a methanol/water (8:2) mixture by spectrophotometric, potentiometric, and NMR titration.[7,8] The attribution of the different pK_as was subject to controversy.[8] In water and M1, the absorption spectra of NbA vary with pH to attain a first plateau at about pH 5 and a second one in the vicinity of pH 9. The analysis of the spectrophotometric data by the SPECFIT32 program allowed us to determine 7 pK_a values (eqs 1−7) and the absorption spectra of the different prototropic NbA species (Figure 1).

$$L^{7-} + H^+ \rightleftharpoons LH^{6-} \quad (1)$$

$$LH^{6-} + H^+ \rightleftharpoons LH_2^{5-} \quad (2)$$

$$LH_2^{5-} + H^+ \rightleftharpoons LH_3^{4-} \quad (3)$$

$$LH_3^{4-} + H^+ \rightleftharpoons LH_4^{3-} \quad (4)$$

$$LH_4^{3-} + H^+ \rightleftharpoons LH_5^{2-} \quad (5)$$

$$LH_5^{2-} + H^+ \rightleftharpoons LH_6^{-} \quad (6)$$

$$LH_6^{-} + H^+ \rightleftharpoons LH_7 \quad (7)$$

where L^{7-} is fully deprotonated NbA species and K_{na} = $[H^+][LH_{n-1}^{(8-n)-}]/[LH_n^{(7-n)-}]$ and 1 ≤ n ≤ 7.

In order to attribute these seven pK_a values to the different acid−base centers of NbA, we performed the same experiments

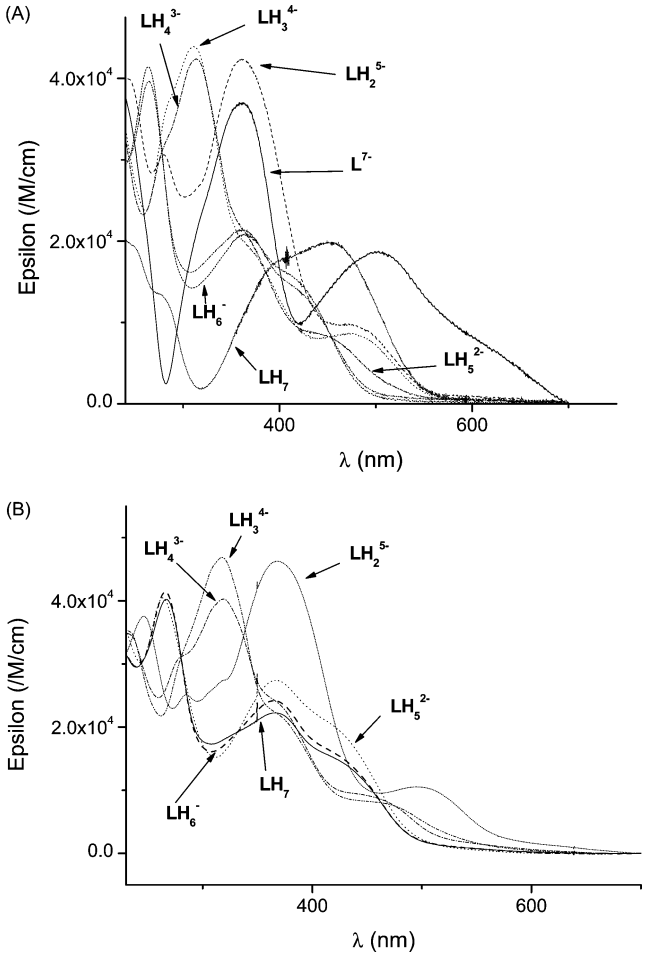


Figure 1. Absorption spectra of the different prototropic species of NbA as determined by the SPECFIT program: (A) in H_2O; (B) in (EtOH/H_2O 9:1).

with the three substructures of Scheme 2. Here also, the absorption spectra vary with pH. The use of the SPECFIT program allowed the determination of the pK_a of the enol involved in C040, the two enols in C041 and the enol and carboxylic acid in C007. The same measurements were also performed in an excess of Cs^+ at $c_1 = 1 \times 10^{-4}$ M or 1×10^{-2} M. In water, the presence of Cs^+ does not affect any of the pK_a values. In M1, the pK_as of C040, C041, and C007 are not affected by the presence of Cs^+ (Table 1). However, with NbA, for $c_1 = 1 \times 10^{-2}$ M, pK_{a7}, pK_{a6}, pK_{a5}, and pK_{a4} are decreased by 0.7, 0.3, 0.3, and 0.4 pK_a unit, while for $c_1 = 1 \times 10^{-4}$ M, only pK_{a7} and pK_{a6} are decreased by 0.5 and 0.3 pK_a unit, respectively.

Complex Formation. In the 2−9 pH range and at fixed pH values, adding Cs^+ to an NbA solution in an aqueous medium does not lead to any change in the absorption spectra. These experiments repeated with C040, C041, and C007 lead to the same results. This does not imply that the Cs^+ complexes do not form with NbA or its three subunits in water. It implies that if a complex is formed, it may not be detected by the spectrophotometric techniques.

These experiments were also performed in M1. With NbA, changes in the absorption spectra are detected. However, these are much too weak to allow an accurate determination of the complexation constants involved. No changes in the absorption are observed with the subunits.

Complex Formation between NbA and Cs^+ in Ethanol. In pure ethanol, adding Cs^+ to a solution of NbA leads to variations in the differential absorption with NbA taken as a reference (Figure 2). No changes are observed with the subunits. The analysis of the spectra by SPECFIT32 shows that, in pure ethanol, two Cs^+ complexes form sequentially with NbA which allows the measurement of two affinity constants (eqs 8 and 9):

$$LH_n^{(7-n)-} + Cs^+ \rightleftharpoons LH_nCs^{(6-n)-} \quad (8)$$

$$LH_nCs^{(6-n)-} + Cs^+ \rightleftharpoons LH_nCs_2^{(5-n)-} \quad (9)$$

with affinity constants, $K_1 = [LH_nCs^{(6-n)-}]/[Cs^+][LH_n^{(7-n)-}]$ and $K_2 = [LH_nCs_2^{(5-n)-}]/[LH_nCs^{(6-n)-}][Cs^+]$.[7,8] In ethanol, $K_{1EtOH} = (1.10 \pm 0.25) \times 10^5$ and $K_{2EtOH} = (2.1 \pm 0.4) \times 10^3$. These values were confirmed by a Benesi and Hildebrand analysis.[30] Furthermore, a potentiometric titration of NbA by Cs^+ was performed. It did not allow the determination of K_{1EtOH} because of the rather high NbA concentration ($c_0 \geq 5 \times 10^{-4}$ M) required for such measurements. However, we determined and confirmed $K_{2EtOH} = (1.6 \pm 0.3) \times 10^3$ (Figure 3).

