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Characterization of the Site-Specific Acid-Base Equilibria of 3-Nitrotyrosine

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The complete macro- and microequilibrium analyses of 3-nitrotyrosine, a biomarker of oxidative stress damage, are presented for the first time. The protonation macroconstants were determined by ^{1}H-NMR-pH titration, while microconstants were elucidated by a combination of deductive and NMR methods, in which properties of the methyl ester derivative as an auxiliary compound were also studied. Combination of the NMR-pH characterization of the title and auxiliary compounds and the pair-interactivity parameters of 3-iodotyrosine provided the sufficient system to evaluate all the microconstants. NMR-pH profiles, macroscopic and microscopic protonation schemes, and species-specific distribution diagrams are included. The phenolate basicity of 3-nitrotyrosine is 500 times below that of tyrosine, and it is even lower than that of 3-iodotyrosine. This phenomenon can be explained by the stronger electron withdrawing and the negative mesomeric effect of the nitro group. Based on our results, 89% of the phenolic OH groups are deprotonated in 3-NT molecules at the pH of the blood plasma.

Keywords: nitrotyrosine, microspeciation, oxidative stress, NMR, pK_a.

Introduction

3-Nitrotyrosine (3-NT, *Figure 1*) is the earliest discovered biomarker of nitrosative stress. It is formed through the nitration of tyrosine (Tyr) by peroxynitrite. Peroxynitrite is not a free radical itself but it can become a source of free radicals by an electron accepting process. Being an isomeric form of nitrate, the lifetime of peroxynitrite is a fraction of a second at physiological pH, thus Tyr nitration represents its most important footprint in biological systems. The formation of peroxynitrite in organisms takes place by diffusion-controlled reaction of NO$^\bullet$ (nitric oxide) and $O_2^{\bullet-}$ (anion superoxide), in various, enzyme-associated process,[1] starting mainly from L-arginine. Some *in vivo* experiments demonstrated its release from nitrones by the action of hydroxyl radical.[2] Peroxynitrite, the deprotonated form of peroxynitrous acid can decompose to $^\bullet$OH and $^\bullet NO_2$, the latter being the most important nitration agent of Tyr.[3] Tyr nitration takes place in two steps: i) production of tyrosyl radical (Tyr$^\bullet$) by one-electron oxidation of the phenolic ring and ii) Tyr$^\bullet$ and $^\bullet NO_2$ radical-radical coupling reaction with a rate constant of 3×10^9 M^{-1}s^{-1}. Oxidants that can achieve the one-electron oxidation of Tyr are: $^\bullet$OH, $^\bullet NO_2$, $CO_3^{\bullet-}$, LOO$^\bullet$, LO$^\bullet$, oxo-metal compounds (O=Mn^n), myeloperoxidase (MPO). Usually, $^\bullet NO_2$ is responsible for solvent-exposed Tyr nitration, while transition metals can lead to nitration of buried Tyr residues.[1]

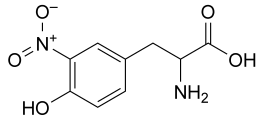

Figure 1. The constitutional formula of 3-nitrotyrosine.

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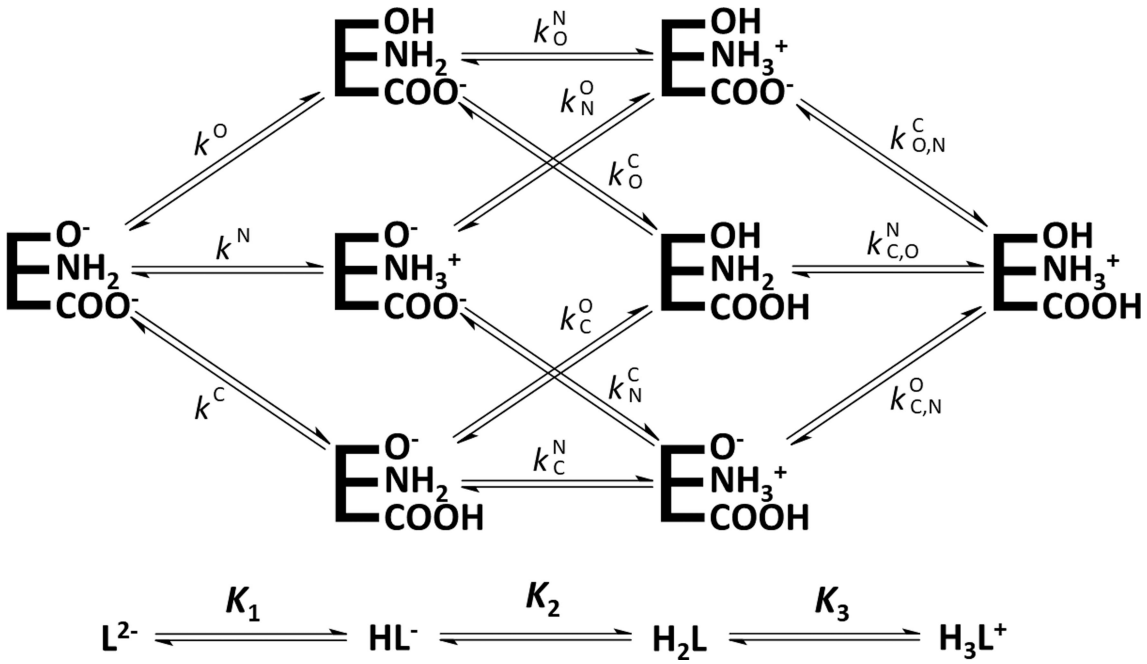

