Study of the protonation (methylation) position and tautomeric structure of thiopyrimidine derivatives by 2D $^{1}H—^{15}N$ NMR HSQC/HMBC. Experimental approach and theoretical modeling*

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Two-dimensional $^{1}H—^{15}N$ NMR HSQC/HMBC experiments enable the unambigous determination of the protonation (methylation) position and tautomeric structure of nitrogen-containing heterocycles. In investigated thiopyrimidines protonation (or methylation) occurs at the N(1) atom of the pyrimidine ring. The tautomeric structures of these compounds were established based on the analysis of $^{1}H—^{15}N$ NMR spectra. *Ab initio* calculations of chemical shifts (GIAO B3LYP/6-31G(d)//HF/6-31G) are in full agreement with experimental values. The stability of various protonated (methylated) and tautomeric species is explained in terms of a thermodynamic approach.

Key words: 2D NMR correlation experiments, ^{15}N, GIAO DFT calculation of chemical shifts, thiocytosine, protonation.

The reactions of proton transfer play a major role in chemical and biomolecular processes.[1,2] Protonated states of chemical compounds, for instance, nucleic bases are directly related to their biomolecular functions. Accordingly, the interest in the protonation phenomenon is kept up, particularly in the determination of its direction and stability of various tautomer forms.[3—5] Protonation can be regarded as a "soft" method of preparation of molecules to the formation of noncovalent and covalent bonds. Furthemore, it attracts attention that many nitrogen-containing biologically active compounds possess at least one charged nitrogen atom.[6] In this connection it is important to determine the position of protonation (alkylation)** and tautomer structure of a charged molecule reliably.

The necessity for the protonation control of such compounds resulted in the development of a whole range of experimental approaches based on spectroscopic methods. UV and IR spectroscopies are widely used for these purposes.[7,8] The same way data on chemical shifts (CS) in ^{1}H NMR spectra are employed for the determination of the protonation position on the basis of their changes. However these methods are lacking in the sensitivity to changes in electronic structure upon a proton addition, and sometimes there is no unambiguous relation between changes in spectral parameters and the protonation position.[9,10] In ^{13}C NMR spectra CS are more sensitive to the protonation of a carbon atom, but not the nitrogen. At the same time ^{15}N NMR could be the most reliable method for studying the protonation at the nitrogen, however, in most cases an ^{15}N one-dimensional spectrum cannot be observed at a natural isotope abundance.[11,12] Revolutionary opportunities opened up with the development of inverse heterocorrelation methods ($^{1}H—^{13}C$ and $^{1}H—^{15}N$) which make it possible to increase the sensitivity by two to three orders of magnitude [13]. This provides a fairly reliable and efficient method of a direct determination of the protonation site.

In recent years new biologically active systems, which are obtained by spacial prearrangement of active components, for example, nucleic bases with the aid of spacers of different rigidity,[14] are being intensively developed. Examples of such systems are macrocyclic derivatives of thiopyrimidines (type **I**). According to preliminary experiments, the protonation plays an important role in the intra- and intermolecular packing of such macrocycles in solutions. It was these considerations that gave rise to this work.

In this paper findings on the predominant position of the protonation of the thiopyrimidine derivatives **1** and **2** are presented based on investigations by inverse heterocorrelation NMR spectroscopy methods. Along with the

* Dedicated to Academician A. I. Konovalov on his 75th birthday.
** For the sake of brevity further on the term "protonation" will only be used, by which methylation is also implicated if not stated otherwise.

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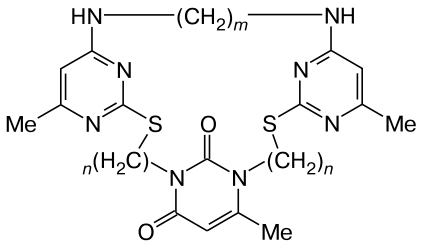
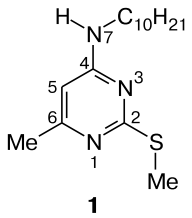


I

determination of the protonation position, we examined the scope and limitations of *ab initio* methods for the calculation of chemical NMR shifts for the identification of a tautomeric structure. This article also performs an analysis of the possibilities of a thermodynamic model for the description of the observed pattern of protonation and tautomeric structure.