Complex Formation between NbA and Cs^+ in Water and M1. The affinity of A2 for Cs^+ and the mechanisms of complex formation with Cs^+, in water, M1, and ethanol, are well-known.[14] These complex formations display important variations in the fluorescence emission spectra of the calixarenes.[14,20,21,25] In a purely aqueous medium, NbA possesses a typical emission spectrum in the vicinity of neutrality as well as around pH 9; adding Cs^+ to the NbA solution does not lead to any variation in the emission. On the other hand, the presence of NbA in a solution of A2 leads to a slight decrease in the fluorescence emission of A2. Adding Cs^+ to this solution shows the expected increase in the fluorescence emission typical of A2 complex formation with Cs^+.[14] A SPECFIT analysis of this increase allows the determination of the two affinity constants related to Cs^+ inclusion in A2 in the absence of NbA.[14]

In M1, NbA also has a typical emission spectrum. The addition of Cs^+ to NbA manifests by a negligible increase in the fluorescence emission. On the other hand, adding NbA to an A2 solution leads to a slight decrease in the fluorescence intensity (8% for an addition of 1×10^{-5} M of NbA to 2×10^{-7} M of A2). Adding Cs^+ to this solution leads to a first increase in the fluorescence intensity, accompanied with the red shift expected for the formation of the 2:1 complex $A2(Cs)_2^{2+}$,[14] to attain a first plateau at about 2×10^{-6} M $\leq c_1 \leq 5 \times 10^{-6}$ M. This first plateau is then followed by a decrease in the fluorescence intensity (with homothetic change in the fluorescence spectrum) down to another plateau at about $c_1 \geq 5 \times 10^{-3}$ M (Figure 4). It should be noted that the spectra in Figure 4 are corrected for the inner filter effect of absorption of both A2 and NbA.[26,27]

The estimated affinity of NbA for the first Cs^+ in water/methanol media is about 10^4-10^6,[7,8] whereas that of A2 is 2.6×10^7 for the first Cs^+ and 8×10^6 for the second.[14] Therefore, under the experimental conditions of Figure 4 ($0 \leq c_0 \leq 1 \times 10^{-5}$ M; $0 \leq c_1 \leq 1 \times 10^{-2}$ M; 2×10^{-7} M $\leq c_2 \leq 4 \times 10^{-7}$ M), A2 becomes saturated with Cs^+ when c_1 is about 2×10^{-6} M. This explains the fluorescence increase to the first plateau and is confirmed by a SPECFIT analysis, which provides two affinity constants in agreement with those recently reported.[14] Subsequently, the decrease in the fluorescence intensity for $c_1 \geq 5 \times 10^{-6}$ M appears to be related to complex formation with

TABLE 1: Protodissociation Constants of LH_7, C007, C040 and C041 in the Absence or Presence of Cs^+ ($c_1 = 1 \times 10^{-2}$ M) in Water, MeOH/H_2O 8:2 (for LH_7 Only),[7] and M1 at 25.0 ± 0.5 °C

	NbA H_2O 100%	NbA MeOH/H_2O 8:2	NbA EtOH/H_2O 9:1 (M1)	NbA with $[Cs^+] = 10^{-4}$ M (M_1)	NbA with $[Cs^+] = 10^{-2}$ M (M_1)	C040 H_2O 100%	C040 EtOH/H_2O 9:1 (M1)	C041 H_2O 100%	C041 EtOH/H_2O 9:1 (M1)	C007 H_2O 100%	C007 EtOH/H_2O 9:1(M1)
pK_{a1}	12.9 ± 0.1	>11.5	>13	>13	>13	–	–	–	–	–	–
pK_{a2}	12.9 ± 0.1	>11.5	>13	>13	>13	–	–	–	–	–	–
pK_{a3}	10.0 ± 0.1	10.15 ± 0.05	12.40 ± 0.05	12.35 ± 0.05	12.35 ± 0.05	–	–	–	–	–	–
pK_{a4}	7.8 ± 0.1	9.1 ± 0.1	8.4 ± 0.2	8.5 ± 0.1	8.0 ± 0.1	–	–	–	–	–	–
pK_{a5}	6.1 ± 0.1	7.7 ± 0.1	7.25 ± 0.05	7.45 ± 0.05	6.9 ± 0.1	–	–	–	–	6.10 ± 0.05	8.95 ± 0.01
pK_{a6}	3.5 ± 0.1	1.90 ± 0.05	4.1 ± 0.1	3.9 ± 0.2	3.8 ± 0.2	3.15 ± 0.05	5.70 ± 0.05	3.15 ± 0.05	6.2 ± 0.1	–	–
pK_{a7}	1.9 ± 0.1	0.7 ± 0.1	2.2 ± 0.1	1.7 ± 0.2	1.5 ± 0.2	–	–	3.15 ± 0.05	5.4 ± 0.1	1.8 ± 0.2	2.75 ± 0.05

NbA. Thus, the quenching of $A2(Cs)_2^{2+}$ by NbA−Cs^+ complex or other complexes constitutes a probe for the spectrophotometric detection of complex formation between NbA and Cs^+. Two series of experiments were undertaken in which the aqueous fraction of M1 was at pH 7.05 or pH 9.31. A SPECFIT analysis leads to one affinity constant [$K_{1M1} = (8 \pm 4) \times 10^4$] for the first series, and two affinity constants for the second series [$K_{1M1} = (1.5 \pm 0.3) \times 10^5$; $K_{2M1} = (4 \pm 2) \times 10^3$]. The K_{1M1} values are within the limits of uncertainty identical in both series. K_1 and K_2 were reported in two different analysis in MeOH/H_2O (8:2).[6,7] K_{1M1} is in agreement with that reported by Garaudée et al., and in disagreement with that recently reported by Kuad et al.. Conversely, K_{2M1} is quite close to the K_2 reported by Kuad et al. and about 4 orders of magnitude lower that that reported by Garaudée et al.[6,7] We were not able to determine any affinity constant in M1 by potentiometric titration. Indeed, in our experimental conditions, the signal-to-noise ratio was much too low to allow accurate measurements of the potential variations with Cs^+ concentrations.

