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Acid–base versus structural properties of an aminoglycoside antibiotic—sisomicin: NMR and potentiometric approach

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Abstract—Aminoglycoside antibiotics constitute a class of the drugs of high interest, whose therapeutic action is based upon the electrostatic interaction with the variety of RNA molecules. The positive charge of these drugs molecules, located at their amino functions, has a prevailing influence on this process. The potentiometry and ^{1}H NMR spectroscopy are applied hereby to achieve the characteristics of the acid–base properties of particular protonating groups. We found that the pK values of deprotonation processes cover a wide values range 6–9.8. The correlation spectra of sisomicin, both COSY and TOCSY, allowed attributing unambiguously individual signals to the corresponding protons. These spectra involve a lot of the cross-peaks originating from the **B** and **C** rings protons, while the analogous signals originating from **A** rings protons are less numerous. Molecular modeling provided that the methylated amino group of **A** ring is located too far from the protonated functions of the remaining rings to affect their pK values. The phenomena observed herein are discussed in line of strength of the analogous processes observed for other aminoglycosides. As the result, four types of amino groups consisted within these antibiotics are distinguished.

1. Introduction

Sisomicin is a popular aminoglycoside antibiotic of fungal origins, produced similarly to gentamicins by *Micromonospora*. It is used in clinical treatment against infections caused by both gram-negative and gram-positive bacteria.[1] In therapy, its higher activity against several bacterial strains was proved in comparison with amikacin, gentamicin or tobramycin.[2] However, sisomicin and other aminoglycosides possess two therapeutically crucial disadvantages. The amount of the mutant strains resistant to these drugs is still increasing. Moreover, the treatment is accompanied by a set of serious toxic side effects, mainly ototoxicity and nephrotoxicity.[3] These factors limit their usage and impose the necessity of permanent monitoring of their level in blood serum.

In the current paper we present the acid–base properties of sisomicin, which differs from other representatives of its group by having unsaturated aminosugar moiety. This structural feature causes higher rigidity of the **C** ring (Scheme 1). Together with the presence of methylated amino function this causes differences in protonation constants when compared to other aminoglycosides, studied by us previously.[4–8] The knowledge of the acid–base character of sisomicin can be useful for understanding its electrostatic interactions with important biomolecules, that is, RNA, which is the main therapeutic target of this drug. Moreover, the results presented hereby may provide hints helpful to study or to design new pharmacologically important derivatives of aminoglycosides, which could be less toxic or more effective in combating the bacterial resistance.

2. Results

Sisomicin possess unsaturated aminosugar moiety, what leads to sp^2 hybridization at two carbon atoms and may have distinct influence on the stereochemical properties of the antibiotic (Scheme 1). Its molecule is a pentaprotic acid in its fully protonated state, consisting of

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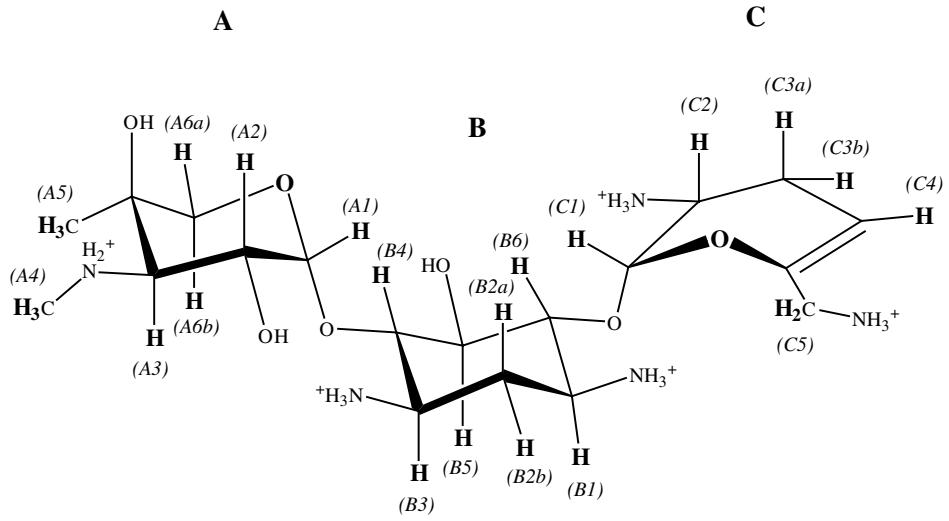

