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ORIGINAL PAPER

Solution-state NMR spectroscopy of famotidine revisited: spectral assignment, protonation sites, and their structural consequences

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Abstract Multinuclear one (1D-) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopic investigations of famotidine, the most potent and widely used histamine H_2-receptor antagonist, were carried out in dimethyl sulfoxide-d_6 (DMSO-d_6) and water. Previous NMR assignments were either incomplete or full assignment was based only on 1D spectra and quantum-chemical calculations. Our work revealed several literature misassignments of the ^{1}H, ^{13}C, and ^{15}N NMR signals and clarified the acid–base properties of the compound at the site-specific level. The erroneous assignment of Baranska et al. (*J. Mol. Struct.* 2001, 563) probably originates from an incorrect hypothesis about the major conformation of famotidine in DMSO-d_6. A folded conformation similar to that observed in the solid-state was also assumed in solution, stabilized by an intramolecular hydrogen bond involving one of the sulphonamide NH_2 protons and the thiazole nitrogen. Our detailed 1D and 2D NMR experiments enabled complete *ab initio* ^{1}H, ^{13}C, and ^{15}N assignments and disproved the existence of the sulphonamide NH hydrogen bond in the major conformer. Rather, the molecule is predominantly present in an extended conformation in DMSO-d_6. The aqueous acid–base properties of famotidine were studied by 1D ^{1}H- and 2D $^{1}H/^{13}C$ heteronuclear multiple-bond correlation (HMBC) NMR-pH titrations. The experiments identified its basic centers including a new protonation step at highly acidic conditions, which was also confirmed by titrations and quantum-chemical calculations on a model compound, 2-[4-(sulfanylmethyl)-1,3-thiazol-2-yl]guanidine. Famotidine is now proved to have four protonation steps in the following basicity order: the sulfonamidate anion protonates at pH=11.3, followed by the protonation of the guanidine group at pH=6.8, whereas, in strong acidic solutions, two overlapping protonation processes occur involving the amidine and thiazole moieties.

Keywords Famotidine · Famotidone · NMR assignment · NMR titration · pK_a · Conformation

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Introduction

Famotidine [{3-[2-guanidino-(1,3-thiazol-4-yl)]methylsulfanyl}-N^2-sulfamoylpropanamidine] is by far the most potent histamine H_2-receptor antagonist on the market [1]. It is also the most frequently used histamine antagonist for the treatment of dyspepsia, gastroesophageal reflux disease, and peptic ulcer in several countries [2, 3]. Famotidine has been known for more than 30 years [4], however, its full and correct nuclear magnetic resonance (NMR) assignment is still lacking. In the solid state, famotidine shows conformational polymorphism: the polymorph "*A*" and "*B*" possess

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an extended and a folded conformation, respectively [5]. Despite the enormous therapeutic significance, its solution state conformation is still unclear; moreover, there is a notable confusion in the literature about the acid–base properties of the drug, especially concerning the identity of its protonation sites.

The structure of famotidine and two related compounds investigated in this work are shown in Fig. 1, along with the numbering that follows the previously used notation [6–9] for a better comparison.

The original chemical patent of famotidine includes incomplete ^{1}H NMR data in dimethyl sulfoxide-d_6 (DMSO-d_6) and reports assignment only for *H25* and *H26,27* [4, 10]. The first paper describing the synthesis and pharmacological behavior of famotidine reports ^{15}N chemical shifts in DMSO-d_6, however, their assignment is inconsistent [1]. Unfortunately, the only complete ^{1}H, ^{13}C, and ^{15}N assignment in DMSO-d_6 published by Baranska et al. also contains some misassignments [6]. Moreover, this misassignment has started to spread over the literature [7, 11, 12] and appeared even in the recent monograph of famotidine in the "Profiles of Drug Substances, Excipients and Related Methodology" in 2009 [9]. Yet another erroneous ^{13}C assignment of famotidine appeared in 2006 [13]. There is also confusion about the solution-state conformation of famotidine: both Ishida et al. [14] and Baranska et al. [6] proposed folded conformers (with different intramolecular hydrogen bonds), while Olea-Azar et al. claimed extended conformers to be more stable [15]. Our work provides complete and correct ^{1}H, ^{13}C, and ^{15}N NMR assignments in DMSO-d_6 and clears up the confusion about the conformation of famotidine in solution.

The assignment given by Baranska et al. relies on the comparison of 1D NMR spectra and quantum-chemical calculations [6]. Although the calculated structure was not published, it is clear from the discussion that a folded conformation similar to that observed in solid state for polymorph "*B*" was assumed to be present in solution. This structure is stabilized by an intramolecular hydrogen bond involving one of the sulphonamide NH_2 protons and the thiazole nitrogen. The authors claim that famotidine assignment can be accomplished without any resort to two-dimensional (2D) NMR experiments as the measured and calculated chemical shifts were in good agreement. However, our detailed experimental work shows that even high level molecular modelling in vacuum cannot substitute experimental 2D NMR for unambiguous assignment of famotidine with several moieties capable of intra- and intermolecular hydrogen bonding and solvent interactions.

The acid–base properties are important physico-chemical parameters of a drug, as they influence both its pharmacokinetic and pharmacodynamic behavior. A proper characterization involves the determination of all macroscopic protonation constants (reported either as stepwise log K or cumulative log β values [16]) that allow either the calculation of the overall electric charge at any pH or the distribution of the differently protonated species. Beyond the log K values, the assignment of protonation sites is also important, especially when specific interactions such as receptor binding are considered. When the successive protonation steps are well-separated (the respective log K values differ by more than four units), the H^+ uptake can be localized to a single group [16, 17]. There are several analytical techniques to identify the protonation centers. Besides UV [18], fluorescence [19], and Raman spectroscopy [20], ^{1}H NMR-pH titration is the most powerful technique since the pH-dependence of chemical shifts of individual nuclei usually provide atomic-level information on the protonation state of the nearby basic centers. In favorable cases, adjacent nuclei may even selectively monitor the H^+ uptake of the group in question [21, 22].

There is no consensus in the literature regarding the aqueous acid–base properties of famotidine. Several sets of

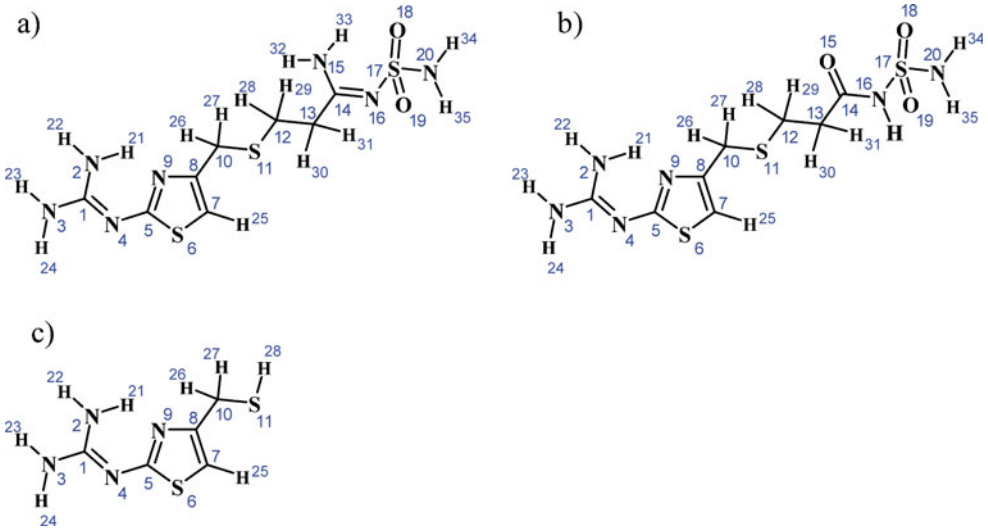

Fig. 1 The structure and numbering of famotidine (**a**), famotidone (**b**), and 2-[4-(sulfanylmethyl)-1,3-thiazol-2-yl]guanidine: STG (**c**)

protonation constants have so far been published [7, 23–25], mostly determined by potentiometry [7, 24, 25]. Identification of protonation sites and even the number of famotidine functional groups protonating in the 0–14 pH range is controversial [15, 24–26]. In addition, famotidine is chemically unstable both in strongly acidic and alkaline media [1, 27, 28] and possesses poor water solubility [23]. NMR titration is among the few techniques not hampered by degradation, as the signals of the parent compound can usually be distinguished from those of the decomposition product(s).