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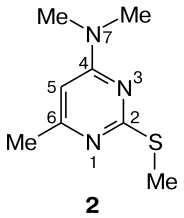

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Experimental

4-(*n*-Decylamino)-6-methyl-2-methylthiopyrimidine (**1**) was synthesized by the following sequence of reactions: 1) the amination of 6-methyl-1,2,3,4-tetrahydropyrimidine-2,4-thione with *n*-decylamine in *n*-nonyl alcohol and 2) the methylation of the S atom at the atom C(2) of the pyrimidine ring of 4-(*n*-decylamino)-6-methylpyrimidine-2-thione produced at the first stage with dimethyl sulfate in water in the presence of NaOH. The composition and structure of the isolated product **1** were proved by elemental analysis, mass spectrometry and NMR spectroscopy data. 4-Dimethylamino-6-methyl-2-(methylthio)pyrimidine (**2**) was prepared according to a known procedure.[15]

The protonation of pyrimidines **1** and **2** was carried out by adding a corresponding amount of freshly distilled trifluoroacetic acid (Lancaster) to a solution of compound **1** or **2** in $CDCl_3$.

The methylation of pyrimidines **1** and **2** was performed by stirring compound **1** or **2** in methyl *p*-toluenesulfonate during 5 h at 100 °C, after which the reaction mixture was cooled down, diethyl ether was added, and the formed precipitate was filtered off.

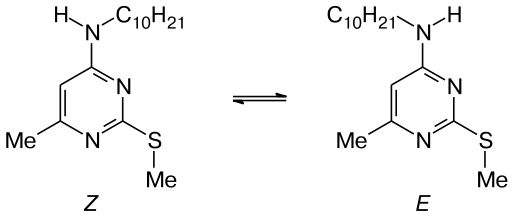
The 1D and 2D NMR experiments (1D NOESY, 2D COSY, 2D HSQC, 2D HMBC) for the studied compounds ($CDCl_3$, C = 0.1 mol L^{-1}) were carried out on a Bruker Avance-600 NMR spectrometer (600.13 (^{1}H), 150.90 (^{13}C) and 60.91 MHz (^{15}N)). The signals of Me_4Si (δ_H 0.00 and δ_C 0.0) and MeCN (δ_N 235.50) were used as the internal standards.

The calculations of ^{13}C and ^{15}N NMR CS were perfomed at the B3LYP/6-31G(d)//RHF/6-31G level with the use of the Gaussian 98[16] software package. The Gibbs free energies were calculated at the same level of theory under standard conditions (T = 298.15 K, P = 1 atm).

Results and Discussion

The determination of the structures of the neutral compounds **1** and **2** and cations produced upon their protonation (H^+-**1**, H^+-**2**) and methylation (Me^+-**1**, Me^+-**2**), was carried out on the basis of ^{1}H, ^{13}C and ^{15}N 1D/2D NMR correlation experiments 2D COSY, HSQC (^{1}H—^{13}C and ^{1}H—^{15}N) and HMBC (^{1}H—^{13}C and ^{1}H—^{15}N). At room temperature for the neutral molecules the conformation exchange is fast in the NMR scale due to rotation around the C(4)—N(7) bond (the rate constant $k = 1.2 \cdot 10^4$ s^{-1} at T = 303 K) (Scheme 1) and, therefore, all experiments were conducted at T = 233 K under the conditions of a slow exchange in the NMR time scale.

Scheme 1



Z *E*

Starting with the H(5) proton the structure of the whole compound was established nonempirically. The basic correlations are presented in Fig. 1. The conformational structure was determined based on NOE experiments, according to which in the dominating (60%) *E* conformer the interactions H(5)—C(8)H_2 take place, whereas in the minor *Z* conformer the H(5)—NH interactions occur. In Table 1 data for the *Z* form are given since it is this form that dominates in charged molecules, its CS were employed in further analysis.