Figure 2. The micro- and macrospeciation schemes of 3-nitrotyrosine, where microconstants with superscript O, N and C belong to the phenolate, amino and carboxylate site, respectively, and K_1, K_2 and K_3 are stepwise macroconstants. The superscript on the microconstant indicates the protonating site, while the subscript (if any) stands for the site(s) already protonated.

The target of electrophilic reagents and/or oxidants in the Tyr structure is the position 3 of the aromatic ring. The nitration of Tyr in proteins in most cases leads to inactivation of the enzymes. Several properties of 3-NT and the proteins, containing it have been described. For example, at low pH, 3-NT is more hydrophobic than Tyr, while at high pH, it is more polar than Tyr.[4,5] Tyr nitration obviously alters the protein structures and concomitantly the physiological effects. These modifications are strongly related to diseases, mainly with degenerative and inflammatory symptoms.

Although the role of 3-NT has been studied in oxidative stress, no previous report was devoted to its microspeciation. Therefore, our aim was to characterize the acid-base properties of 3-NT at the site-specific level. Macroscopic protonation constants (K) are known, based on a previous work which used capillary zone electrophoresis.[6] Nevertheless, we also determined the K values by ^{1}H-NMR-pH titration method. Macroscopic equilibrium constants of multiprotic molecules give information only on the acid-base properties of the compound as a whole. Site-specific, submolecular basicities can be obtained when microconstants are determined. The site-specific acid-base characterization of 3-NT is therefore of fundamental importance to allow the interpretation of its biological functions at site-specific level.

Results and Discussion

3-NT is a triprotic molecule with phenolate, amino and carboxylate protonation sites. As a triprotic molecule, the total number of microspecies and microconstants are 8 and 12, respectively. The macroscopic- and microscopic protonation schemes are shown in *Figure 2*.

The relationships between the micro- and macroconstants which has been used for the calculation of microspeciation are as follows:

$$K_1 = k^O + k^N + k^C \tag{1}$$

$$\beta_2 = K_1K_2 = k^Ok^N_O + k^Ok^C_O + k^Nk^C_N = k^Nk^O_N + k^Ck^O_C + k^Ck^N_C \tag{2}$$

$$\beta_3 = K_1K_2K_3 = k^Ok^N_Ok^C_{O,N} = k^Nk^C_Nk^O_{N,C} = \ldots \tag{3}$$

Equation 3 can be written in six different and equivalent ways based on the Hessian-relationship of protonation constants which means that the sum of