Thus, our study also aimed at acid–base characterization of famotidine by NMR spectroscopy. The number of protonation steps, the corresponding equilibrium constants, and their assignment to functional groups are deduced from NMR-pH titrations involving both 1D ^{1}H spectra and 2D $^{1}H/^{13}C$ heteronuclear multiple-bond correlation (HMBC) spectra. To augment the acid–base study of famotidine as well as to help assigning the protonation sites, a related model compound, 2-[4-(sulfanylmethyl)-1,3-thiazol-2-yl] guanidine (STG, see Fig. 1), was also investigated.

Materials and methods

Materials

Famotidine and STG were prepared at Gedeon Richter Plc., while the sample of famotidone hydrochloride was obtained as CRS reference standard from EGC Promochem (Fig. 1). Deuterated solvents (D_2O 99.9% D, 99.96% D for D-exchange experiments and DMSO-d_6 99.9% D) and reagents (20% DCl), as well as the internal reference materials sodium-3-trimethylsilyl-propanesulphonate (DSS) and tetramethylsilane (TMS) used for the NMR experiments were obtained from Sigma-Aldrich Co. The pH-indicator dichloroacetic acid (99%) was purchased from Fluka, while azeotropic HCl (20.25%) was a gift from Eötvös Loránd University. All materials were used without further purification, and stock solutions were prepared using purified Millipore water with conductivity of 1.1 μS cm^{-1}.

NMR experiments

General NMR conditions

The ^{1}H NMR titration of STG was performed on a Bruker Avance spectrometer (^{1}H resonance frequency, 250.13 MHz) equipped with a 5-mm SB dual $^{1}H/^{13}C$ probe and XWinNMR software. For the multinuclear assignment of famotidine in DMSO-d_6, the spectra were measured on a Varian Unity Plus spectrometer (499.77 MHz for ^{1}H, 125.67 MHz for ^{13}C, and 50.65 MHz for ^{15}N) equipped with a $^{1}H\{^{13}C/^{15}N\}$ 5-mm PFG triple resonance ^{13}C enhanced cold probe and VnmrJ 2.1B. All other NMR experiments were conducted on a Varian VNMRS spectrometer (599.93 MHz for ^{1}H, 150.87 MHz for ^{13}C) with an IDPFG probe and VnmrJ 2.2C software. All experiments were carried out at 25 °C in 5-mm tubes using standard pulse sequences, 1D experiments: ^{1}H and $^{13}C\{^{1}H\}$, selective dpfgse-NOE/ROE: NOESY1D (mixing time, 300 and 500 ms), ROESY1D (mixing time, 200 ms; spinlock, 3.2 kHz); 2D experiments: NOESY (mixing time, 300 ms), $^{1}H/^{13}C$ gHSQC and gHMBC (optimized to 8 Hz), $^{1}H/^{15}N$ gHSQC, and gHMBC (optimized to 5 Hz). For the NMR-pH titrations in 95% H_2O/5% D_2O, the solvent peak was suppressed by presaturation.

^{1}H chemical shifts in DMSO-d_6 were referenced to TMS, while those in D_2O or H_2O, to internal DSS. In strongly acidic solutions (pH<1), the 3.35 ppm CH_3 singlet of 0.2 mM methanol was used as ^{1}H reference. ^{13}C NMR spectra were referenced to the DMSO-d_6 peak at 39.5 ppm or to DSS (0.0 ppm) in D_2O/H_2O. ^{15}N chemical shifts were referenced to external neat nitromethane with δ=380 ppm for a better comparison with literature data. The MestReNova 5.3.1 (http://www.mestrec.com, accessed on 14 Feb 2011) software was also used for spectral processing.

Experiments on the decomposition of famotidine in acidic solutions

Some 4.7 mg famotidone hydrochloride was dissolved in 3 ml of D_2O (pH^* 0.49,[1] adjusted with 20% DCl) containing 0.5% *w/v* DSS, yielding 4.64 mM famotidone solution. Some 650 μl was immediately transferred to an NMR tube, and the 1D ^{1}H and ^{13}C as well as 2D $^{1}H/^{13}C$ gHMBC spectra were recorded. Subsequently, 4.4 mg of famotidine was dissolved in 1.5 ml of the above famotidone solution, and then 650 μl was immediately added to the former NMR sample, sonicated for 20 s, resulting in a solution containing 4.64 mM famotidone and 4.35 mM famotidine, on which all the above experiments were repeated.

^{1}H NMR-pH titrations

A pH=2.2 stock solution of 2.0 mM STG and equimolar DL-dithiothreitol (as antioxidant) was prepared. The desired pH values were set by adding 1 M KOH or 2 M HCl solutions.

The 40.3 mg famotidine was suspended in 45 ml water and sonicated in ultrasound bath (50 °C, ~1 h) until dissolution. The resulting solution was cooled; DSS, KCl,

[1] pH* refers to the value measured in deuterium oxide by a glass electrode calibrated with aqueous buffer solutions. According to Gross-Butler-Purlee theory [29], the pD value can be calculated from pH* values by addition of a factor of 0.44.

dichloroacetic acid, and 2.5 ml D_2O (5%) were added, and it was diluted to 50 ml with H_2O. Final concentrations in the stock solution were as follows—2.39 mM famotidine, 0.15 M KCl, 1.09 mM DSS, and 0.2 mM dichloroacetic acid. The pH of individual aliquots was adjusted with additions of minute amounts of distilled HCl or 10 M KOH. This experimental design eliminated the need for acidic or alkaline famotidine stock solutions, in which a significant proportion of famotidine would have decomposed during the titration process [1, 27, 28]. Apart from extreme values of pH, the ionic strength was kept constant at I=0.15.

pH was determined with Metrohm 6.0234.110 combined glass electrode at 25 °C, calibrated using four NIST buffer solutions in H_2O to the H^+ activity scale. The measured samples contained 5% *v/v* D_2O for the lock, which shifted the pH-scale within the deviation limit of ca. 0.02 pH units, according to the Gross–Butler–Purlee theory [29]. To avoid the well-known uncertainty of the glass electrode in highly acidic medium, pH was calculated between 1 and 0 from the measured chemical shift of dichloroacetic acid [30] according to the modified Henderson–Hasselbalch equation [22]. The acidity of HCl solutions more concentrated than 1 M was characterized by the negative logarithm of the molar HCl concentration.

Titration curves were evaluated with nonlinear least-square parameter fitting on the measured points of all the observed nuclei using the Origin 6 and Origin Pro 8 SR0 programs (http://www.originlab.com/index.aspx?go=Downloads/OriginEvaluation, accessed on 14 Feb 2011). For famotidine, three protonation steps were fitted for carbon-bound protons using the following function,

$$\delta^{\mathrm{obsd}} = \frac{\delta_{\mathrm{L}} + \delta_{\mathrm{HL}} K_1 a_{\mathrm{H}} + \delta_{\mathrm{H_2L}} K_1 K_2 a_{\mathrm{H}}{}^2 + \delta_{\mathrm{H_3L}} K_1 K_2 K_3 a_{\mathrm{H}}{}^3}{1 + K_1 a_{\mathrm{H}} + K_1 K_2 a_{\mathrm{H}}{}^2 + K_1 K_2 K_3 a_{\mathrm{H}}{}^3}, \quad (1)$$

where δ^{obsd} is the measured chemical shift (in parts per million), while δ_{L}, δ_{HL}, $\delta_{\mathrm{H_2L}}$, and $\delta_{\mathrm{H_3L}}$ are the chemical shifts of the individual unprotonated, mono-, di-, and triprotonated species, respectively. a_{H} is the activity of hydrogen ions in the solution, while K_1, K_2, and K_3 are the stepwise protonation macroconstants, e.g.,

$$K_1 = \frac{[\mathrm{HL}]}{a_{\mathrm{H}}[\mathrm{L}]}, \quad (2)$$

These quantities are so-called "mixed" equilibrium constants, containing both concentration and activity values.