In order to confirm these results, kinetic runs were performed.

Kinetics of Cs^+ Exchange between NbA and A1 or A2, Complex Formation between NbA and Cs^+. Two techniques were used for the kinetic runs: stopped-flow mixing and T-jump

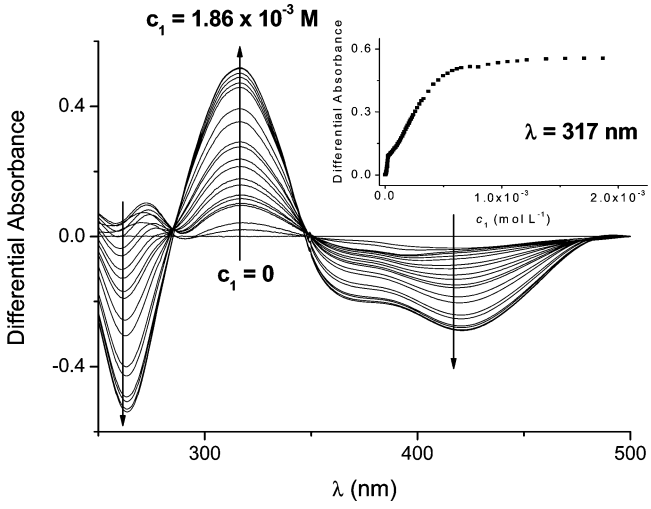


Figure 2. Differential absorption spectra of NbA solution in ethanol at different Cs^+ concentrations with NbA taken as reference, with $c_0 = 2.5 \times 10^{-5}$ M and $0 < c_1 < 1.86 \times 10^{-3}$ M at 25.0 ± 0.5 °C.

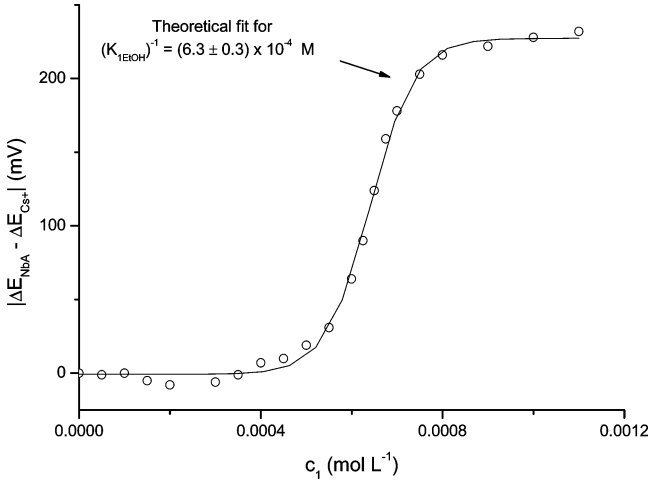


Figure 3. Differential potentiometric titration of NbA by Cs^+ in ethanol, with $\Delta E_{NbA} = E_{0NbA} - E_{NBA}$ and $\Delta E_{Cs} = E_0 - E_{Cs}$; E_{0NbA} and E_{NBA} are the values of potential of a solution of NbA in the absence or presence of Cs^+; E_0 and E_{Cs} are the values of potential in the absence of NbA and in the absence or presence of Cs^+. Reported at 25 ± 1 °C for $c_0 = 4 \times 10^{-5}$ M and $0 \le c_1 \le 1.2 \times 10^{-3}$ M.

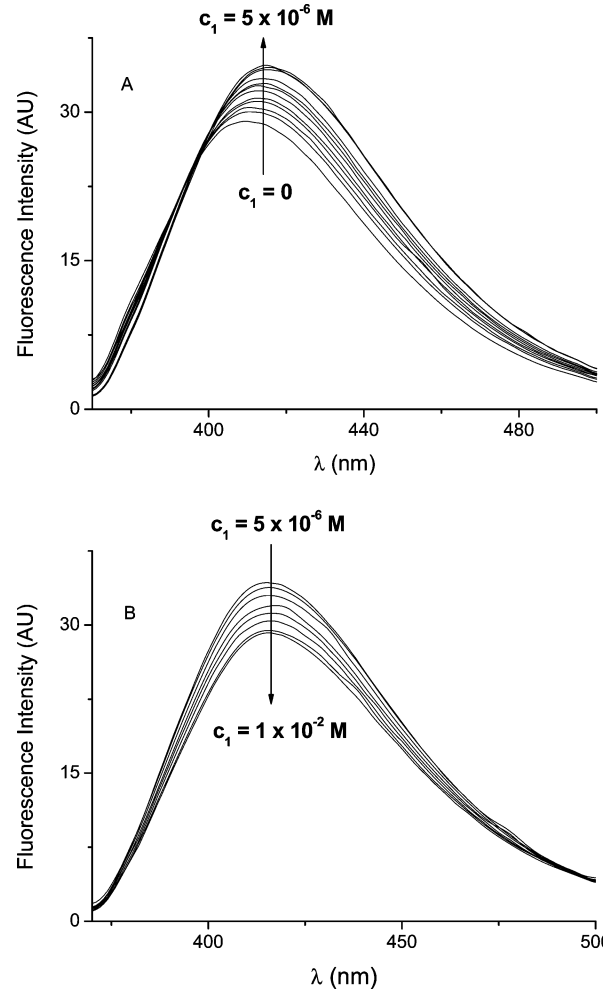


Figure 4. Emission spectra of solution of A2 and NbA in M1 at different Cs^+ concentrations at 25 ± 0.5 °C for $\lambda_{ex} = 340$ nm, $c_0 = 5 \times 10^{-6}$ M, $c_2 = 2 \times 10^{-7}$ M: (A) $0 \le c_1 \le 5 \times 10^{-6}$ M; (B) 5×10^{-6} M $\le c_1 \le 1 \times 10^{-2}$ M.