Scheme 1. The molecule of sisomicin in its fully protonated form.

four primary amino groups and a secondary one, as the only functions possessing acid–base properties. Those groups exist within the antibiotic as substituents at both aminosugar rings: garosamine and 2,6-diamino-2,3,4,6-tetradeoxohexa-4-enose[9] (**A** and **C**, respectively) and a central aminocyclitol (ring **B**). They differ from the chemical point of view, what influences their protonation constants. The potentiometric data indicate that the macroconstants obtained from titrations are typical for the values of amino groups deprotonations within aminosugars and their derivatives. One of pK_a values is very low (6.09) while the remaining ones fit in the characteristic range 7–9.5[10–12] (Table 1). Figure 1 shows the protonation species distribution diagram of the sisomicin molecule. All six antibiotic forms are represented therein by their concentration curves depending on the pK_a values of the consecutive deprotonation processes.

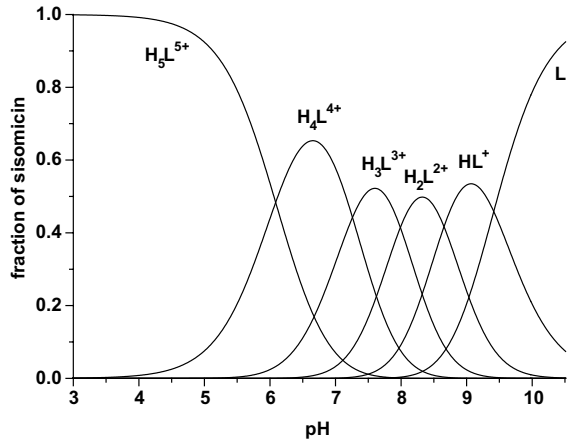

Figure 1. Species distribution diagram for sisomicin (10 mM). H_5L^{5+} represents the fully protonated species.

For precise characteristics of acidity of particular functions, the ^{1}H NMR technique was used. The correlation spectra of sisomicin, both COSY and TOCSY (Fig. 2) produced at pH 10.5 allowed us to attribute univocally particular signals to the corresponding protons of the **A**, **B** and **C** rings of sisomicin molecule (see Scheme 1). ^{1}H NMR titrations provided the full insight into the dissociation process of the antibiotic. The chemical shifts determined both for completely protonated form (below pH 5.0) and deprotonated one (above pH 11.6) are presented in Table 2 along with pK_g values (group constants calculated on the basis of changes in chemical shifts of particular protons) and Hill indices (n) calculated with application of Eq. 1.

$$\delta = \delta_p \times \frac{H^n}{H^n + K_g^n} + \delta_u \times \frac{K_g^n}{H^n + K_g^n} \qquad (1)$$

Table 1. Stability and protonation constants of sisomicin

Species	Log β	pK^a	pK^{*b}	p$K_{NMR}{}^c$	p$K_{NMR+I}{}^d$
HL	9.414(2)	9.414	9.75(8)	9.45(7)	9.40(7)
H_2L	18.069(1)	8.655	8.84(9)	8.63(8)	8.60(8)
H_3L	26.058(1)	7.989	8.09(9)	7.93(9)	7.91(9)
H_4L	33.317(1)	7.259	7.45(9)	7.34(9)	7.33(9)
H_5L	39.404(1)	6.087	6.12(7)	6.11(6)	6.11(6)

[a] Calculated on the basis of log β values.
[b] Evaluated from ^{1}H NMR data on the basis of pH*.
[c] Generated from NMR spectra and corrected for isotopic effect.[18]
[d] Generated from NMR spectra and corrected for isotopic and ionic effect.[18]