In the case of STG, a two-step protonation profile was fitted with K_3=0 in Eq. 1 [22].

The pH dependence of the ^{13}C chemical shifts of famotidine was determined by 2D $^1H/^{13}C$ gHMBC and $^1H/^{13}C$ gHSQC NMR-pH titrations.

Quantum-chemical calculations

Quantum-chemical calculations were carried out using the Gaussian 03 program (http://www.gaussian.com/g_misc/g03/citation_g03.htm, accessed on 14 Feb 2011), with DFT method at the B3LYP/6-31++G(2d,p) level *in vacuo* for geometry optimizations and energies, while chemical shift calculations were carried out applying the same basis at Hartree–Fock level. For STG, the Gibbs free energies ΔG (tot, gas) were calculated as the sum of electronic energies ΔE(gas) and thermal free energy corrections at 298 K and 1 atm ΔG(298, 1 atm, gas). The relative values in contrast to the lowest energy protonation form were compared.

The Marvin program (http://www.chemaxon.com/marvin/sketch/index.jsp, accessed on 14 Feb 2011) was applied for pK_a prediction of famotidine and STG.

Results and discussion

The assignment of famotidine NMR signals in DMSO-d_6

Our complete ^1H, ^{13}C, and ^{15}N NMR assignment is based on ^1H signal integrals, multiplicity patterns, and heteronuclear single quantum coherence (HSQC)/HMBC correlations. As the assignment is a routine task, only those points are emphasized which are crucial to the unambiguous assignment of the previously incorrectly assigned peaks.

The ^1H NMR spectrum of famotidine in DMSO-d_6 (Fig. 2) contains triplets at 2.46 and 2.69 ppm (^3J (^1H,^1H)~7.6 Hz), while all other famotidine peaks are singlets of varying linewidth. Carbon- and nitrogen-bound protons were unambiguously assigned by using $^1H/^{13}C$- and $^1H/^{15}N$ HSQC spectra (Fig. 3). Assignments of the carbon-bound *H25* (6.49 ppm singlet) and the *H26,27* methylene protons (3.62 ppm singlet) are obvious, and their HSQC correlation peaks identify *C7* (104.5 ppm) and *C10* (31.3 ppm), respectively. The two triplets are assigned using the $^1H/^{13}C$ HMBC spectrum in Fig. 4: The protons at 2.46 ppm exhibit two multiple-bond correlations (at 27.9 and at 165.0 ppm), while the ones at 2.69 ppm show three cross-peaks (at 31.3, 36.2, and 165.0 ppm), allowing their respective assignment to *H30,31* and *H28,29*. The HMBC spectrum enables the assignment of all but one quaternary carbon (see Table 1). The remaining guanidine *C1* is separated by more than three covalent bonds from all the CH_n protons and is unlikely to give correlation to the broad *H21-24* signal either. Nevertheless, its chemical shift (157.0 ppm) is unambiguously determined from the 1D ^{13}C spectrum (Fig. S1 in Electronic supplementary material).

The eight nitrogen-bound protons give four signals above 6.5 ppm with relative integrals of 1H, 1H, 4H, and 2H, respectively (Fig. 2). Since the dissolved famotidine was

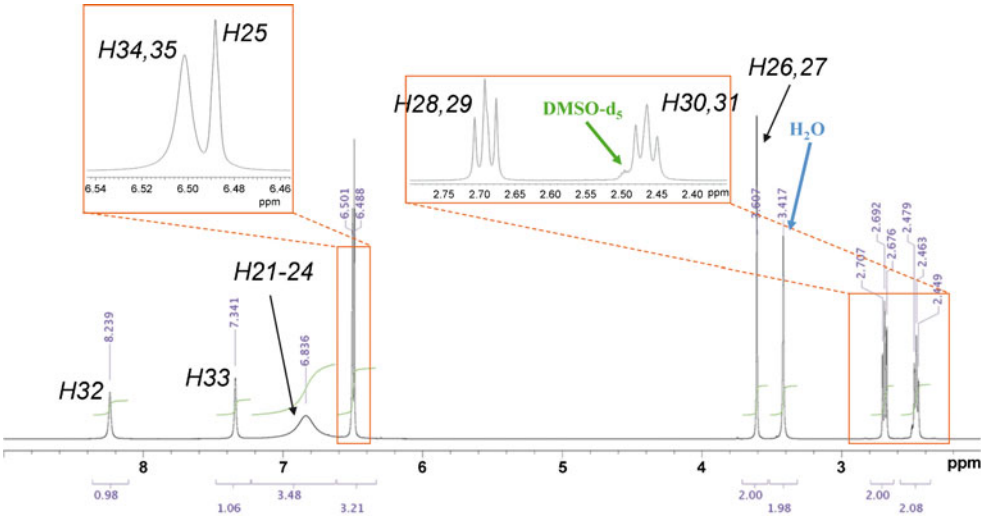

Fig. 2 The 500 MHz ^{1}H NMR spectrum of famotidine in DMSO-d_6

neutral, the ^{1}H/^{15}N HSQC (Fig. 3) spectrum proves the presence of four NH_2 groups and thus, the tautomeric state depicted in Fig. 1, in accordance with previous results [1, 6]. Assignment of the sp^2 nitrogen atoms (*N16* and *N9*) follows directly from the ^{1}H/^{15}N HMBC correlations (Fig. 5). *N4* was detected by correlation due to the long-range ^{4}J(^{15}N, ^{1}H) couplings to *H25*. Unambiguous assignment of *N15* relies on the ^{1}H/^{15}N HMBC correlation with *H30,31* as well as on the ^{1}H/^{13}C HMBC correlation of the attached *H32* and *H33* protons to *C13* and *C14*. Assignment of the remaining *N2*, *N3*, and *N20* nitrogens is based on the ^{1}H intensities of the directly attached protons at 6.84 ppm (4H) and 6.50 ppm (2H), respectively. It should be noted that *H21-H24* protons of the guanidine moiety give one broad signal due to fast exchange on the NMR chemical shift timescale, and the ^{15}N signals of *N2* and *N3* are also averaged. On the contrary, the *H32* and *H33* protons (chemical shifts, 8.24 and 7.34 ppm) attached to *N15* are chemically non-equivalent due to restricted rotation (see below). The *H30,31* as well as *H28,29* protons give much stronger NOE to the proton at 8.24 ppm than to the proton at 7.34 ppm, which provide evidence for their assignment to the *H32* and *H33* protons, respectively (1D selective dpfgse-NOE/ROE as well as 2D ^{1}H/^{1}H NOESY spectra are shown in Electronic supplementary material Figs. S2 and S3).

Comparison of the measured and calculated chemical shifts with previous assignments

The measured and calculated ^{1}H, ^{13}C, and ^{15}N chemical shifts of famotidine in this work and in the work of Baranska et al. [6] are compared in Table 1. (Table S1 in Electronic supplementary material also contains previous literature assignments from Yanagisawa et al., Hirata et al. (1980, 1981), and Miodragovic et al. [1, 4, 10, 13].)