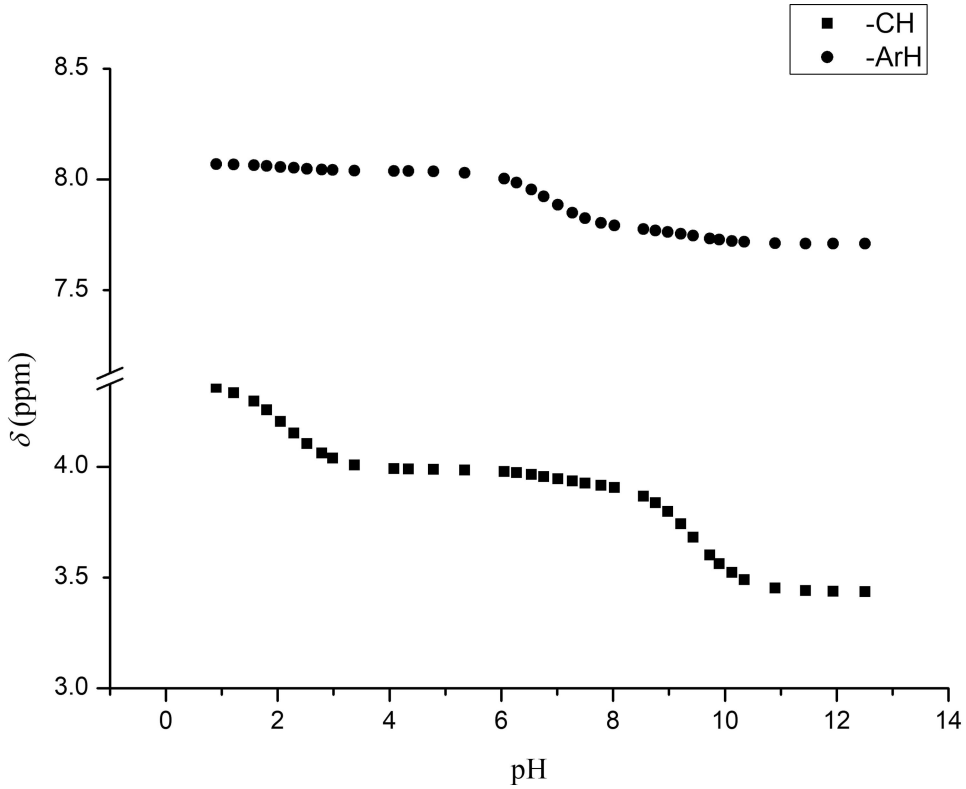

Figure 3. Chemical shifts of the observed protons in 3-NT as a function of pH.

protonation constants in logarithmic units is constant between the same start and end-points regardless of the path of protonation.

Determination of Protonation Macroconstants

Evaluation of the protonation constants from ^{1}H-NMR-pH titration curves was based on the principle that non-exchanging NMR nuclei near the basic site sense different electronic environments upon protonation, and change their chemical shifts accordingly. All carbon-bound protons could be observed.

The protonation macroconstants of 3-NT and its methyl ester derivate were determined by investigating the chemical shift changes of the CH proton and the aromatic proton next to the nitro group in ^{1}H-NMR-pH titrations (*Figure 3*). Since the effect of protonation on the chemical shifts diminishes along with increasing distance from the site of protonation, the aromatic proton signals were much more sensitive for the protonation of the phenolate group, while the chemical shifts of the aliphatic protons changed more upon the protonation of the amino and the carboxylate groups. Some representative NMR spectra at different pH values were depicted in the *Supporting Information* (*Figure S1*).

The protonation constants were determined by nonlinear curve fitting of *Equation 19* to the collected data. The values are shown in *Table 1*.

The macroconstant values determined by capillary electrophoresis earlier and our values are in good agreement.

Complete Microspeciation of 3-Nitrotyrosine

For the determination of all microconstants, the macroconstants of 3-NT and its carboxymethyl deriva-

Table 1. Logarithmic values of macroscopic protonation constants of 3-nitrotyrosine and methyl 3-nitrotyrosinate (mean ± SD, $n = 3$)

	3-Nitrotyrosine* (CZE)	3-Nitrotyrosine	Methyl 3-nitrotyrosinate
$\log K_1$	9.54 ± 0.01	9.452 ± 0.002	7.570 ± 0.006
$\log K_2$	6.82 ± 0.01	6.859 ± 0.003	6.371 ± 0.01
$\log K_3$	2.06 ± 0.01	2.154 ± 0.002	–

The data in column marked with * are from the capillary zone electrophoresis-pH studies of Ren et al.[6]

Table 2. Pair-interactivity parameters derived from 3-iodotyrosine with the values of standard deviations based on Gaussian-propagation of uncertainty.

$\log E^{O-N}$	0.53 ± 0.04	$\log E^{N-C}$	2.04 ± 0.04	$\log E^{O-C}$	0.29 ± 0.05

tive, and the pair-interactivity parameters of 3-iodotyrosine from our previous work were used.[7]

Protonation of a basic site reduces the basicity of the other basic sites in the molecule. This basicity-reducing effect is strong if the sites are in nearby positions, but it gradually fades away along with the increasing distance. The basicity-modifying effect between two moieties can be quantified by the pair-interactivity parameter. For a triprotic molecule, three different pair-interactivity parameters are defined. For example, the interactivity parameter (E) in log units between the amino and the carboxylate sites is:

$$\log E^{N-C} = \log k^{N} - \log k^{N}_{C} = \log k^{C} - \log k^{C}_{N} = \ldots \quad (4)$$

The pair-interactivity parameter is the most invariant and best transferable parameter between molecules having analogous moieties.[8] Due to the similarity between the nitro- and iodo-group, the pair-interactivity parameter of 3-iodotyrosine can be used in the microspeciation of 3-NT as well. The three pair-interactivity parameters were calculated in a previous work,[7] while the standard deviations were based on the Gaussian propagation of uncertainty (*Table 2*).