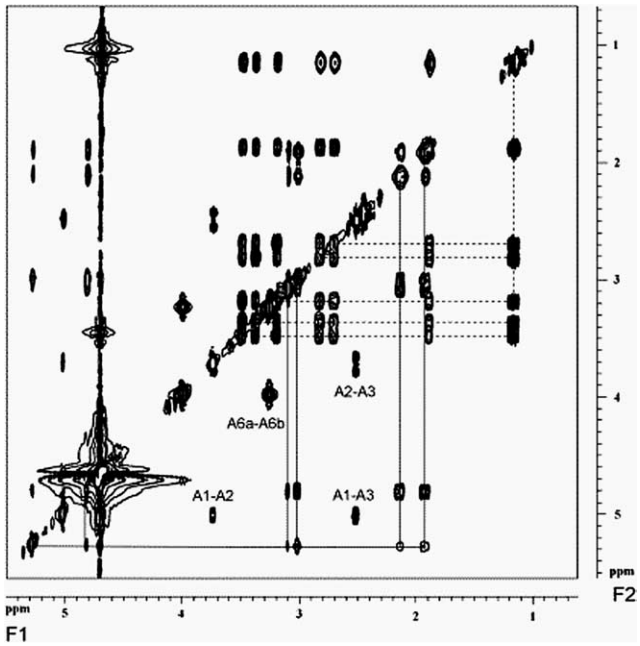

Figure 2. ^{1}H–^{1}H TOCSY spectrum of sisomicin at pH 10.5 and concentration of 10 mM. Solid lines represent connections between protons in **C** ring, dash lines between protons in **B** ring.

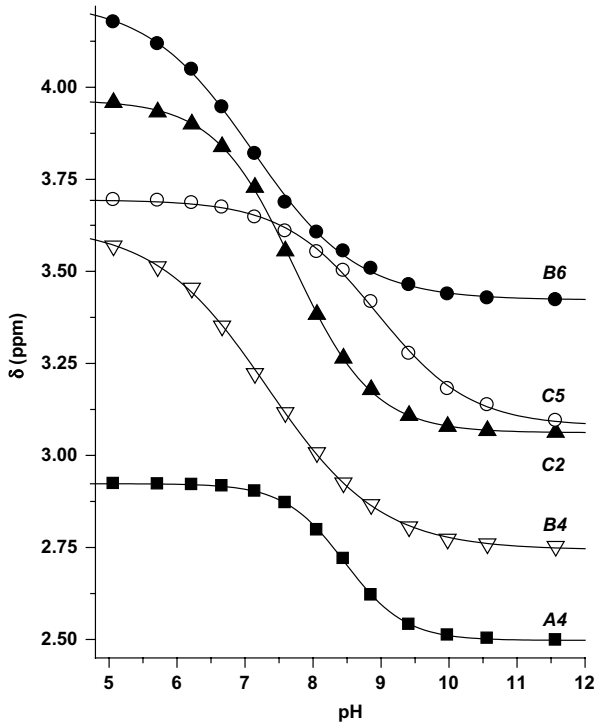

Figure 3. pH titration curves derived from chemical shifts (from TSP) in ^{1}H NMR spectra of sisomicin for A4, B4, B6, C2 and C5 protons.

The estimation of macroconstants on the basis of NMR results has been carried out with respect to the chemical shifts of the protons nearest to the dissociating groups. The pH profiles of the chemical shifts of A4, B4, B6, C2 and C5 protons courses are shown in Figure 3. For these calculations, B4 and B6 protons were chosen instead of B1 and B3 ones. Conversion of the shifts, using Eq. 2, to ionization fractions of particular groups (F), allows for direct determination of group constants, which are listed in Table 2. Parameter δ represents experimental average of chemical shifts, while δ_p and δ_u (Eqs. 1 and 2) are chemical shift values of particular protons for fully protonated or deprotonated molecule, respectively.[13,14]

$$F = (\delta - \delta_p)/(\delta_u - \delta_p) \quad (2)$$

recently[18] in order to compare their values with the ones given by the standard potentiometric titrations (pK). This conversion allows to eliminate the isotopic effect (pK_{NMR}) and the effect of ionic strength (pK_{NMR+I}) (Table 1, columns 6 and 7).[18]