The calculations of Baranska et al. using a HF/6-31G (d)//B3LYP/6-31G(d) method underestimated the chemical shift value of *C13* by more than 10 ppm resulting in

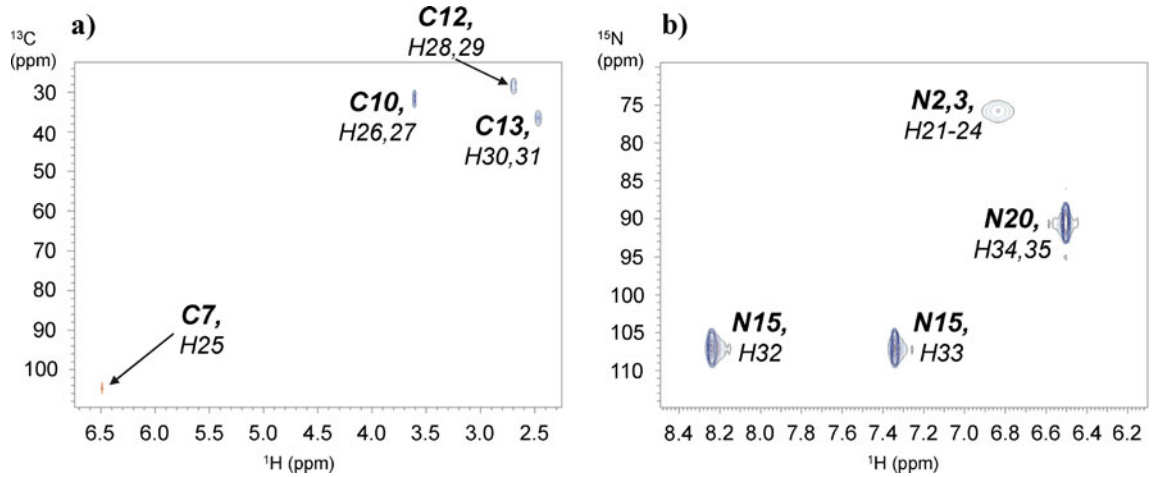

Fig. 3 500 MHz ^{1}H/^{13}C (**a**) and ^{1}H/^{15}N (**b**) phase-sensitive gHSQC spectra of famotidine in DMSO-d_6

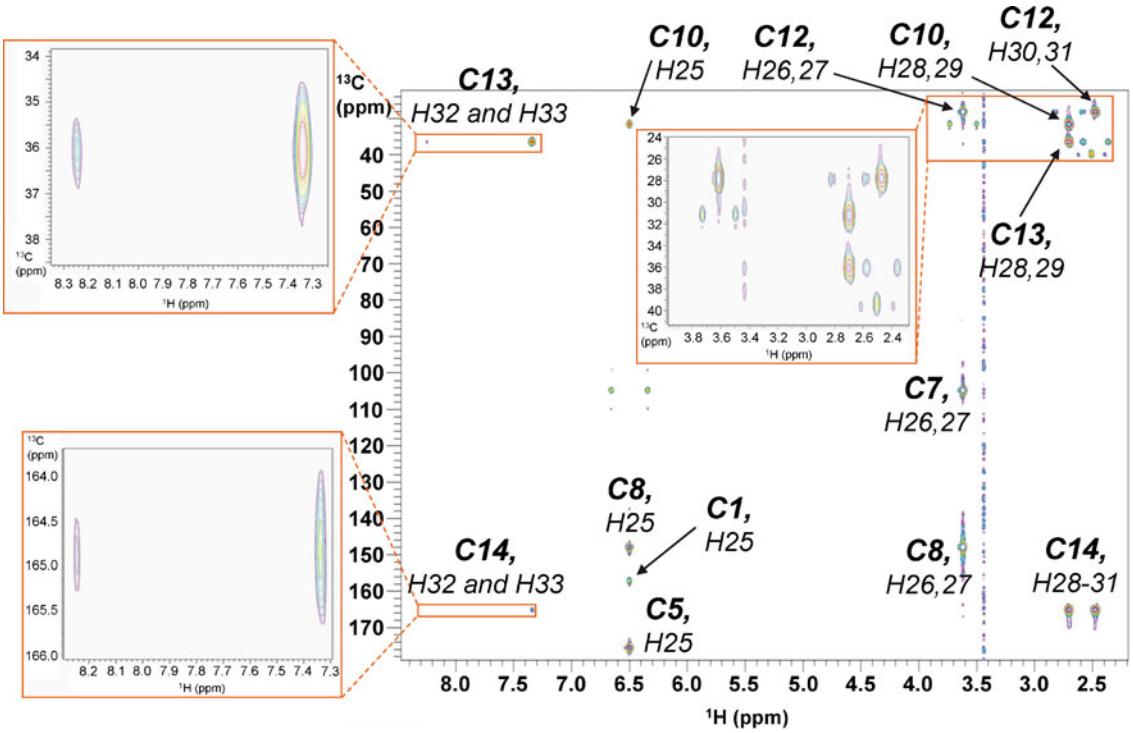

Fig. 4 600 MHz $^{1}H/^{13}C$ gHMBC spectrum of ca. 0.4 M famotidine in DMSO-d_6. Regions of the aliphatic signals and the peaks confirming the assignment of *H32* and *H33* are enlarged in *grey boxes*

erroneous assignment of all the aliphatic ^{13}C signals. Using HF/6-31++G(2d,p)//B3LYP/6-31++G(2d,p), our calculated chemical shifts matched the correct order of all aliphatic ^{13}C signals (Table 1). The difference probably originates form the optimized geometries: Starting from the folded conformation of the known crystal structure of polymorph "*B*", our geometry optimization resulted in an extended conformation (see Electronic supplementary material Fig. S4) as opposed to the folded conformer in the work of Baranska et al. [6].

Several different calculated conformers of famotidine have been published. On the basis of their pharmacophore model, Ishida et al. suggested a folded conformation with an intramolecular hydrogen bond between the *N15* amidine and *N9* thiazole nitrogens [14]. In their paper, the temperature-dependence of the *H25* thiazole proton in D_2O was also ascribed to this connection, however, the *N2/3-N9* hydrogen bond is more likely the cause of this phenomenon. The semi-empirical calculations of Olea-Azar et al. established the latter interaction as the structures with *S6-C5-N4-C1* dihedral angle=180° were the most stable [15]. On the other hand, their calculation disproved the amidine–thiazole hydrogen bond as variation of the *C14-C13-C12-C11* dihedral angle showed that extended conformers are the most stable. However, in the abstract of Baranska's paper [6], the optimized geometry was declared to be similar to the folded structure of famotidine in its "*B*" polymorphic form, where one of the sulfonamide NH_2 protons, *H34* or *H35* is involved in an intramolecular hydrogen bond with the *N2* or *N3* guanidine nitrogen atoms [5]. As their assignment is based explicitly on calculation, the interchanged assignment of the *N15* and *N20* nitrogens along with the corresponding *H32,33* and *H34,35* protons could be a result of the invalid structural hypothesis.

In our calculation, the order of the ^{13}C, ^{15}N, and most ^{1}H chemical shifts are in good agreement with our *ab initio* NMR assignments, although the calculated values generally underestimate the measured chemical shifts. Comparison of the two quantum-chemical calculations (see Table 1) shows that the calculated chemical shifts are significantly influenced by the optimized geometry. Moreover, solvent effects as well as the contribution of minor conformational states can also play a significant role. Thus, *in vacuo* calculation methods cannot be a substitute of NMR correlation experiments which provide unambiguous, *ab initio* assignment of famotidine possessing high number of hydrogen bond donor and acceptor atoms as well as considerable conformational freedom.

Consequences of the new assignment on the structure of famotidine in DMSO-d_6

Provided that famotidine exists predominantly in a folded conformer, this should be stabilized by hydrogen bond involving one of the *H34*/*H35* protons such as seen in the

Table 1 Experimental (in DMSO-d_6) and calculated (in vacuum) ^{1}H, ^{13}C, and ^{15}N chemical shifts of famotidine compared with values from literature [6]

	Measured	Calculated	Baranska et al. [6] calculated	Baranska et al. [6] measured
Reference	TMS	TMS	TMS	TMS
H30,31	2.46 (t. 2H)	2.21	2.68	2.47 (t. 2H)
H28,29	2.69 (t. 2H)	2.48	2.90	2.70 (t. 2H)
H26,27	3.61 (s. 2H)	3.50	3.82	3.62 (s. 2H)
H25	6.49 (s. 1H)	7.66	6. 95	6.59 (s. 1H)
H34,35	6.50 (s. 2H)	3. 88	**8.78** and **9.18**	**7.35 (s. 1H)** and **8.26 (s. 1H)**
H21-24	6.84 (brs. 4H)	5. 59	6. 83	6.84 (brs. 4H)
H33	7.34 (s. 1H)	8. 30	**7.04**	**6.56 (s. 2H)**
H32	8.24 (s. 1H)	6. 05	**7.04**	**6.56 (s. 2H)**
Reference	DMSO-d_6	TMS	TMS	DMSO-d_6
C12	27.9	21. 4	**28.2**	**31.8**
C10	31.3	30. 1	**29.1**	**36.7**
C13	36.2	35. 4	**25.0**	**28.4**
C7	104.5	110. 2	105. 5	105.7
C8	147.8	139. 1	137. 9	148.3
C1	157.0	157. 2	155. 9	157.5
C14	165.0	168. 4	166. 8	166.1
C5	175.4	183. 2	182. 9	177.5
Reference	CH_3NO_2, external, 380 ppm	CH_3NO_2	CH_3NO_2	ND
N2,3	76	69	72 and 79	78 (t)
N20	91	94	**107**	**110 (t)**
N15	107	95	**95**	**93 (t)**
N4	156	157	163	156 (s)
N16	212	210	215	210 (s)
N9	261	263	265	265 (s)