Theoretically in the context of prototropy the existence of four tautomers of compound **1** (Scheme 2) is possible, which are in euqilibrium. According to the ^{1}H—^{15}N HMBC correlations between C(8)H_2, C(9)H_2 and N(7), between H(5) and N(3), between C(6)H_3, H(5) and N(1) corresponding nitrogen atoms were unambigously determined. Next from the presence of a cross-peak between the proton of the NH fragment and the N(7) atom in the ^{1}H—^{15}N HSQC spectrum and the absence of corresponding cross-peaks with the atoms N(1), N(3), and C(5) (in the ^{1}H—^{13}C HSQC spectrum), the structure of the dominated tautomer of compound **1** was unambiguously determined (**A**, Scheme 2), while the presence of other tautomers (**B—D**) in solution was not detected.

The protonation of compound **1** with trifluoroacetic acid in $CDCl_3$ (the ratio of **1** : CF_3COOH is 1 : 1) resulted in an appreciable change in the ^{1}H NMR spectrum. A strong shift of the NH proton band to the area of low fields is observed (from 5 to 9 ppm). At low temperature (T = 233 K) in the ^{1}H NMR spectrum a new signal pre-

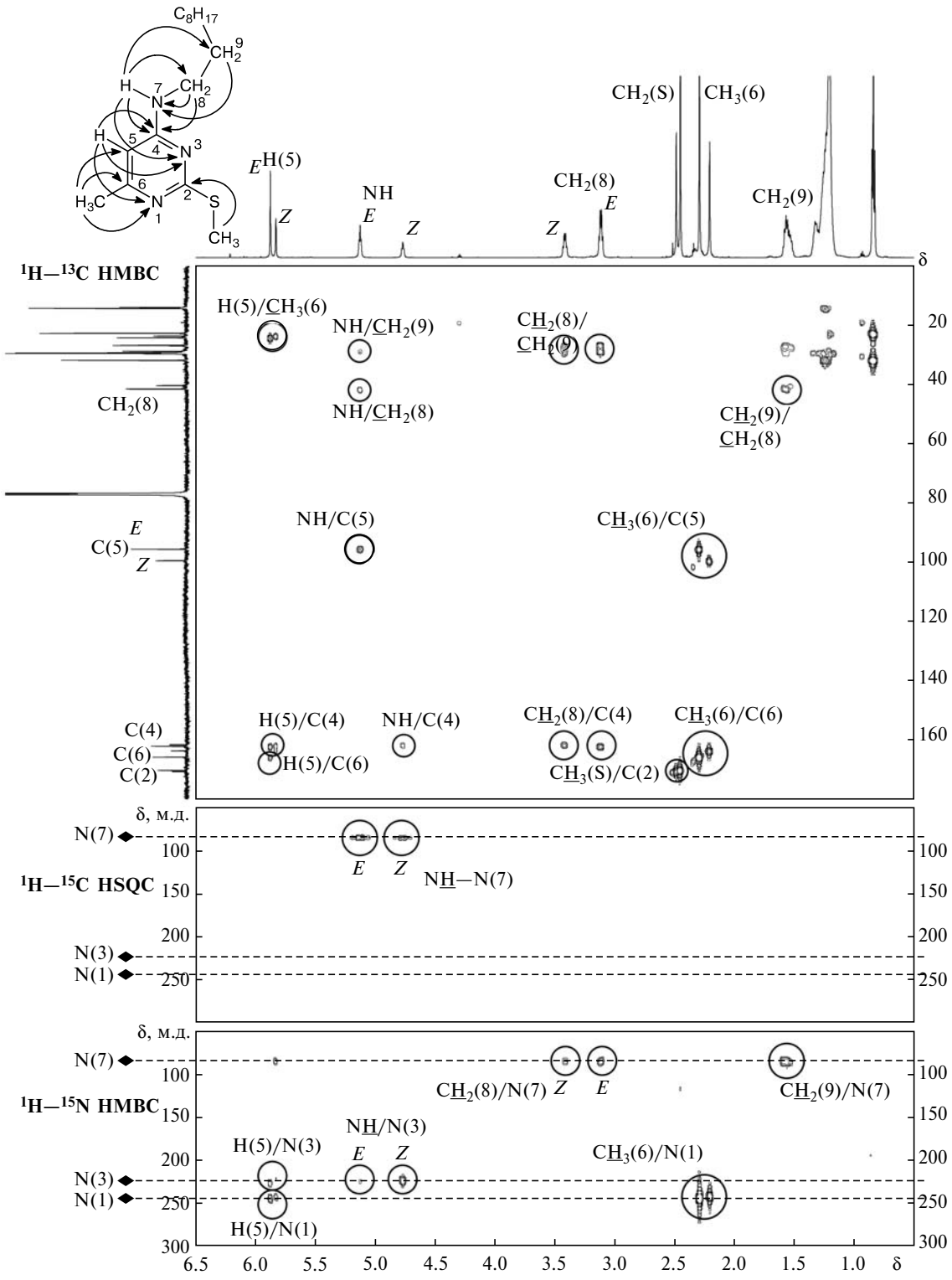


Fig. 1. 2D HMBC (^{1}H—^{13}C/^{15}N), HSQC ^{1}H—^{15}N spectra and key correlations for the neutral molecule **1** in the *Z* conformation.

sumably related to the N^+H proton appears in the region of 14 ppm. The methylation of compound **1** with methyl tosilate (MeOTs) (1 : 1) in $CDCl_3$ the same way affects the shift of the NH proton signal to low fields up to 10 ppm The influence of protonation (methylation) is also observed in the CS of ^{13}C NMR.