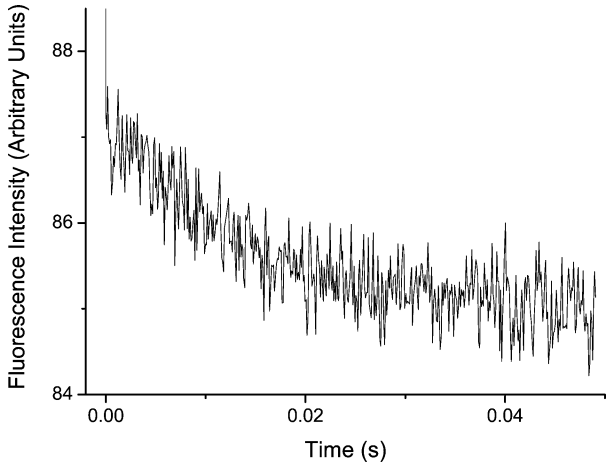


Figure 5. Evolution of fluorescence emission with time for $\lambda_{em} \geq 400$ nm at $\lambda_{ex} = 365$ nm after stopped-flow mixing of solution containing NbA ($c_0 = 2.5 \times 10^{-5}$ M) and Cs^+ ($c_1 = 2 \times 10^{-7}$ M) with solution of A1 ($c_2 = 3.5 \times 10^{-6}$ M) in ethanol at 25.0 ± 0.5 °C.

by Joule effect for the processes occurring in >5 ms and <5 ms, respectively. The detection was fluorimetric with an excitation wavelength of $\lambda_{ex} = 365$ nm (an emission peak of the HBO/XBO lamp) and detection at $\lambda_{em} \geq 400$ nm.[14]

EtOH. When a solution of NbA and Cs^+ and a solution of A1 in ethanol are mixed by stopped-flow, a single kinetic process is detected. It is an exponential decrease in the fluorescence intensity with time which occurs in the 5−50 ms range (Figure 5). The experimental reciprocal relaxation times associated with this process are dependent on c_0 and c_2 and independent of c_1. The same experiments repeated with the three pulvinic acid subunits only showed Cs^+−A1 complex formation.[14] Therefore, with these subunits, all occurs as with A1 only. This indicates that, if a Cs^+ complex is formed, the affinities involved are much too weak to allow its detection.

Cs^+ exchange between NbA and A1 can be expressed by eqs 8, 10, and 11:

$$LH_n^{(7-n)-} + Cs^+ \rightleftharpoons LH_nCs^{(6-n)-} \quad (8)$$

$$A1 + Cs^+ \rightleftharpoons A1Cs^+ \quad (10)$$

$$LH_nCs^{(6-n)-} + A1 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A1Cs^+ + LH_n^{(7-n)-} \quad \text{(rate limiting)} \quad (11)$$

with

$$K_{1A1} = [A1Cs^+]/[A1][Cs^+],$$
$$K_{exchange} = [LH_nCs^{(6-n)-}][A1]/[A1Cs^+][LH_n^{(7-n)-}],$$
$$\text{and } K_1 = K_{1A1}K_{exchange}$$

The dependence of the reciprocal relaxation times on c_0 and c_2 and their independence of c_1 led us to assume that this process may be associated with eq 11. Apart from some particular cases,[14] complex formation with Cs^+ is an extremely fast process.[15−18,32] We, therefore assume that eq 11 is rate-limiting. In this case, the reciprocal relaxation time equation associated with this process can be expressed as eq 12 (Supporting Information):

$$\tau_1^{-1} = k_1\{[LH_n^{(7-n)-}] + [A1Cs^+][LH_n^{(7-n)-}]/(K_{1EtOH}^{-1} + [LH_n^{(7-n)-}])\} + k_{-1}\{[LH_nCs^{(6-n)-}] + [A1][LH_n^{(7-n)-}]/(K_{1EtOH}^{-1} + [LH_n^{(7-n)-}])\} \quad (12)$$

Under our experimental conditions, solutions containing 1×10^{-4} M $\leq c_0 \leq 1 \times 10^{-5}$ M and 2×10^{-7} M $\leq c_1 \leq 4 \times 10^{-7}$ M were mixed with solutions containing 1×10^{-6} M $\leq c_2 \leq 5 \times 10^{-6}$ M. Since $K_{1EtOH} \ll K_{A1EtOH}$,[8,14] eq 12 simplifies to eq 13:

$$(c_0 + K_{1EtOH})\tau_1^{-1}/c_2c_0 = (c_0 + K_{1EtOH})k_1/c_2 + k_{-1} \quad (13)$$

A very good linear least-squares regression of the data against eq 13 was obtained (Figure 6). From the slope and intercept of the best line, $k_{1EtOH} = (3.1 \pm 0.1) \times 10^7$ M^{-1} s^{-1} and $k_{-1EtOH} = (2.8 \pm 1.0) \times 10^5$ M^{-1} s^{-1} were determined. The Cs^+ exchange constant, $K_{exchange} = k_{-1EtOH}/k_{1EtOH} = (9 \pm 4) \times 10^{-3}$, allowed the measurement of $K_{1EtOH} = (7.5 \pm 3.0) \times 10^4$. This last value is within the limits of uncertainty equal to that determined from Figure 2.

M1. Mixing solutions of NbA and Cs^+ with A1 in M1 leads to kinetic phenomena with very low signal-to-noise ratio, which does not yield any useful results. Mixing the same solutions with A2 shows the occurrence of very fast processes (<1 ms). On the other hand, when a T-jump is performed on a solution of NbA, A2, and Cs^+, at least two very fast kinetic phenomena are detected. The first occurs as a sharp quenching of the fluorescence emission (<3 μs), whereas the second is a weak but quite detectable exponential increase in the fluorescence emission which occurs in the 20−100 μs range (Figure 7). The first signal is observed in the absence or presence of Cs^+, whereas the second is not detected in the absence of Cs^+. Furthermore, this second signal becomes much too fast to be acquired by the T-jump technique for c_0 and/or $c_2 \geq 5 \times 10^{-5}$ M. The first fast process depicts the temperature rise from 20 to approximately 25 °C. This was checked by acquiring the fluorescence emission spectra of the NbA and A2 solution in the absence of Cs^+ at the initial and final temperatures, respectively. Regardless of their nature, fluorescence quenching processes are usually extremely fast when compared to chemical reactions occurring in the fundamental state,[27] such as proton transfers or complex formation with Cs^+.[15−18,32] In Figure 7, heating takes place in about 3 μs. Hence, it rate-limits fluorescence quenching. Under our experimental condition ($c_1 \gg c_2 \geq 2 \times 10^{-7}$ M) A2 is always completely saturated by Cs^+. We, therefore, linked the first fast process of Figure 7 to eqs 14 and 15, and the second kinetic process to complex formation between Cs^+ and NbA (eq 8):