The *ND* abbreviation means that the data in question was not disclosed, while bold characters denote erroneous literature assignments

crystal structure of polymorph "*B*" [5] and supposed in the work of Baranska et al. [6]. Our unambiguous ^{1}H and ^{15}N assignment of famotidine, however, disproves the presence of the intramolecular hydrogen bond between the *H34* or *H35* and *N2* or *N3* atoms. Signals of two chemically non-equivalent NH_2 protons belong to the amidine *H32* and *H33* nuclei while *H34* and *H35* give one single sharp peak at relatively low chemical shift. In the case of an intramolecular hydrogen bond, they are expected to be either anisochronous or averaged out at a significantly larger chemical shift compared with the measured value. All this points to that the major conformer in DMSO-d_6 cannot be a folded structure. Also, the *H28,29* as well as *H30,31* protons appear as triplets ($^{3}J(^{1}H,^{1}H) \sim 7.6$ Hz) indicating that the rotation is free about the *C12-C13* bond which would not be expected in the folded structure.

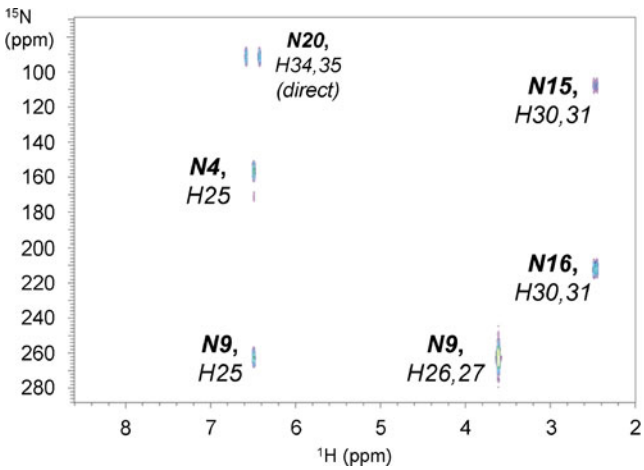

Fig. 5 500 MHz $^{1}H/^{15}N$ gHMBC spectrum of famotidine in DMSO-d_6

As the *H32* and *H33* protons give two signals (they are in slow exchange), the rotation about the *C14-N15* bond is slow on the chemical shift timescale. The lower frequency shift of *H33* relative to *H32* is probably caused by the shielding effect of the *N15-C14-N16* conjugated bonds. Our calculation suggests that this shielding effect would be compensated if *H33* participates in an intramolecular hydrogen bond with *O18* (see Electronic supplementary material Fig. S4) as the calculated chemical shifts of *H33* are larger

than that of *H32* (see Table 1). Therefore, our opposite assignments disagree with this H-bond being a strong and dominant interaction. Moreover, all the NH protons are in fast exchange on the relaxation timescale giving negative crosstalk in the NOESY spectrum (see Electronic supplementary material Fig. S3). Furthermore, deuterium exchange experiments could not differentiate between *H32* and *H33* both having immediate deuterium exchange to substantially the same degree (see Electronic supplementary material Fig. S5). One would expect significantly slower deuterium exchange with protons in strong H-bond [31].

Both literature data [5, 14, 15, 32] and our quantum-chemical calculations suggest the presence of an intramolecular hydrogen bond between the *N9* thiazole nitrogen and one of the guanidine NH protons, in which case, an energetically favorable six-membered ring would form. One might expect that the hydrogen involved has a higher chemical shift compared with the other guanidine protons. However, the guanidine NH_2 groups give one broad signal over coalescence, which indicates that rotations about the *C1-N4, C1-N2* and *C1-N3* bonds are fast on the chemical shift timescale in DMSO-d_6 at 25 °C. Thus, the lifespan of this hydrogen bond has to be short on the NMR timescale according to our experimental results.

The 2-guanidinothiazole moiety possesses widespread conjugation, one manifestation of which are the enhanced distant $^{5}J(^{13}C, ^{1}H)$ and $^{4}J(^{15}N, ^{1}H)$ couplings of *H25* shown by long-range correlations between *H25* and *C1* or *H25* and *N4* observed in the $^{1}H/^{13}C$ HMBC and $^{1}H/^{15}N$ HMBC spectra (see Figs. 4 and 5).

NMR studies of famotidine in aqueous solution

Islam and co-workers published first a protonation constant of famotidine (log $K \sim 6.9$) at 23 °C, based on spectrophotometric, solubility, and partitioning experiments [23]. At 37 °C and 0.5 M ionic strength, they published a slightly decreased value of 6.60, however, further protonation steps or the sites involved were not mentioned. Two years later, Crisponi et al. determined log $K=6.87$ by potentiometry for the same protonation step [24]. The supposed site of protonation was the thiazole *N9* atom based on ^{1}H NMR spectra measured in D_2O at different pD values. This conclusion was drawn from the chemical shift changes of *H25* and *H26,27* being larger than those of *H28,29* and *H30,31*, which were hardly affected. Duda et al. published three protonation constants of famotidine at 25 °C and $I=0.1$, and they also assigned them to basic centers—log $K_1=11.12$, sulphonamidate (*N20*); log $K_2=6.71$, thiazole (*N9*); log $K_3=1.48$ guanidine (*N2* or *N3*) [25]. This is somewhat surprising as guanidine itself is more than 10 log *K* units stronger base than thiazole (protonation constants are 13.6 [33] and 2.5 [34, 35], respectively). They supposed the above sequence of protonation on the basis of previous literature data dealing with the effect of substitution on the basicity of guanidine [36, 37] and ^{13}C NMR spectra measured in D_2O at two different pH^* values. In their published spectrum at $pH^*=0.49$ two ^{13}C signal sets arise, the largest splitting being observed for the peak around 171 ppm. They assumed this peak to be the *C1* guanidine carbon atom and supposed that two famotidine isomers exist in strongly acidic solution as a consequence of guanidine protonation. However, our aqueous ^{13}C HMBC experiment (see Electronic supplementary material Fig. S6) reveals that the peak around 171 ppm belongs to *C14* instead of *C1*. Furthermore, the duplication of all ^{13}C signals could result from formation of a degradation product owing to the hydrolysis of famotidine during their experiment lasting for several hours. The decomposition process of famotidine to *famotidone* (Fig. 1) in acidic medium has been described in several papers [1, 27, 28].

There are literature data suggesting a different protonation order of famotidine and related structures. Button et al. declared the *N4* guanidine and *N9* thiazole nitrogen atoms as basic centers in 2-(4-methyl-1,3-thiazol-2-yl)guanidine with protonation constants of log $K_1=7.05$ and log $K_2=-0.55$, respectively [26]. These results are in agreement with the semi-empirical calculations of Olea-Azar et al. [15] for 2-(1,3-thiazol-2-yl)guanidine and famotidine as well as the crystal structure of famotidine hydrochloride known since 1987 [14, 32].

It is noteworthy that none of these papers are concerned with the protonation of the amidine group of the molecule, which was first considered as a basic center of famotidine by Baranska et al., where the first protonation step was assigned to the sulfonamidate or to the amidine group [7].