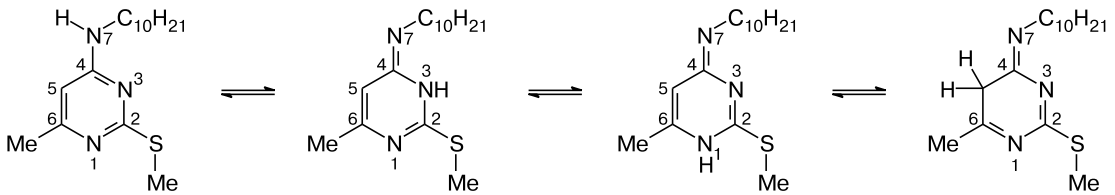
The proof of the structure of the cation generated upon the protonation of compound **1** was obtained from a

Table 1. Chemical shifts in the ^{1}H, ^{13}C and ^{15}N NMR spectra of the neutral compounds **1**, **2** and cations produced upon their protonation (methylation) in $CDCl_3$[a]

Nucleus	δ					
	1	H^+-**1**	Me^+-**1**	**2**	H^+-**2**	Me^+-**2**
H(C(5))	5.83	6.28	7.06	5.99	6.22	6.80
H(N(7))/CH$_3$(8)	4.77	8.89	9.76	3.02	3.26	3.24
CH$_3$´(8)				3.24	3.42	3.28
CH$_3$(C(6))	2.22	2.27	2.41	2.32	2.44	2.58
CH$_3$(S)	2.49	2.53	2.59	2.53	2.60	2.58
H($^+$N(1))/CH$_3$($^+$N(1))		14.15	3.64		14.18	3.67
C(2)	170.63	165.87	167.06	169.81	165.82	166.06
C(4)	161.67	160.81	158.91	161.57	160.45	158.26
C(5)	99.64	100.67	104.37	96.54	97.20	101.35
C(6)	163.76	152.48	153.63	164.46	155.98	157.10
CH$_3$(C(6))	23.65	18.23	20.12	24.06	18.85	21.18
CH$_3$(S)	14.09	13.48	15.47	14.12	13.49	15.85
CH$_3$($^+$N(1))			35.81			36.45
N(1)	241.50	154.50	146.67	238.00	155.98	149.44
N(3)	223.30	218.20	223.32	226.12	224.00	—[b]
N(7)	84.00	112.20	116.14	67.39	93.74	94.65

[a] The chemical shifts of the *Z* form, **1**, H^+-**1**, **2**, H^+-**2** at $T = 233$ K; Me^+-**1**, Me^+-**2** and ^{13}C for H^+-**2** at $T = 303$ K.
[b] The cross-peak for this line has not been detected, seemingly due to a low signal/noise ratio .