$$A2(Cs)_2^{2+} \overset{h\nu}{\rightleftharpoons} A2(Cs)_2^{2+*} \quad (14)$$

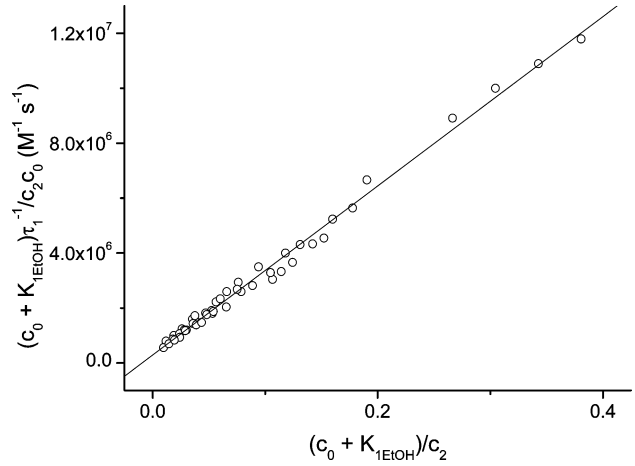


Figure 6. Plot of $(c_0 + K_{1EtOH})\tau_1^{-1}/c_2c_0$ against $(c_0 + K_{1EtOH})/c_2$ in EtOH. Intercept, $(2.8 \pm 0.8) \times 10^6$ M^{-1} s^{-1}; slope, $(3.1 \pm 0.8) \times 10^7$ M^{-1} s^{-1}; $r = 0.99\,631$.

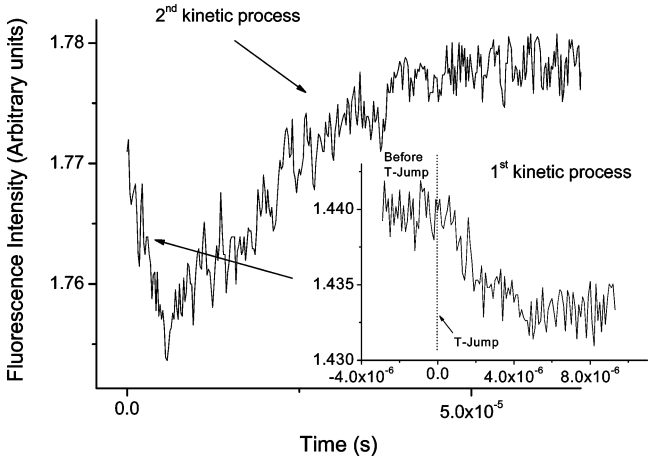


Figure 7. Evolution of fluorescence emission with time for $\lambda_{ex} = 365$ nm after a fast (~3 μs) T-jump, from 20 to about 25 ± 2 °C, performed on M1 solution containing NbA ($c_0 = 2.5 \times 10^{-5}$ M) and A2 ($c_2 = 2 \times 10^{-7}$ M) in the presence of Cs^+ ($c_1 = 4 \times 10^{-7}$ M) and 0.4 M NH_4Cl.

$$A2(Cs)_2^{2+}* + LH_nCs^{(6-n)-} \rightleftharpoons A2(Cs)_2^{2+}* - LH_nCs^{(6-n)-} \quad (15)$$

$$LH_n^{(7-n)-} + Cs^+ \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} LH_nCs^{(6-n)-} \quad \text{(rate limiting)} \quad (8)$$

Since reaction 8 is slow as compared to eqs 14 and 15, the reciprocal relaxation time associated with this process will be expressed by eq 16:[22,23]

$$\tau_2^{-1} \approx k_2([Cs^+] + [LH_n^{(7-n)-}]) + k_{-2} \quad (16)$$

Since $[LH_n^{(7-n)-}] \gg [Cs^+]$, eq 16 simplifies to eq 17:

$$\tau_2^{-1} \approx k_2c_0 + k_{-2} \quad (17)$$

A good least-squares linear regression of the experimental τ_2^{-1} against c_0 is obtained (Figure 8). From the slope and intercept of the best line: $k_{-2} = (1.3 \pm 0.4) \times 10^4$ s^{-1}, $k_2 = (1.80 \pm 0.15) \times 10^9$ M^{-1} s^{-1} and $K_{1M1} = (1.4 \pm 0.5) \times 10^5$. This value is within the limits of uncertainty equal to that determined from Figure 4.

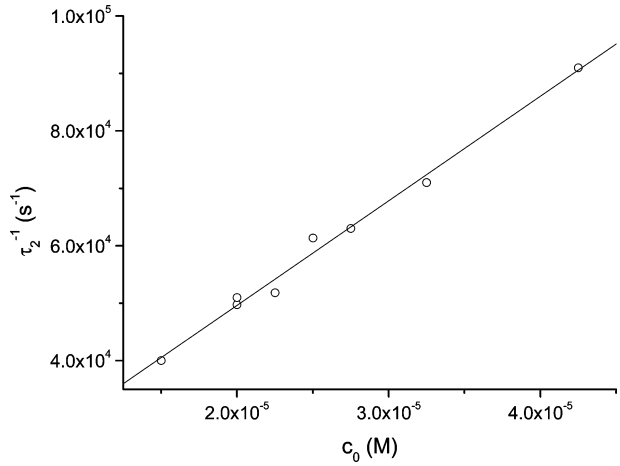


Figure 8. Plot of τ_2^{-1} against c_0. Intercept, $(1.3 \pm 0.2) \times 10^4$ s^{-1}; slope, $(1.80 \pm 0.15) \times 10^9$ M^{-1}; $r = 0.99\,519$.

The T-jump experiments repeated with the three pulvinic acid subunits did not show any detectable signal.

Water. In water, mixing solutions of excess NbA and Cs^+ with solutions of A2 leads to the formation of the A2−Cs^+ complex by the mechanism, we recently reported.[14] This implies that the affinity of NbA for Cs^+ is lower than 10^3. T-jump experiments did not show any process occurring in less than 1 ms. The same experiments were repeated with the three NbA subunits. This also led to the A2−Cs^+ complex.