Decomposition of famotidine under acidic conditions

In order to rule out the existence of a famotidine isomer in acidic solutions as suggested by Duda et al. , 1D ^{1}H and ^{13}C as well as ^{13}C gHMBC spectra were measured on famotidone itself and on its mixture with famotidine at pH^* 0.49. The resulting ^{13}C spectra are shown in Fig. 6. The observed ^{13}C chemical shifts of the mixture were in good agreement with the two set of signals detected at pH^* 0.49 in the work of Duda et al. [25], and the pure famotidone spectrum allows assignment of the respective signals. Our results prove that the additional set of signals in the work of Duda et al. [25] arose from famotidone, i.e., the acidic decomposition product of famotidine. The decomposition process was also confirmed by 1D ^{1}H NMR experiments shown in Electronic supplementary material Fig. S7. Considering the acidic degradation and limited solubility of famotidine, recording a series of HMBC spectra (see section "The protonation sites in famotidine") is more feasible to observe its

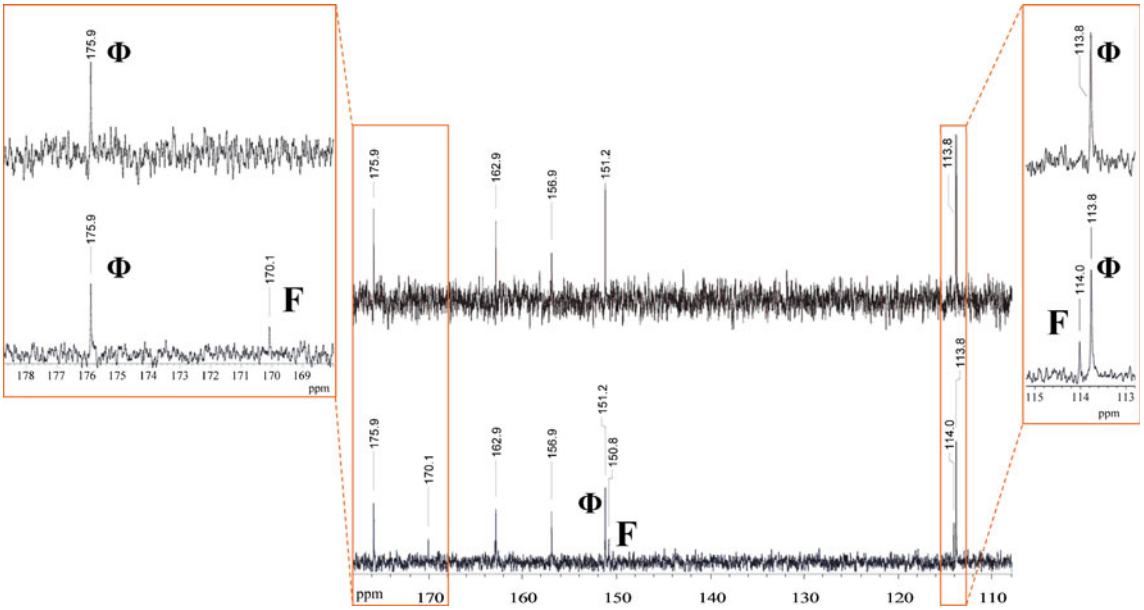

Fig. 6 Higher frequency regions of the 150.9 MHz ^{13}C spectra of famotidone (*upper trace*) and its mixture with famotidine (*lower trace*), peaks of the compounds are indicated with the symbols *Greek letter phi* (famotidone) and *F* (famotidine)

pH-dependent ^{13}C NMR behavior than a time-consuming series of ^{13}C spectra.

pH-dependence of famotidine ^{1}H spectra in aqueous medium

In water above pH 5, the famotidine ^{1}H NMR spectrum consists of only four peaks corresponding to the carbon-bound protons, since the NH protons are in fast exchange with the solvent.

Assignment of the four carbon-bound protons was carried out in analogous way as described for DMSO-d_6 solutions (section "The assignment of famotidine NMR signals in DMSO-d_6"). The measured chemical shifts at different pH values are shown in Fig. 7, together with computer-fitted titration curves while the derived protonation constants are listed in Table 2.

On the basis of Fig. 7, two types of chemical shift-pH profiles can be distinguished: Protons on the two sides of *S11* sulfur atom exhibit markedly different pH-dependent behavior. The thioether sulfur atom separates the molecule into two parts in terms of protonation effects, so the ^{1}H chemical shifts on the one side are barely affected by the protonation processes of the other side. These two constituents of famotidine are hereafter referred as units "*S*" and "*G*" containing the sulfamoyl amidine and the guanidinothiazole moieties, respectively (see Fig. 7).

Chemical shifts of the two methylene groups in unit *S* (*H28,29* and *H30,31*) are affected significantly by the protonation step characterized by the macroconstant log K_1= 11.27. This protonation step can therefore be assigned to the *N*-sulfamoyl amidine moiety. The chemical shift-pH profiles of the *G* (*H25* and *H26,27*) proton signals are overwhelmingly influenced by the protonation step with the macroconstant log K_2=6.80, thus involving the 2-guanidinothiazole moiety. Figure 7 shows that these two protonation steps are separated not only from each other (by 4.5 pH units) but also from the subsequent steps in acidic milieu.

Although the respective carbon-bound protons are highly selective for the acid–base reactions within the *S* or *G* units, small protonation shifts still occur upon "remote" protonations as well (see Table 3 for protonation shifts). *H25* and *H26,27*, for example, exhibit a small inflection upon the first protonation step at pH=11.27, but the observed $\Delta\delta$ value is significantly larger in the second step. The opposite trend is found for *H28,29* and *H30,31*. The direction of the 'minor' protonation shifts is remarkable, since all of these are changes to lower frequencies with decreasing pH except for *H30,31*. Such "wrong-way" shifts are not without precedent, especially when oxygen is the basic center [38–44]. In famotidine, however, all the donor atoms are nitrogens.

The third and fourth protonation steps of famotidine occur in highly acidic medium. All carbon-bound protons have significant chemical shift changes in this pH-range (see Fig. 7). Chemical shift changes of *H28,29* and *H30,31* start at pH~2 and tend to reach a plateau in solutions more concentrated than 2 M HCl. For *H25* and *H26,27*, however, the chemical shift change begins around pH=1, and the measured values increase steeply even in 4 M HCl solutions. These observations suggest that two protonation processes occur in an overlapping manner: in the *N*-sulfamoyl amidine

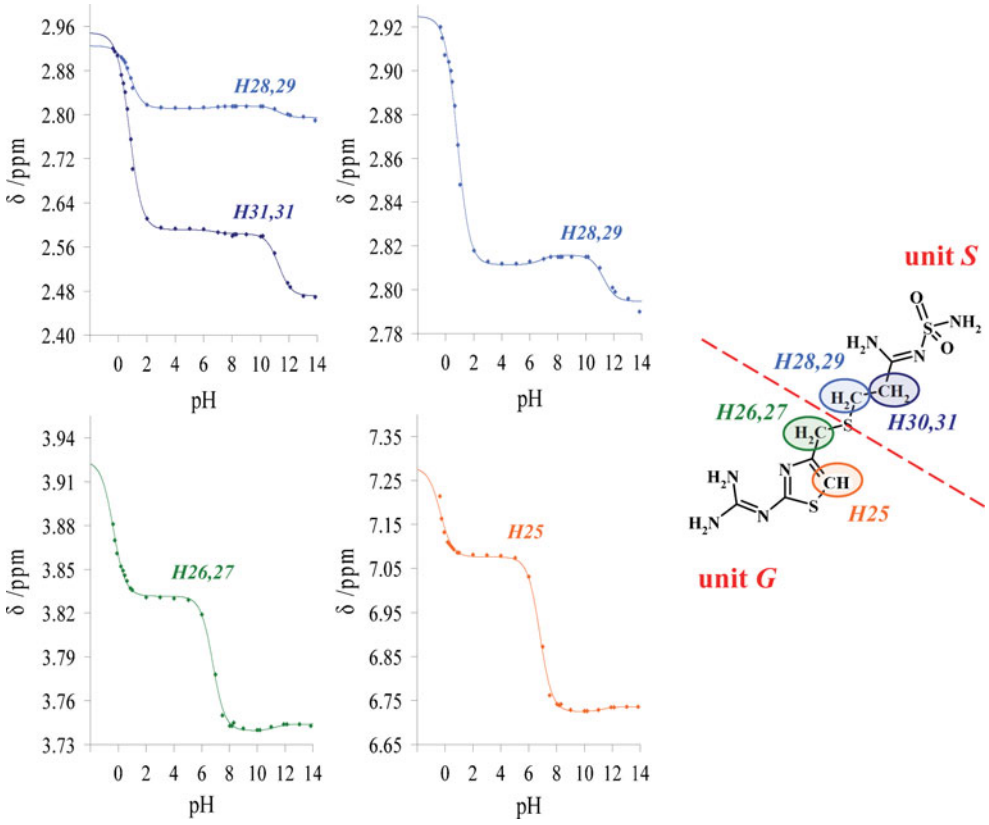

Fig. 7 Chemical shift versus pH profiles of the carbon-bound protons of famotidine in H_2O/D_2O 95/5 *v/v*.% at 25 °C and 0.15 ionic strength