Scheme 2



data collection of 2D experiments $^{1}H—^{15}N$ HSQC (direct spin-spin coupling constants) and HMBC (remote spin-spin coupling constants). First, from the presence of the $^{1}H—^{15}N$ HMBC correllations from the protons C(8)H$_2$, C(9)H$_2$, H(5), Me(C(6)), and N(H) the signals of all the three nitrogen atoms in the molecule were identified (Fig. 2). Further, from the $^{1}H—^{15}N$ HSQC spectrum data the existence of a covalent bond between the proton with CS at 14 ppm and the N(1) nitrogen atom was unambiguously established. The key $^{1}H—^{13}C/^{1}H—^{15}N$ HMBC correlations are summarized in Fig. 2. It was unambiguously demonstrated that compound **1** is protonated at the position N(1).

In addition, ^{15}N NMR CS were measured, and it was shown that in the case of the methylated thiopyrimidine derivative the key correlations occur from N—Me to the vicinal carbon atoms C(2)/C(6) in the HMBC spectrum (Fig. 3).

The presence of a cross-peak between 6-Me and N(1) in the $^{1}H—^{15}N$ HMBC spectrum permits an unequivocal statement that the line of N(1) was assigned correctly. On the basis of these facts it was concluded that the given compound is methylated also at the position N(1).

In an analogous manner compound **2** was analyzed as well as its protonated (methylated) derivatives (see Table 1). For this compound protonation (methylation) occurs at the position N(1) as well.

Analysis of chemical shifts. Overally, as expected, the effect of protonation (methylation) is maximum for the CS of ^{15}N (see Table 1), and a strong high field shift is observed for the nitrogen atom at which the protonation occurs; to a lesser extent and in the opposite direction the shift for the exocyclic nitrogen atom occurs. These changes in CS provide evidence for the protonation (methylation) at the position N(1).

At the same time, for carbon and hydrogen atoms the effects are significantly smaller and, what is most important, these alterations in CS are hard to directly correlate with the attachment position. Accordingly, it is impossible to draw a conclusion only on the basis of a qualitative analysis of the changes.