In classical Tyr microspeciation works,[9] UV-pH titrations were successfully used, owing to the overlapping protonations of the phenolate and amino sites, and the selective pH-dependence of the phenolate UV-absorption. For 3-NT, however, this method should not work, due to the negligible overlap between the phenolate and amino protonation regions.

Using the Hessian relationship with the macroconstants of 3-NT and methyl 3-nitrotyrosinate, $\log k^{C}$ was calculated as follows:

$$\log k^{C} = (\mathbf{\log K_1} + \mathbf{\log K_2} + \mathbf{\log K_3}) - (\log K_1 + \log K_2) \quad (5)$$

where the protonation macroconstants of 3-NT are in bold, the protonation macroconstants of methyl 3-nitrotyrosinate are in normal fonts. After the value of $\log k^{C}$ is determined, $\log k^{C}_{O}$, $\log k^{C}_{N}$ and $\log k^{C}_{O,N}$ can be calculated by using the pair-interactivity parameters and the appropriate form of *Equation 4*:

$$\log k^{C}_{O} = \log k^{C} - \log E^{O-C} \quad (6)$$

$$\log k^{C}_{N} = \log k^{C} - \log E^{N-C} \quad (7)$$

$$\log k^{C}_{N,O} = \log k^{C}_{O} - \log E^{N-C} \quad (8)$$

Applying *Equations 1* and *3* for methyl 3-nitrotyrosinate:

$$K_1 = k^{O}_{C} + k^{N}_{C} \quad (9)$$

$$\log K_1 + \log K_2 = \log k^{O}_{C} + \log k^{N}_{C,O} \quad (10)$$

Furthermore, $\log k^{N}_{C,O}$ can be expressed from *Equation 4*:

$$\log k^{N}_{C,O} = \log k^{N}_{C} - \log E^{O-N} \quad (11)$$

Then, $\log k^{N}_{C,O}$ was introduced into *Equation 10*:

$$\log K_1 + \log K_2 = \log k^{O}_{C} + \log k^{N}_{C} - \log E^{O-N} \quad (12)$$

Equations 9 and *12* constitute a system of equations with two unknown parameters ($\log k^{O}_{C}$ and $\log k^{N}_{C}$), resulting in values of 7.06 and 7.41 in log units. *Equations 9* and *12*, however, do not contain information on the assignments, i.e., which of the above values belong to $\log k^{O}_{C}$ and $\log k^{N}_{C}$. The chemical shift-pH profiles (*Figure 3*), chemical evidences and microconstant values in *Table 3* unequivocally indicate that insertion of the electron-withdrawing nitro group in the aromatic ring reduces the basicity of the phenolate to a much greater extent than that of the amino.

Taking also the low diversity of the $\log k^{N}_{C}$values into account, the two parameters could be identified, which also afforded the calculation of the remaining six protonation microconstants using the pair-interactivity parameters and the appropriate form of *Equation 4*:

Table 3. The $\log k^{O}_{C}$ and $\log k^{N}_{C}$ microconstants of tyrosine, 3-iodotyrosine and diiodotyrosine from ref. [7].

Parameter	Tyrosine	3-Iodotyrosine	Diiodotyrosine
$\log k^{O}_{C}$	9.76	8.42	6.54
$\log k^{N}_{C}$	7.58	7.53	7.41

Table 4. Microconstants of 3-nitrotyrosine in log*k* units with the standard deviations based on the Gaussian propagation of uncertainty.