moiety affecting the chemical shifts of *H28,29* and *H30,31;* and in the 2-guanidinothiazole moiety, for which the *H25* and *H26,27* protons are sensitive. Computer fitting provided the protonation macroconstants of log K_3=0.81 and log K_4=−0.35, respectively. Note that, in Fig. 7, the measured NMR titration curves are plotted against pH, however, in solutions with [HCl]>1 M (where hydrogen ion concentration and activity deviate significantly), the depicted value means –log[HCl] rather than the usual activity-based pH. In the latter solutions, therefore, K_4 is a concentration constant while K_3 to K_1 are "mixed" constants (see Eq. 2). The value of K_4 agrees with the literature constant (−0.55) for the second protonation step of 2-(4-methyl-1,3-thiazol-2-yl) guanidine [26].

Table 2 The protonation constants of famotidine determined from ^{1}H NMR-pH titrations with nonlinear parameter fitting for three steps

	Fitted constant	Error	Predicted pK_a values
logK_1	**11.27**	±0.09	9.29 (*N20*)
			Diff., −2.07
logK_2	**6.80**	±0.09	8.37 (*N4*)
			Diff., +1.57
logK_3	0.79 (*H30,31*)	±0.09 (*H30,31*)	0.64 (*N16*)
	0.83 (*H28,29*)	±0.12 (*H28,29*)	Diff., −0.17
	Average, 0.81		
logK_4	−0.35 (*H26,27*)	±0.30 (*H26,27*)	–
	−0.34[a] (*H25*)	±0.09 (*H25*)	
	Average, −0.35		

The logK_1 and logK_2 values were declared as common for all the four nuclei, while a third constant was fitted independently. Predicted centers of protonation and pK_a values (in the case of basic groups, for the conjugated acid) using the Marvin program are also listed

[a] The acidic limiting chemical shift value of this nucleus was fixed to 7.280 ppm during the fitting process

Since *S11* largely inhibits the inductive communication between the *S* and *G* units, the *H30,31* and *H28,29* nuclei are only slightly responsive to the fourth, while *H25* and *H26,27* to the third step, although these steps are very close in terms of log *K* values. These facts also imply that determination of the famotidine protonation macroconstants would be ill-conditioned by using a four-step fitting even if all the four NMR-pH datasets were applied simultaneously. Instead, a three-step fitting was carried out where the first two constants were declared as common values for all nuclei, while the third one was fitted independently. The latter value fitted for *H30,31* and *H28,29* agreed within error (see Table 2); their average was considered as log K_3. The fitted values for *H26,27* and *H25* are also in perfect agreement thus giving log K_4. (The limiting acidic plateau of the *G* structural unit could not be reached experimentally, resulting in the

Table 3 The measured and fitted ^{1}H chemical shifts in certain protonation states ("limiting chemical shifts") of famotidine and the fitted chemical shift changes ($\Delta\delta$)

Nucleus	δ_L/ppm	δ_{HL}/ppm	δ_{H_2L}/ppm	δ_{H_3L}/ppm	δ_{H_4L}/ppm
H30,31 meas.	2.469	2.583	2.593	2.920	–
H30,31 fitted	2.471±0.004	2.584±0.003	2.591±0.003	2.948±0.005	–
$\Delta\delta$	–	+0.113	+0.007	+0.357	
H28,29 meas.	2.790	2.815	2.812	2.920	–
H28,29 fitted	2.795±0.003	2.816±0.002	2.811±0.003	2.925±0.005	–
$\Delta\delta$	–	+0.021	−0.005	+0.114	
H26,27 meas.	3.743	3.740	3.831	–	–
H26,27 fitted	3.744±0.003	3.740±0.002	3.832±0.003	–	3.924±0.037
$\Delta\delta$	–	−0.004	+0.092	–	+0.092
H25 meas.	6.736	6.726	7.079	–	–
H25 fitted	6.736±0.003	6.724±0.003	7.077±0.002	–	7.280 (fixed)
$\Delta\delta$	–	−0.012	+0.353	–	+0.203

Because of the three-step fitting method detailed in the text, no values were obtained for logK_4 or logK_3 in the case of *H28-31* and *H25-27*, respectively

considerably big error of log K_4 for *H26,27*. The calculated uncertainty of *H25* acidic shift is much lower, however, its supposed limiting value had to be fixed during the fitting process.) Figure 7 shows that all four titration curves could be fitted adequately using this evaluation strategy.

The protonation sites in famotidine

Although the $\Delta\delta$ ^{1}H NMR protonation shifts and chemical considerations suggest the protonation sites, ^{13}C NMR-pH titration and pK_a predictions were also performed for an unequivocal characterization. The ^{13}C NMR-pH titration was carried out by recording a pH-dependent series of 2D ^{1}H/^{13}C HMBC spectra. The chemical shift-pH profiles which show the largest protonation shifts are depicted in Fig. 8. The log$K$ values obtained by computer fitting between pH 13 and 2 match the ones measured in this range by ^{1}H titration (corresponding data are listed in Electronic supplementary material Table S2).

The first and third protonation steps involve the *N*-sulfamoyl amidine moiety. The $\Delta\delta$ protonation shifts of *H28,29* and *H30,31* are larger in the acidic (third) step than in the alkaline (first) one about five and three times, respectively (see Table 3). Thus, log K_1=11.27 belongs to the farther located sulfonamidate (*N20* NH^-) group (reflected also by *C14* in Fig. 8), while log K_3=0.81 refers to the closer amidine moiety (*N16* or *N15*). Chemical considerations and pK_a prediction (*N15*: pK_a=−8) indicate that protonation of *N15* is probable in concentrated acids only. Hence, the third H^+ is attached most probably to *N16*.

The second and fourth protonation steps occur on the 2-guanidinothiazole part of famotidine. Its two formal constituents are the thiazole ring (expected to protonate on *N9*) and the guanidine group (expected to protonate on *N4*). The

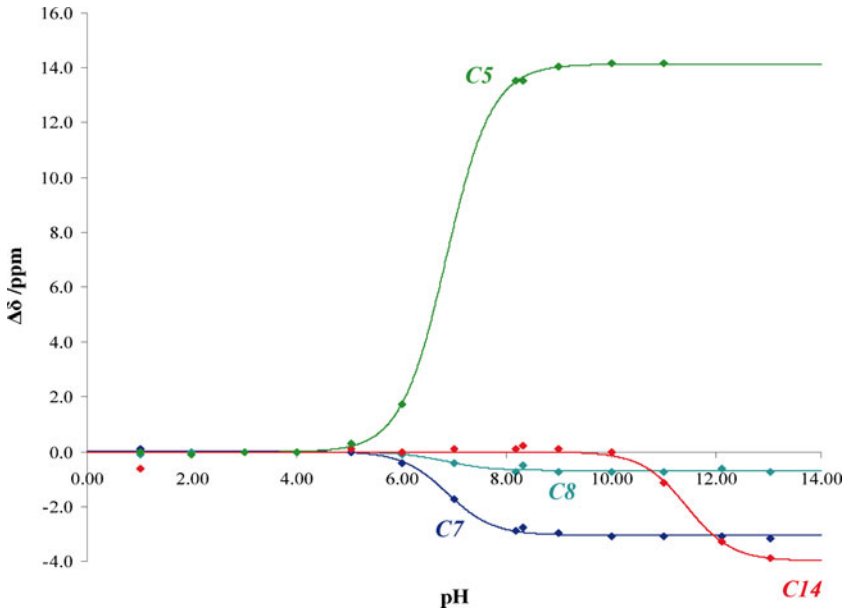

Fig. 8 pH-induced ^{13}C chemical shift changes (relative to pH=4) of selected famotidine carbons