Discussion

Our starting material is the acidic form of NbA (LH_7). The affinities of NbA for Na^+ and K^+ were recently reported by Kuad et al. They are weak to very weak when compared to those for Cs^+ reported in this work.[6] They, however, were determined in media and experimental conditions different from ours. Therefore, in order to avoid any interference with other alkali cations, our experiments, excluding those dealing with protodissociations, were performed, when possible, in their absence. $[Na^+]$ present in the medium was about 2×10^{-6} M including that brought by the tetrasulfonated A2 salt ($4 \times c_2 = 8 \times 10^{-7}$ M), which is always lower than $c_1 \geq 5 \times 10^{-6}$ M and 2 orders of magnitude lower than dissociation constant we reported for first complex formation with NbA in M1 $[(K_{1M1})^{-1} = 6.7 \times 10^{-4}$ M)]. However, to make sure of the lack of interference of Na^+ with Cs^+ complex formation, experiments were performed at $[Na^+] = 5 \times 10^{-3}$ M and the results were, within the limits of the experimental uncertainty, identical to those obtained from Figure 4.

For the kinetic runs, we used NH_4Cl as an electrolyte. In order to be sure of its innocuousness, the affinity constants of NbA for Cs^+ were measured again in the presence of the ammonium salt. They were within the limits of uncertainty identical to those measured in its absence.

Tables 1 and 2 present a summary of our results. The affinity constants were determined spectrophotometrically with or without the use of the two calixarene probes and kinetically with A1 in EtOH and A2 in M1.

In EtOH, spectrophotometric detection allowed us to measure directly, by absorption variation via data analysis by SPECFIT, two affinity constants for complex formation of NbA with Cs^+ (Table 2, eqs 8 and 9).

TABLE 2: Kinetic and Thermodynamic Data Related to Complex Formation between NbA and Cs^+ in EtOH and M1 at 25 ± 0.5 °C

		EtOH			M1		
mechanism		affinity const	2nd-order rate const (M^{-1} s^{-1})	1st-order rate const (s^{-1})	affinity const[a]	2nd-order rate const (M^{-1} s^{-1})	1st-order rate const (s^{-1})	affinity const[a]
$LH_5^{2-} + Cs^+ \rightleftharpoons LH_5Cs^-$	(18a)	–	–	–	$(8 \pm 4) \times 10^4$	–	–	–
$LH_3^{4-} + Cs^+ \rightleftharpoons LH_5Cs^{3-}$	(19)	$(1.10 \pm 0.25) \times 10^5$	–	–	$(7.5 \pm 3) \times 10^4$	$(1.5 \pm 0.3) \times 10^5$	$(1.80 \pm 0.15) \times 10^9$	$(1.3 \pm 0.4) \times 10^4$	$(1.4 \pm 0.5) \times 10^5$
$LH_3Cs^{3-} + Cs^+ \rightleftharpoons LH_3Cs_2^{2-}$	(20)	$(2.1 \pm 0.4) \times 10^3$	–	–	–	$(4 \pm 2) \times 10^3$	–	–	–
$LH_3Cs^3 + A1 \rightleftharpoons LH_3^{4-} + A1Cs^+$	(11)	–	$(3.1 \pm 0.1) \times 10^7$	$(2.8 \pm 1) \times 10^5$	$(9 \pm 4) \times 10^{-3}$	–	–	–	–

[a] Related to kinetic determination of K_1 values.

Figure 9. Normalized distribution of NbA−cesium complex species with log[Cs^+].

In M1, we were not able to measure these affinity constants directly by absorption spectroscopy. This was achieved in the presence of A2 and was based on the fluorescence emission quenching of A2 by NbA (eqs 14 and 15, Table 2). The SPECFIT analysis reveals two Cs^+ complexes which form successively, the first with $K_{1M1} = 1.5 \times 10^5$ and the second with $K_{2M1} = 4 \times 10^3$. This implies that the first Cs^+ complex forms at $c_1 \leq 1 \times 10^{-4}$ M and that the bicomplex is completely formed at $c_1 \geq 2.5 \times 10^{-3}$ M (Figure 9).

In the MeOH/water (8:2) medium, NbA exists in four different configurations, two of which form with Cs^+ complexes of comparable stability.[8] On the other hand, molecular dynamics and quantum mechanics simulation implied that only a NbA species with enolates and carboxylates (LH_3^{4-}) can form a bicomplex and that the species with enolates and carboxylic acids (LH_5^{2-}) can only form a single Cs^+ complex.[9] Further, the proton loss from one of the enols leads to a hydrogen bond, which is assumed to bridge both carboxylic and enolic groups of each pulvinic moiety.[8,9] Breaking this hydrogen bond by protodissociation is considered as a prerequisite for complex formation with alkali, such as Cs^+, K^+, and Na^+ and leads to lower affinities for Cs^+ than those accounted for earlier.[6,8] At room temperature, hydrogen bonds breakage and formation are normally extremely fast processes, unless they are involved in a sterically hindered environment or in a rigid structure, which is not particularly the case of NbA.[14,33−36] They should not, therefore, control rate of Cs^+ uptake and should not affect the kinetic analysis. Furthermore, our experiments were performed in water, ethanol, and ethanol/water (9:1) at NbA concentrations (1×10^{-6} M $\leq c_0 \leq 5 \times 10^{-4}$ M) and at Cs^+ (5×10^{-7} M $\leq c_1 \leq 1 \times 10^{-2}$ M), which cover the range of concentrations in which we measured the two dissociation constants involved in complex formation [Figure 9, $(K_{1EtOH})^{-1} = 9.1 \times 10^{-6}$ M; $(K_{2EtOH})^{-1} = 4.8 \times 10^{-4}$ M; $(K_{1M1})^{-1} = 7 \times 10^{-6}$ M; $(K_{2M1})^{-1} = 2.5 \times 10^{-4}$ M]. These experimental conditions differ from those reported recently by Kuad et al. in methanol/water (8:2), where the used techniques (calorimetric, NMR, and potentiometric) required NbA concentrations (10^{-3}−10^{-2} M) 1−4 orders of magnitude higher than ours.[6−8] To illustrate our purpose, we performed a potentiometric titration of NbA by Cs^+ in EtOH and M1 ($c_0 = 5 \times 10^{-4}$ M and $0 \leq c_1 \leq 2 \times 10^{-3}$ M). In M1, the variations in the potential were too weak to allow any accurate measurement. In EtOH, we were not able to detect first complex formation. Indeed, with our technique and in order to measure K_{1EtOH}, NbA concentration should be in the vicinity of $(K_{1EtOH})^{-1}$. In these conditions (c_0, $c_1 \approx 1 \times 10^{-5}$ M), the

variations in the potential are extremely weak and their measurements are flawed with more than 200% of uncertainty. Nonetheless, the second complex was detected with an affinity constant [$K_{2EtOH} = (1.6 \pm 0.3) \times 10^3$], which within the limits of uncertainty is identical to that measured by spectrophotometric titration (Figures 2 and 3, Table 2).