3. Discussion

The protonation constants obtained from potentiometric titration for sisomicin are presented in Table 1. Four of them are typical for aminosugars, while the first constant is significantly lower. Low logarithmic values of the first deprotonation constant were found for all

$$U = \frac{10^{\mathrm{pH}-pK_1} + 2\times 10^{2\times\mathrm{pH}-pK_1-pK_2} + 3\times 10^{3\times\mathrm{pH}-pK_1-pK_2-pK_3} + 4\times 10^{4\times pH-pK_1-pK_2-pK_3-pK_4} + 5\times 10^{5\times\mathrm{pH}-pK_1-pK_2-pK_3-pK_4-pK_5}}{1 + 10^{\mathrm{pH}-pK_1} + 10^{2\times\mathrm{pH}-pK_1-pK_2} + 10^{3\times\mathrm{pH}-pK_1-pK_2-pK_3} + 10^{4\times\mathrm{pH}-pK_1-pK_2-pK_3-pK_4} + 10^{5\times\mathrm{pH}-pK_1-pK_2-pK_3-pK_4-pK_5}} \quad (3)$$

The sum (U) of all five protonation ionization fractions (F_{A4}, F_{B4}, F_{B6}, F_{C2} and F_{C5}), calculated from the chemical shifts of the protons proximal to the deprotonating groups of sisomicin in pH function, allowed fitting the macroconstants, with use of Eq. 3. All the estimated values are put together in Table 1 where they were compared with potentiometric data. Evaluation of Eq. 3 was presented previously by Rabenstein and Sayer[15] and Noszal.[16,17] The protonation constants obtained in D_2O at low ionic strength (pK^*) were converted according to the formula published by us

other aminoglycosides studied to date. These values were 6.0 for geneticin[8] and 6.19 for kanamycin A,[5] which carry the +4 charge at low pH, and ca. 5.7 for kanamycin B[6] and tobramycin,[7] which have the +5 charge. Amikacin, on the other hand, the only antibiotic with one of amino groups at the deoxystreptamine ring acylated, is also a +4 ion and has a much higher value of this pK_a −6.8.[4] We previously concluded, that the reason for the lowering of the first pK_a in aminoglycosides is a local electrostatic interaction between the neighbouring NH_3^+ groups of ring **B**, rather than the overall

Table 2. Chemical shifts (δ, DSS) of particular protons in protonated and deprotonated sisomicin molecule

Proton	δ_p	δ_u	pK_g	n
A1	5.1307(5)	5.0711(6)	8.50(2)	1.12(5)
A2	4.214(4)	3.777(4)	8.32(2)	0.83(3)
A3	3.471(5)	2.546(7)	8.36(1)	0.88(3)
A4	2.923(1)	2.498(2)	8.46(1)	0.98(2)
A5	1.3449(7)	1.203(1)	8.43(1)	0.93(3)
A6a	4.0516(7)	3.970(1)	8.56(2)	1.04(5)
A6b	3.521(7)	3.295(6)	7.95(7)	0.56(6)
B1	3.86(2)	3.241(8)	7.38(5)	0.66(5)
B2a	3.64(2)	2.870(6)	7.23(4)	0.60(3)
B2b	2.21(2)	1.210(5)	7.09(3)	0.60(2)
B3	2.56(2)	1.953(8)	7.36(6)	0.63(5)
B4	3.625(9)	2.744(5)	7.33(2)	0.53(1)
B5	3.98(1)	3.539(4)	7.28(5)	0.60(3)
B6	4.23(1)	3.422(6)	7.11(4)	0.56(2)
C1	5.631(9)	5.330(7)	8.19(5)	0.72(5)
C2	3.965(4)	3.062(3)	7.72(1)	0.75(1)
C3a	2.715(5)	2.158(3)	7.72(2)	0.66(2)
C3b	2.425(5)	1.966(3)	7.70(2)	0.87(3)
C4	5.204(6)	4.79(2)	8.97(7)	0.59(5)
C5	3.694(5)	3.080(9)	8.94(3)	0.63(3)

charge of the whole molecule. In the case of sisomicin, this pK_a value is quite higher than for other drugs with the same charge +5. This phenomenon may result from the structural differences within the unsaturated aminosugar ring (**C**) and methylation of the only amine function in garosamine ring (**A**), what affects its basicity.