Phenolate microconstants		Amino microconstants		Carboxylate microconstants	
$\log k^{O}$	7.35 ± 0.05	$\log k^{N}$	9.45 ± 0.05	$\log k^{C}$	4.53 ± 0.01
$\log k^{O}_{N}$	6.82 ± 0.06	$\log k^{N}_{O}$	8.92 ± 0.05	$\log k^{C}_{O}$	4.24 ± 0.05
$\log k^{O}_{C}$	7.06 ± 0.07	$\log k^{N}_{C}$	7.41 ± 0.03	$\log k^{C}_{N}$	2.49 ± 0.04
$\log k^{O}_{C,N}$	6.53 ± 0.04	$\log k^{N}_{C,O}$	6.88 ± 0.05	$\log k^{C}_{O,N}$	2.20 ± 0.07

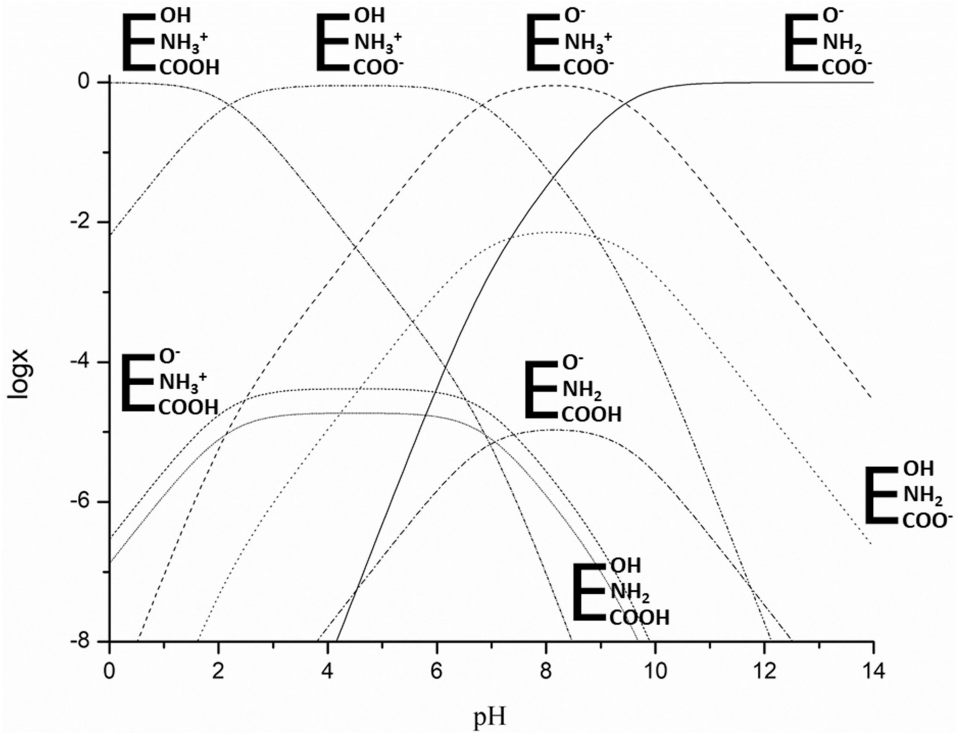

Figure 4. Logarithmic distribution diagram for all the microspecies of 3-nitrotyrosine.

$$\log k^{N} = \log k^{N}_{C} + \log E^{N-C} \quad (13)$$

$$\log k^{N}_{O} = \log k^{N} - \log E^{O-N} \quad (14)$$

$$\log k^{N}_{C,O} = \log k^{N}_{C} - \log E^{O-N} \quad (15)$$

$$\log k^{O} = \log k^{O}_{C} + \log E^{O-C} \quad (16)$$

$$\log k^{O}_{N} = \log k^{O} - \log E^{O-N} \quad (17)$$

$$\log k^{O}_{C,N} = \log k^{O}_{C} - \log E^{O-N} \quad (18)$$

After all the microscopic protonation constants of 3-NT were determined, some of the values could be verified by the self-consistence of the system. The value of K_1 can be calculated using *Equation 1*; additionally, $K_3 \approx k^{C}_{O,N}$, since the protonation along the $k^{C}_{O,N}$ arrow overwhelmingly predominates over the $k^{O}_{C,N}$ and $k^{N}_{C,O}$. The results of both checking procedure were within the margin of error. All 12 microconstants are shown in *Table 4*.

The pH-dependent distribution of the eight microspecies (*Figure 4*) was calculated based on the protonation microconstants in *Table 4*.

Comparing the microconstants of Tyr, 3-iodotyrosine and 3-NT, it is found that the basicity of the carboxylate and amino sites are similar, unlike the phenolate. This is straightforward to interpret, considering how distant the iodo or nitro group is from the protonation sites. The microconstants describing the phenolate basicity in three compounds are collected

Table 5. The phenolate microconstants of tyrosine, 3-iodotyrosine and 3-nitrotyrosine.