In water and M1, we report the protodissociation constants of the different acid−base functions present in NbA (Table 1). Comparison of these values with those of C007, C040, and C041 allowed us to attribute them to each of the acid−base centers (Table 1, Scheme 1). These attributions are in agreement with those reported by Garaudée et al.[7] They, however, differ from those of Kuad et al. performed by NMR titration in the methanol/water medium. In these, the lowest values (pK_{a7} and pK_{a6}) are attributed to the carboxylic acid, whereas pK_{a5} and pK_{a4} are attributed to the enols.[8]

In the presence of a Cs^+ concentration favoring the presence of the first complex and largely excluding that of the second ($c_1 \leq 1 \times 10^{-4}$ M), decreases in the apparent pK_{a7} and pK_{a6} are observed. With a larger excess of Cs^+ ($c_1 = 1 \times 10^{-2}$ M), two new decreases in the apparent pK_{a5} and pK_{a4} occur (Table 1). pK_{a7} and pK_{a6} are related to the enols, whereas pK_{a5} and pK_{a4} are related to the two carboxylic acids (Table 1, Scheme 1). This implies that the two enols are involved in the single NbA−Cs^+ complex, whereas the enols and carboxylates are involved in the bicomplex. Indeed, when a complex forms with a ligand, the apparent pK_a of the ligand is decreased by the so-called coordination effect (eqs 18−21).[30,37] The larger is this pK_a difference ($\Delta pK_a = pK_{a\text{-free ligand}} - pK_{a\text{-complexed ligand}}$), the higher is the affinity for the metal.[30,37] Hence, first Cs^+ complex involves the two enols of the LH_5^{2-} and LH_3^{4-} species, whereas the bicomplex occurs with the two enols and the two carboxylates of the LH_3^{4-} species (eqs 18−20).

$$LH_5^{2-} + Cs^+ \rightleftharpoons LH_5Cs^- \quad \text{(neutral media)} \qquad (18)$$

$$LH_3^{4-} + Cs^+ \rightleftharpoons LH_3Cs^{3-} \quad \text{(slightly basic media)} \qquad (19)$$

$$LH_3Cs^{3-} + Cs^+ \rightleftharpoons LH_3Cs_2^{2-} \qquad (20)$$

This confirms the proposals of Kuad et al. concerning the involvement of both carboxylates and enolates in the Cs^+ as it also confirms the quantum mechanical calculations of Shurhammer et al.[8,9] However, K_1 is in agreement with that reported in methanol/water by Garaudée et al. and in disagreement with that recently reported by Kuad et al. (eq 18, Table 2).[6,7] It was also assumed that bicomplex formation takes place with LH_5^{2-} and that the affinity of the first Cs^+ complex (LH_5Cs^-) for a second Cs^+ is higher than that of the free LH_5^{2-} ligand ($K'_2 > K_1$ eqs 18, 21).[7]

$$LH_5Cs^- + Cs^+ \rightleftharpoons LH_5Cs_2 \qquad (21)$$

With

$$K'_2 = [LH_5Cs_2]/[LH_5Cs^-][Cs^+]$$

This allostericity implies that, in the methanol/water media, the first Cs^+ complex induces conformation changes (allosteric effect) which enhance the formation of the bicomplex as the thermodynamic species (eq 21 and eq 22 in the Supporting Information).[7] This means that, in the presence of Cs^+, the major NbA complex will almost always be the bicomplex species $LH_3Cs_2^{2-}$.[7] We did not observe any bicomplex formation with LH_5Cs^- in M1 and EtOH (eqs 18 and 21, Table 2). Moreover, with LH_3^{4-}, the distribution of the concentrations of the NbA species with [Cs^+] clearly indicates that $LH_3Cs_2^{2-}$ starts to become the major species at [Cs^+] $\geq 4 \times 10^{-5}$ M (Figure 9), as observed recently by Kuad et al.[6]

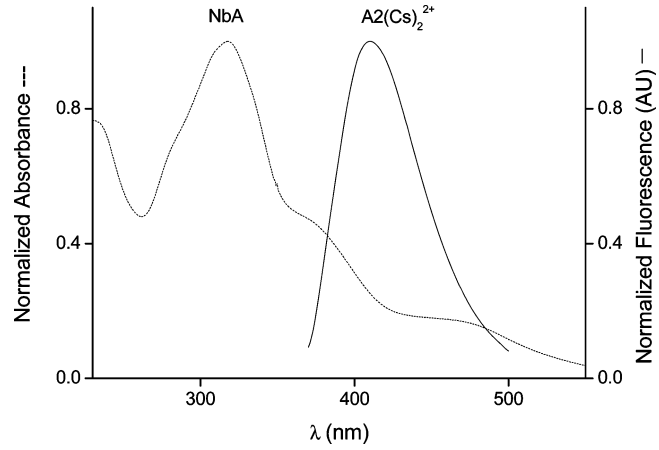


Figure 10. Overlap between normalized absorption spectrum of NbA and emission spectrum of $A2(Cs)_2^{2+}$ for $\lambda_{ex} = 340$ nm.