The next three deprotonations, observed during potentiometric measurements, are separated by only 0.67–0.72 log units (Table 1). This phenomenon is reflected by the low molar fractions of the consecutive protonation species from H_3L^{3+} to HL^{+} (Fig. 1). The statistics of deprotonation of bifunctional molecules (the statistical $\log K_1 - \log K_2 = 0.6$)[19] or subunits, as in our case, indicates that these three processes actually run in parallel. Therefore, these macroconstant values, which correspond to the amino groups deprotonating in the successive steps, cannot be assigned individually by potentiometric titration.

Taking the basicity and chemical properties of amino functions in aminoglycoside antibiotics into account, four types of them may be distinguished. They can be divided into the groups as follows: the primary ones, bound directly to the aminosugar ring (RNH_2); the aminomethylene functions (RCH_2NH_2); the primary amines in the aliphatic aglycon chains and the secondary ones, attached to the sugar rings. Studies on their acid–base properties, performed for aminoglycosides and simple aminosugars with use of potentiometry and ^{1}H NMR, suggest that the most acidic ones are the primary amines, situated directly at the sugar rings. The most basic ones, however, are the side chains functions.

The dissociation constants collected for the range of aminoglycoside antibiotics possessing various amounts and types of amines, prompted us to undertake the statistical analysis of their acid–base properties. Confrontation of the summary stability constants $\log \beta\ H_xL$ ($\sum pK_x$) for six antibiotics, built up of three rings, and application of the multiple linear regression allowed for estimation of the average values of protonation constants for the groups, which differ with its chemical character (Table 3). The value of pK for the 1° amines is on average 7.2(2), for the 2°–8.6(2) and for the primary aminomethylene functions and aliphatic: 9.3(8) and 9.8(3), respectively. In fact, these data represent the average values of the group dissociation constants; despite they originate from the potentiometric data based on the chemical composition of the substance.

Essential for signals attribution to particular protons on the ^{1}H NMR spectra were the correlation experiments. Figure 2 presents the TOCSY spectrum of sisomicin at pH 10.5, along with the signals from **B** and **C** rings depicted. What is of interest, is there are no cross-peaks originating from A4 and A5 connections.[20,21]

The group constants obtained for sisomicin, as well as the chemical shifts of both protonated and deprotonated antibiotic forms (Table 2), portray the effects for the particular protons, which result from the amines dissociations. Apparently, the most explicit effects can be observed for the protons neighbouring to the deprotonating functions and as such, are burdened in the least degree by the influence of other, more distant dissociation centers. The B4 and B6 protons, however, are taken into account as more sensitive for deprotonation of both amines at the deoxystreptamine moiety, instead of B1 and B3 protons, from which the signals overlap. This process results from their proximity to both dissociating RNH_3^+ groups in this ring. The calculated average values of the group constants, referring to the chemically similar functions within sisomicin, are 7.39 and 8.49 for the 1° and 2° amines, respectively, and 8.94 for the aminomethylene substituent, what ideally fits the values provided by the application of multiple linear regression

Table 3. Comparison of the summary stability constants $\log \beta\ H_xL$ ($\sum pK_x$) for selected aminoglycoside antibiotics with the number of amino groups of particular types indicated

Antibiotic	Log β (H_xL)	x	1°	2°	RCH_2NH_2	Aglycon	Ref.
Amikacin	33.433	4	2	0	1	1	4
Kanamycin A	30.80	4	3	0	1	0	5
Kanamycin B	37.637	5	4	0	1	0	6
Tobramycin	38.121	5	4	0	1	0	7
Geneticin	30.127	4	3	1	0	0	8
Sisomicin	39.404	5	3	1	1	0	This work
pK_{av}[a]	—	—	7.2(2)	8.6(2)	9.3(3)	9.8(3)	

[a] pK_{av}: negative logarithm of average dissociation constant.

to the potentiometry derived data (Table 3). These results correlate well with the values previously obtained by ^{1}H NMR and potentiometry for amikacin,[4] recalculated here to give 7.26 for the primary amines attached directly to the ring and 8.85 the aminomethylene function. ^{1}H NMR data for the NH_3^+ group at the aliphatic aglycon of amikacin **A** ring were not provided.[4]

The detailed analysis of the group constants and the differences in chemical shifts values between the protonated and completely deprotonated studied molecule, but most of all the Hill indices (n), indicates the presence of the cooperative effects in amines dissociation at each of three component rings. The average n values are: 0.9(1), 0.60(4) and 0.7(1) for rings **A**, **B** and **C**, respectively. It allows for the conclusion that the deviation of average n value, with respect to the unit value for **B** ring, is a sum of analogous factors deviations in case of both rings **A** and **C**. Actually, it signifies that low n values for the aminocyclitol ring are influenced mainly by the **C** ring, while the impact of ring **A** is almost negligible. These effects remains in direct relation with the number and type of amino functions in rings **A** and **C**, as well as with interactions between the positive charge placed on these groups and the oxygen atoms among the all rings.