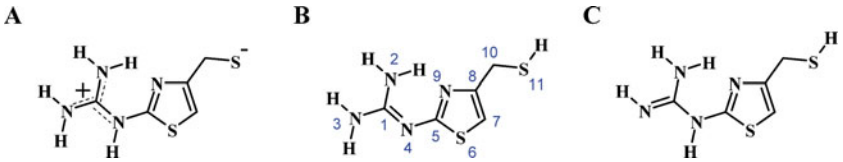

Fig. 9 Structure and numbering for 2-[4-(sulfanylmethyl)-1,3-thiazol-2-yl]guanidine (STG), the model compound of the *G* structural unit of famotidine in its neutral form (**B**), along with the other two possible tautomeric forms being formed in its first protonation process: the zwitterionic (**A**) and 'tau-neutral' forms (**C**). (The optimized geometries of the different tautomers are shown in Electronic supplementary material Fig. S9)

conjugation effects in this complex functional group remarkably influence the basicity of the individual donor atoms of the parent groups. ^{1}H NMR titration data cannot unequivocally identify these basic sites, for example, the fitted $\Delta\delta$ in the fourth step for *H26,27* is identical to that in the second (see Table 3). The ^{13}C titration profile yields more detailed information about the *G* structural unit, although it is known that the relationship between the extent of protonation-induced ^{13}C chemical shift changes and the distance from the protonation site cannot always be so easily interpreted as in ^{1}H NMR [45–47]. Between pH 13 and 2, chemical shift-pH profiles (see Fig. 8) of the thiazole carbon atoms (*C8*, *C7*, and *C5*) were found to be selective to the second protonation step. In the course of protonation, *C8* and *C7* show moderate chemical shift change to higher frequencies (0.7 and 3.0 ppm, respectively), while *C5* shifts 14.1 ppm to lower frequency (see Fig. 8 and Electronic supplementary material Table S2). This large shift shows that the second protonation occurs at *N4* as the protonation of *N9* cannot result in an order of magnitude difference in the shifts of the neighbouring *C8* and *C5* atoms. In conclusion, protonation with log K_2=6.8 occurs at *N4* and log K_4~ −0.35 refers to protonation at *N9*.

This basicity order is in agreement with the predicted pK_a value for *N4* (see in Table 2) and reveals that conjugation of the guanidine with the thiazole ring results in a moiety of highly communicative atomic arrangement. The above assignment of the second protonation site disproves the corresponding data published by Crisponi et al. [24], Duda et al. [25], and Baranska et al. [7]. However, it is in accordance with the crystal structure of famotidine hydrochloride [14, 32], the results on 2-(4-methyl-1,3-thiazol-2-yl)guanidine [26], and semi-empirical calculations on the 2-(1,3-thiazol-2-yl)guanidine molecule by Olea-Azar et al. [15].

Table 4 Relative energies of the different protonation isomers of STG, in kilocalorie per mole units

Species	ΔE(gas)	ΔG(298, gas)	ΔG(tot, gas)
Zwitterionic	42.71	0.29	43.00
'Tau-neutral'	12.55	−0.14	12.41
Neutral	0	0	0

Our results regarding STG, a model compound of the *G* structural unit of famotidine (Fig. 1), provide further evidence for the above protonation sequence, including the *N4* and *N9* basic sites. According to ^{1}H NMR titrations of STG carried out between pH 1 and 13, the compound shows two protonation steps with log *K* values of 9.25 and 6.69. The former value can be assigned unambiguously to the thiolate, based on the measured chemical shift versus pH profiles (see Electronic supplementary material Fig. S8). Provided that the second step (log *K*=6.69) belongs to the guanidine group, the thiazole nitrogen should protonate at pH<1. Alternatively, assuming that log *K*=6.69 characterizes the thiazole ring than the guanidine should protonate at pH> 13.0. Since both alternatives occur outside the studied pH interval, no decision can be made experimentally.

To resolve this ambiguity, energies of the three possible tautomers (Fig. 9) formed in the first protonation step were computed and compared. The neutral form is proved to be the most stable (see Table 4), with 12.41 and 43.00 kcal/mol less free energy than the 'tau-neutral' and zwitterionic forms, respectively. The zwitterionic form is energetically the least favorable, since the electrostatic orientation effect of thiolate anion turns the guanidine group out of the plane of the thiazole ring (the *N9-C5-N4-C1* dihedral angle is 43.8°). Contrary to that, in the neutral tautomers, the guanidine part is almost co-planar with the thiazole ring—the *N9-C5-N4-C1* dihedral angle is 4.7° and 0.9° in the 'tau-neutral' and neutral forms, respectively. Comparing these dihedral angles with the corresponding energy levels in Table 4, the huge effect of co-planarity on the stability of the 2-guanidinothiazole moiety is obvious.

These results show that, in STG, the thiolate is the most basic site and the guanidine group protonates at lower pH, in accordance with the predicted pK_a values (9.85 for the thiole, 8.60 for *N4*). Thus, the STG log K_2=6.69 is assigned to *N4*, confirming our suggested protonation sequence for the parent famotidine.

Conclusions

Although famotidine has been known for a long time, its previous NMR spectral assignments were either incomplete

or incorrect. Based on 1D and 2D NMR experiments, complete *ab initio* multinuclear assignments are given here, rectifying the errors that infiltrated into the literature. We have shown that two-dimensional NMR correlation experiments are essential for unambiguous assignment of famotidine since calculated chemical shifts are strongly affected by the optimized geometry. Moreover, as the molecule contains numerous hydrogen bond acceptor and donor moieties, solvent effect also plays a significant role which is indicated by the differences between the *in vacuo* calculated and measured chemical shifts.

The previous invalid hypothesis regarding the solution state conformation of famotidine led to interchanged assignment of the *H32*, *H33* and *H34*, *H35* protons. The present assignment is based solely on NMR experiments, and it does not need structural presumptions. In fact, on the contrary, it indicates the conformation in DMSO. Our results show that the *H34,35* sulfonamide hydrogens give a common signal, and they do not participate in an intramolecular hydrogen bond, which also disproves a folded major conformation proposed earlier. Therefore, the molecule exists predominantly in an extended conformation.

According to our new assignment, the *H32* and *H33* protons are anisochronous due to slow rotation about the *C14-N15* bond. *In vacuo* calculations indicate an intramolecular hydrogen bond involving *H33* and *O18*. However, neither chemical shift considerations nor deuterium exchange experiments support this interaction. Another finding is that the lifespan of the previously established intramolecular hydrogen bond between the *N9* thiazole nitrogen and one of the guanidine NH protons is short on the NMR chemical shift timescale.

Our work revealed that famotidine has four protonation steps in aqueous medium, of which no complete characterization was given previously. The molecule bears a negative charge above pH=11.3, where the sulfonamidate moiety protonates. The compound remains uncharged until pH=6.8, where the second protonation occurs. In this step, the *N4* guanidine nitrogen protonates and famotidine carries one positive charge until pH=0.8. Consequently, at physiological pH values, the compound exists in two protonation forms, one with the uncharged form being predominant over pH=6.8 while at lower pH the other species of positive charge at *N4* prevails. Thus, both species could exist in different tissues, cells, or subcellular organelles depending on their actual pH. Therefore, it is probable that the protonation or deprotonation of the *N4* nitrogen has an important role in the binding of famotidine to the H_2 histamine receptor and its activation. In strongly acidic medium, both the *N16* amidine and the *N9* thiazole nitrogens protonate, the former process representing the major protonation pathway. However, these two steps overlap, so the speciation of the two H_3Famo^{2+} protonation isomers could be properly characterized only using microscopic equilibrium constants.

The previous incorrect conclusion stating that due to protonation at extreme pH values two famotidine isomers are formed and detected simultaneously in ^{13}C NMR spectra is refuted. Our 1D ^{1}H,^{13}C and 2D $^{1}H/^{13}C$ HMBC experiments show that decomposition of famotidine to famotidone in strongly acidic medium is responsible for the two sets of NMR signals.

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