It was impossible to analyze by direct spectrophotometric detection the kinetics of complex formation between NbA and Cs^+. We, therefore, used the two calixarenes A1 and A2 as probes. Our aim was to form the NbA complex (eqs 8 and 9, Table 2) and then analyze the kinetics of Cs^+ depletion by A1 or that of Cs^+ distribution between NbA and A2 (eq 11, Table 2). In water A2 and its Cs^+ complex both have typical fluorescence emission spectra.[20] Complex formation between A2 and Cs^+ is a fast process, which was detected and analyzed by stopped flow.[14] During the experiments performed by mixing solutions of NbA and Cs^+ with A2 solutions, all occurred as in the absence of NbA, and the kinetic and thermodynamic constants involved were identical to those reported for complex formation between Cs^+ and A2.[14] This implies that, if a complex is formed in water, the affinity of NbA for Cs^+ should be at least 1 order of magnitude lower than that of A2 ($K_{1H2O} \leq 1 \times 10^3$).

In M1, the affinity of A2 for Cs^+ is higher than that reported for first complex formation with NbA, whereas that of A1 is of the same order of magnitude. A kinetic analysis was not possible with A1. Nonetheless, the quenching of $A2(Cs)_2^{2+}$ by NbA−Cs^+ complexes allowed the determination of the thermodynamics, and of the T-jump kinetics of complex formation between NbA and Cs^+. Although, we observe a linear relationship between the decrease in the ratio of the initial fluorescence intensity of A2 in the absence of NbA over that observed in its presence with the concentration of NbA (not shown), the nature of this quenching is not easy to predict.[27] Nevertheless, in view of the significant overlap between the fluorescence spectrum of $A2(Cs)_2^{2+}$ and the absorption spectrum of NbA (in the presence or absence of Cs^+, Figure 10), this quenching may be possibly attributed to a Förster resonance energy transfer (FRET).[27]

Adding Cs^+ to an A2 solution containing NbA, leads first to complex formation between A2 and two Cs^+, which is confirmed by the expected increase in emission accompanied by the also expected red shift (Figure 3A).[14] When the A2

complex is formed, the addition of Cs^+ manifests then by a homothetic decrease in the fluorescence emission down to a plateau at $[Cs^+] \approx 5 \times 10^{-3}$ M (Figure 3B). Complex formation between Cs^+ and NbA in M1 does not lead to noticeable variations in the emission or absorption spectra of NbA. Therefore, this complex formation should not lead to any variation in the emission spectra of A2, unless the concentration of the NbA quencher is modified by its complexation with Cs^+. This led us to ascribe the decrease in fluorescence observed in Figure 4 to complex formation between NbA and Cs^+ (eqs 8 and 9), permitted the measurement of K_{1M1} by SPECFIT and the confirmation of our proposals by T-jump kinetics (Table 2, eq 8). The second-order rate constant $k_1 = 1.80 \times 10^9$ M^{-1} s^{-1} is in agreement with the values reported for complex formation with crown ethers. Moreover, the K_{1M1} value determined kinetically (k_{-1M1}/k_{1M1}) is, within the limits of uncertainty, equal to that determined spectrophotometrically (Table 2). We were not, however, able to analyze bicomplex formation because it requires concentrations of Cs^+ ($c_1 > 1 \times 10^{-3}$ M) at which the kinetic processes became much too fast to be measured by the T-jump technique.

In EtOH, where A2 is not soluble, a Cs^+ exchange between LH_3Cs^{3-} and A1 was observed by stopped flow (eq 11, Table 2). This allowed us to determine two second-order rate constants for Cs^+ exchange between NbA and A1, and to confirm the K_{1EtOH} value revealed by absorption spectroscopy. However, we were not able to detect the formation of the second complex with Cs^+.

NbA is until further notice assumed to be among the major Cs^+ ligands in the environment, as it is supposed to bear a certain responsibility in the accumulation of ^{137}Cs emitted during the Chernobyl nuclear accident.[2] However, complex formation with alkali best occurs with structures carrying inclusion cavities with oxygenated heterocycles, such as crown ethers or calixarenes.[14,17,18,20,21,25,38] Indeed, with such molecules, the affinities of the ligand for Cs^+ can be quite high, as they vary from 10^3 to about 10^8. With NbA, complex formation occurs between two Cs^+ and the enolates and/or carboxylates (Scheme 2). However, the tetronic and pulvinic acid derivatives C040, C007, and C041 bearing enols and/or carboxylic acid moieties do not show any affinity for Cs^+ in water, EtOH or M1. This implies that enolates and/or carboxylates are poor ligands for Cs^+. Therefore, a high affinity of NbA for Cs^+ is not easy to understand. This affinity can be compared to that of the Cs^+-specific crown ether and calixarene ligands.[14,20,21,25] Therefore, despite a structure which does not seem a priori adequate for complex formation with Cs^+, stable complexes occur. What is then so particular about this structure of NbA that it provides such a good chelator for one or two Cs^+? This affinity for Cs^+ can come from the flexibility of the two pulvinic acids, which allows them to adopt the best conformation for complex formation. This can as shown by quantum mechanics modeling take place with the Cs^+ close to the oxygen coordination sites of the ligands in the pseudocavity formed by the two pulvinic arms.[9] This cavity can mimic that of crown ethers or calixarenes and, therefore, promotes complex formation with the oxygens of the carboxylate group and those of enolates and/or the ether of the lactone.

Conclusion

NbA forms strong complexes with Cs^+ in alcohol and aquo-alcoholic mixtures. The affinities involved are surprisingly high and renders NbA as efficient as calixarenes or some crown ethers in Cs^+ uptake in alcohol or alcohol−water mixtures. Cs^+ complex formation occurs with the two enolates and the two carboxylates of NbA. It, therefore, depends on the protonation state of the ligands and takes with one Cs^+ when only the enols are in the ionized state and with two Cs^+ when both the enol and the carboxylic acid moieties are deprotonated. However, in water, which is considered to be the main biological medium, NbA does not possess an important affinity for Cs^+. If such complexes occur, they are extremely weak. Therefore, unless other factors are involved, such as the particular microenvironment in the mushroom or the association with other molecules, the enigma related to NbA as one of the species responsible for Cs^+ accumulation in the environment remains.

Acknowledgment. This work is dedicated to the memory of Dr. Charles Mioskowski, who was the head of the CEA (Commissariat à l'Energie Atomique) isotope labeling laboratory and at the origin of our interest in norbadione A. It was supported by DGA (Délégation Générale pour l'Armement) grant PEA PROPERGAL No. 06.70.110. The authors are grateful to Dr John S. Lomas for helpful discussions.

Supporting Information Available: The derivation of reciprocal relaxation time eq 12 is described. This material is available free of charge via the Internet at http://pubs.acs.org.

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JP1060232