From the sterical reason, the secondary amino function of **A** ring ($n = 0.98$) exerts markedly no influence on the deprotonation process of the amino groups within rings **B** and **C**. Analogous conclusion may also be drawn for the A3 and A5 protons on the basis of their n factor values (0.88 and 0.93, respectively). Additionally the lack of any interactions between amino group of **A** ring and the surrounding protons may be also proven by the absence of cross-peaks on the TOCSY spectrum for A4 and A5 protons (see Fig. 2). Even though the spectrum in Figure 2 was obtained at pH 10.5, such patterns were observed on the correlation spectra of the fully protonated molecule alike. Similar effects originating from the cooperative action of amino groups, which undoubtedly influence the macroconstants values, were also revealed for amikacin in our previous study.[4]

The molecular modeling calculations resulted in a few conformers of the lowest potential energy. Apparently, the ring **B** seems to be the most stable from all three moieties, preceding **C** and **A** rings, respectively. These both rings are the terminal moieties and as such, they are very flexible. However, higher stability of the **C** ring is induced by the presence of a double bond within this residue, forcing thereby its rigid structure. Probably, this structural feature influences on the acid–base properties of sisomicin, in the similar way, like the presence of aglycon in amikacin molecule, exemplifying the steric hindrance, has impact on its analogous properties.

4. Conclusions

In the current study, the detailed discussion of the protonation equilibria in the sisomicin molecule is presented. On the basis of comparison to other three-ring representatives of aminoglycosides we distinguish four types of amino functions in the molecules of these drugs. Three of them are present in sisomicin and their group dissociation constants, calculated hereby, are in accordance with the analogous constants obtained in separate studies for amikacin, which contains all four types of amines. The NMR TOCSY experiment, as well as the calculations of the Hill index values, clearly indicates that the amino groups deprotonations within rings **B** and **C**, are influenced by one another. The molecular modeling confirmed, that the amino function of **A** ring is sterically unavailable for any interactions with the remaining groups attached to rings **B** and **C**. These studies also revealed the higher stability of the **C** ring, than **A** one, resulting from its unsaturated character.

The results obtained may appear useful in prediction of acid–base properties of another, not studied but chemically similar compounds and may be very helpful in their NMR investigations. They may also contribute to the better understanding of the origins of electrostatic in vivo interactions of the drug with its target sites.

5. Experimental

5.1. Materials

Sisomicin sulfate, NaOD (40% D_2O solution) and other simple chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). KNO_3, DCl (20% D_2O solution) and D_2O were purchased from Cambridge Isotope Laboratories (Cambridge, UK).

5.2. Potentiometry

Potentiometric titrations of sisomicin in the presence of 0.1 KNO_3 were performed at 25 °C using pH-metric titrations over the pH range 3–11.5 (Molspin automatic titrator, Molspin Ltd, Newcastle upon Tyne, UK) with 0.1 M NaOH as titrant. Changes in pH were monitored with a combined glass–Ag/AgCl electrode (InLab 422, Mettler-Toledo Sp. z o.o. Warsaw, Poland), calibrated daily in hydrogen ion concentrations by HNO_3 titrations.[22] Sample volumes of 2 mL and sisomicin concentration of 1 mM were used. These data were analyzed using SUPERQUAD program.[23] Standard deviations computed by SUPERQUAD refer to random errors only.