	Tyrosine	3-Iodotyrosine	3-Nitrotyrosine
$\log k^{O}$	10.04	8.71	7.35
$\log k^{O}_{N}$	9.65	8.18	6.82
$\log k^{O}_{C}$	9.76	8.42	7.06
$\log k^{O}_{C,N}$	9.37	7.89	6.53

in *Table 5* (while all of the microconstants in Tyr, 3-iodotyrosine and 3-NT were summarized in the *Supporting Information*, *Table S1*).

The substitution of tyrosine with iodine decreases all the respective phenolate microconstants by some 1.4 log units. Changing the iodo group to nitro group results in greater changes and even lower protonation constants. Compared to tyrosine, the decrease is around 2.7 log units. This phenomenon can be explained by the stronger electron withdrawing effect and the negative mesomeric effect of the nitro group. Due to the phenolate microconstants, 89% of the phenolic OH groups are deprotonated in 3-NT molecules at the pH of the blood plasma (7.40).

Conclusions

Proteins with 3-NT obtain an extra negative site at physiological pH, with several intra- and intermolecular consequences: the 'new' anionic site attracts cationic counterparts, such as arginine guanidinium, lysine, ornithine, terminal ammonium, histidine imidazolium moieties, typically present in the same peptide or protein molecule, causing inevitable conformational modifications. Also, the anionic phenolate is prone to act as an anchor unit to associate with other biomolecules of cationic loci, and to bind metal ions. These changes in conformation, association and composition may all bring about alterations in the biological behavior.[3,4,10,11]

Experimental Section

Materials

3-Nitro-L-tyrosine ((2*S*)-2-amino-3-(4-hydroxy-3-nitrophenyl)propanoic acid), 3-nitrotyrosine methyl ester (methyl (2*S*)-2-amino-3-(4-hydroxyphenyl)propanoate) and the internal NMR-pH indicators (imidazole, sarcosine, acetone oxime, sodium acetate) were obtained from Sigma-Aldrich. Deuterium oxide (D_2O) and methanol were purchased from Merck. All reagents were of analytical grade, obtained from commercial suppliers. The deionized water was prepared with a Milli-Q Direct 8 Millipore Water Purification System.

NMR Spectroscopy Measurements

All NMR measurements were carried out on a Varian 600 MHz spectrometer with a dual 5 mm inverse-detection gradient probe head at 25 °C. The NMR-pH titrations were performed in a mixture of 5% D_2O/95% H_2O (v/v). For titration at 0.15 M ionic strength acidic and basic stock solutions were prepared with concentrations of 0.1 M HCl/NaOH and 0.05 M KCl to ensure 0.15 M ionic strength. The spectra were referenced to internal DSS (sodium 3-(trimethylsilyl)-1-propanesulfonate). The sample volume was 600 µl, the titrant and pH indicator concentrations were 5 mM. In the ^{1}H-NMR experiments, pH values were determined by internal indicator molecules optimized for NMR.[12,13] The concentration of the analytes was 1 mM for the titration. The water resonance was diminished by a double pulse field gradient spin echo sequence (number of transients = 16, number of points = 16384, acquisition time = 3.33 s, relaxation delay = 1.5 s).

Data Analysis

For the analysis of NMR titration curves of proton chemical shifts versus pH, the software Origin Pro 8 (OriginLab Corp., Northampton, MA, USA) was used. In ^{1}H-NMR-pH titrations the non-linear curve fitting regression analysis option was used with the following function:[8]

$$\delta_{obs(pH)} = \frac{\delta_{L^{2-}} + \sum_{i=1}^{n} \delta_{H_iL^{i-2}} \times 10^{\log\beta_i - i\times pH}}{\sum_{i=0}^{n} 10^{\log\beta_i - i\times pH}} \quad (19)$$

where $\delta_{L^{2-}}$ is the chemical shift of the unprotonated ligand (L^{2-}), $\delta_{H_iL^{i-2}}$ values stand for the chemical shifts of successively protonated species of L^{2-}, n is the maximum number of protons that can bind to the unprotonated ligand, β is the cumulative protonation macroconstant. The standard deviations of logβ values from the regression analyses were used to calculate the Gaussian propagation of uncertainty for the other equilibrium constants.

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Author Contribution Statement

G. Tóth and B. Noszál designed the study. T. Pálla and E. Fogarasi performed the NMR-pH titration. T. Pálla evaluated the data. All authors contributed equally to draft and revise the manuscript.

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