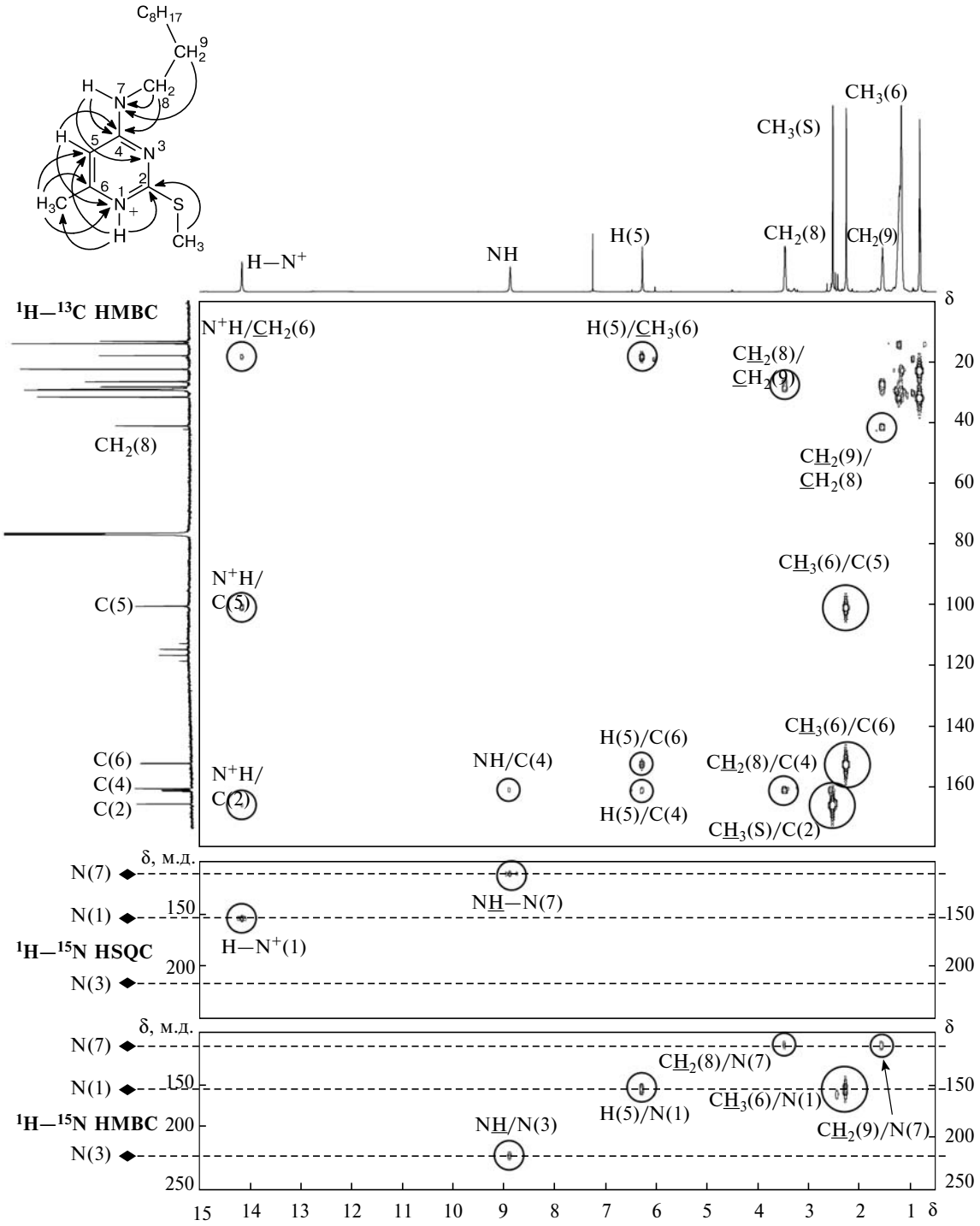


Fig. 2. The key correlations and 2D NMR ^{1}H—^{13}C HMBC, ^{1}H—^{15}N HSQC, and ^{1}H—^{15}N HMBC NMR spectra of the compound H^+-**1** at 233 K.

Recently *ab initio* estimation of CS for the analysis of these or those structural pecularities including tautomer structures[17,18] finds ever increasing diagnostic application. Hence it seems interesting to analyze the possibilities and limitations of such methods for the determination of the methylation (protonation) site and tautomeric composition in our case as well.

We carried out calculations of CS by a combination of the B3LYP/6-31G(d)//HF/6-31G methods (theory levels for the calculation of CS//geometry optimization) as