5.3. NMR spectroscopy

^{1}H NMR (1D and 2D) spectra of 10 mM sisomicin samples in D_2O were obtained at 25 °C. Antibiotic samples (10 mM) were recorded at 25–70 °C on a Bruker AMX-300 and AMX-500 spectrometer (Karlsruhe, Germany). TSP (sodium (3-trimethylsilyl)-2,2,3,3-tetradeuterio-1-propanesulfonate) was used as an internal standard. R_{pH^*} (pH-meter readings in D_2O using glass

electrode calibrated with standard buffers in H_2O) was corrected for isotopic effect and transformed into pH according formula: $R_{pH} = 0.929 \times R_{pH^*} + 0.42$.[18]

5.4. Molecular modeling (processing of NMR data)

Processing of NMR spectra, geometry optimization, molecular dynamics and conformational space analysis were performed by PERCH NMR software (PERCH solutions Ltd, Finland). Conformations were calculated using the simulated annealing protocol. Electrostatic interactions were included to calculations. The initial structure generation was followed by 20 ps of high-temperature (900 K) molecular dynamics and 10 ps cooling process, until temperature reached 100 K. A total of 50 structures were calculated and the 10 lowest energy structures were analyzed.

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References and notes

1. Davis, B. D. *Microbiol. Rev.* **1987**, *51*, 341.
2. Sanders, W. E.; Danders, C. C. *Rev. Infect. Dis.* **1980**, *2*, 182.
3. Gale, E. F.; Cundliffe, E.; Reynolds, P. E.; Richmond, M. H.; Waring, M. J. In *The Molecular Basis of Antibiotic Action*, 2nd ed.; Wiley Interscience: London, 1981; pp 278–288.
4. Jeżowska-Bojczuk, M.; Bal, W. *J. Chem. Soc., Dalton Trans.* **1998**, 153.
5. Szczepanik, W.; Kaczmarek, P.; Sobczak, J.; Bal, W.; Gatner, K.; Jeżowska-Bojczuk, M. *New J. Chem.* **2002**, *26*, 1507.
6. Jeżowska-Bojczuk, M.; Bal, W.; Kozłowski, H. *Inorg. Chim. Acta* **1998**, *275–276*, 541.
7. Jeżowska-Bojczuk, M.; Karaczyn, A.; Kozłowski, H. *Carbohydr. Res.* **1998**, *313*, 265.
8. Jeżowska-Bojczuk, M.; Karaczyn, A.; Bal, W. *J. Inorg. Biochem.* **1998**, *71*, 129.
9. Cooper, D. J.; Jaret, R. S.; Reimann, H. *J. Chem. Soc., Chem. Commun.* **1971**, 285.
10. Gyurcsik, B.; Nagy, L. *Coord. Chem. Rev.* **2000**, *203*, 81.
11. Whitfield, D. M.; Stojkovski, S.; Sarkar, B. *Coord. Chem. Rev.* **1993**, *122*, 171.
12. Jeżowska-Bojczuk, M.; Kozłowski, H.; Pettit, L. D.; Micera, G.; Decock, P. *J. Inorg. Biochem.* **1995**, *57*, 1.
13. Dorčàk, V.; Krężel, A. *Dalton Trans.* **2003**, 2253.
14. Krężel, A.; Bal, W. *Org. Biomol. Chem.* **2003**, *1*, 3885.
15. Rabenstein, D. L.; Sayer, T. L. *Anal. Chem.* **1976**, *48*, 1141.
16. Noszal, B. *J. Phys. Chem.* **1986**, *90*, 6345.
17. Noszal, B. *J. Phys. Chem.* **1986**, *90*, 4104.
18. Krężel, A.; Bal, W. *J. Inorg. Biochem.* **2004**, *98*, 161.
19. Noszal, B. In *Biocoordination Chemistry—Coordination Equilibria in Biologically Active Systems*; Burger, K., Ed.; Ellis Harwood: Chichester, 1990; pp 18–55.
20. Homans, S. W. In *NMR of Macromolecules. A Practical Approach*; Roberts, G. C. K., Ed.; Oxford University Press: Oxford, 1993; pp 289–311.
21. Karst, N.; Jacquinet, J. C. *J. Chem. Soc., Perkin Trans. 1* **2000**, 2709.
22. Irving, H.; Miles, M. G.; Pettit, L. D. *Anal. Chim. Acta* **1967**, *38*, 475.
23. Gans, P.; Sabatini, A.; Vacca, A. *J. Chem. Soc., Dalton Trans.* **1985**, 1195.