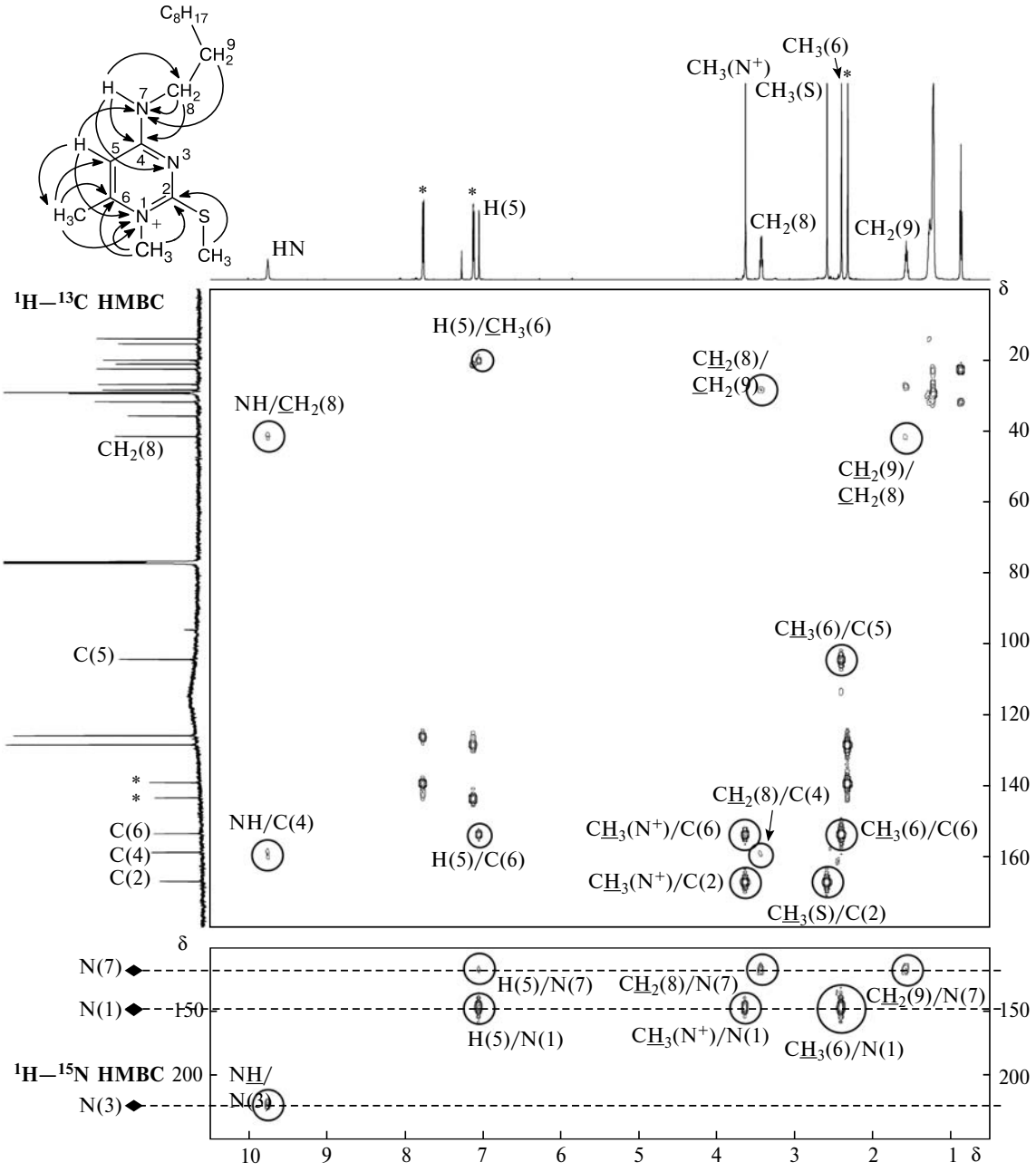


Fig. 3. The $^{1}H—^{13}C$ and $^{1}H—^{15}N$ NMR HMBC spectra of the cation Me^{+}-**1** in $CDCl_3$ (T = 303 K) and key correlations $^{1}H—^{13}C$ and $^{1}H—^{15}N$ for the methylated form. * Signals of the tosylate anion.

it was found earlier that such a combination gave a good quality for the determination of CS for a comparatively short computation time.[19] Results of a correlation analysis upon the comparison of experimental and calculated CS are presented in Tables 2 and 3.

Generally, the correlation coefficients (R^2) for the protons are close to each other for the three hypotheses (N(7)/N(3)/N(1)-protonation), therefore if solely proton CS had been used, it would have been virtually impossible to distinguish these protonation positions. Moreover, these coefficients also hardly differ for various tautomer forms (see Table 3), which does not allow their use as a tool for the determination of a tautomeric structure.

In this respect the use of CS in the ^{13}C NMR spectrum is considerably more efficient. In the case of protonation R^2 values clearly demonstrate that the N(1)-protonated form is preferable. At the same time the direction of methylation cannot be determined only on the basis

Table 2. The values of correlation coefficients R^2 [a] for products of protonation (methylation) neutral compounds **1** and **2** at different positions

Compound	Nucleus	R^2			
		N(1)	N(3)	C(5)	N(7)
1	^{15}N	0.970	0.011	0.190	0.419
		(0.993)	(0.003)	(0.070)	(0.287)
	^{13}C	0.992	0.978	0.812	0.966
		(0.991)	(0.990)	(0.842)	(0.975)
	^{1}H	0.988	0.985	0.039	0.994
		(0.981)	(0.992)	(0.022)	(0.996)
2	^{15}N	0.937	0.073	0.280	0.495
		(—)[b]	(—)	(—)	(—)
	^{13}C	0.997	0.982	0.849	0.983
		(0.994)	(0.995)	(0.873)	(0.982)
	^{1}H	0.998	0.986	0.003	0.997
		(0.988)	(0.991)	(0.030)	(0.981)

[a] R^2 — the correlation coefficient of theoretical CS with experimental ones.
[b] Due to a low solubility not all CS were available for the computation of R^2.

Table 3. The values of R^2 for the different tautomeric forms of compound **1** and products of its protonation (methylation) at the atom N(1)

Compound	Nucleus	R^2			
		A (N(7))	**B** (N(3))	**C** (N(1))	**D** (C(5))
1	^{15}N	0.999	0.134	0.436	0.613
	^{13}C	0.993	0.994	0.988	0.842
	^{1}H	0.995	0.996	0.987	0.075
H^+-**1**	^{15}N	0.970	0.467	0.053	0.225
	^{13}C	0.992	0.971	0.969	0.738
	^{1}H	0.988	0.989	0.977	0.124
Me^+-**1**	^{15}N	0.993	0.381	0.009	0.118
	^{13}C	0.991	0.974	0.976	0.754
	^{1}H	0.981	0.978	0.956	0.149

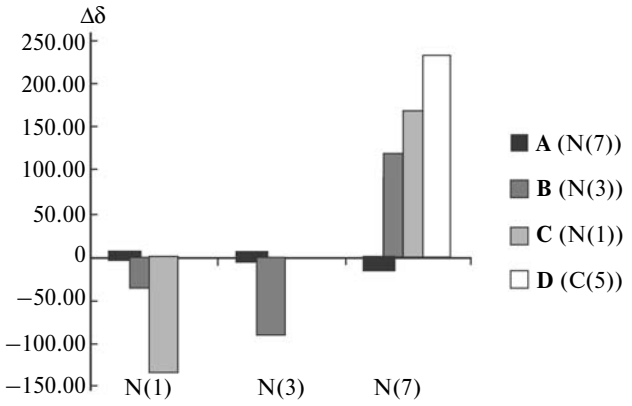


Fig. 4. The difference of calculated and experimental ^{15}N CS for four possible tautomers **A—D**.

of ^{13}C NMR spectrum data since experimental CS correlate equally well with calculated CS both for N(1)-, and for N(3)-metylated forms (R^2 = 0.991 and 0.990). Furthermore, the utilization of ^{13}C CS for the determination of the tautomeric composition of a neutral molecule in this case would lead to an erroneous outcome (N(7) and N(3) forms (see Table 3).

Beyond any doubt, ^{15}N CS are most sensitive to protonation and also to the tautomeric structure. Only for the correct structure of a tautomeric form R^2 values are close to unity (0.97—0.99), whereas for a wrong hypothesis these values are less than 0.6. Consequenly the analysis of the ^{15}N CS is the most reliable tool for the solution of such structural problems.

A good numerical agreement of the calculated and experimental values of ^{15}N CS (Fig. 4) is also very important for practice. It offers the possibility of using the analysis of ^{15}N CS also in the case of an equilibrium between two tautomeric species on a fast exchange in the NMR scale.

The preferred position of protonation (or methylation). Theoretical examination. The knowledge of the tautomeric structure is essential in view of taking into account possible interactions governing the recognition and structure of the complex of a sought compound with a biological target. In this regard the question arises as to whether it is possible to reliably predict the protonation site purely theoretically and, therefore, take a real step in the rational design of compounds with a desired tautomeric structure.

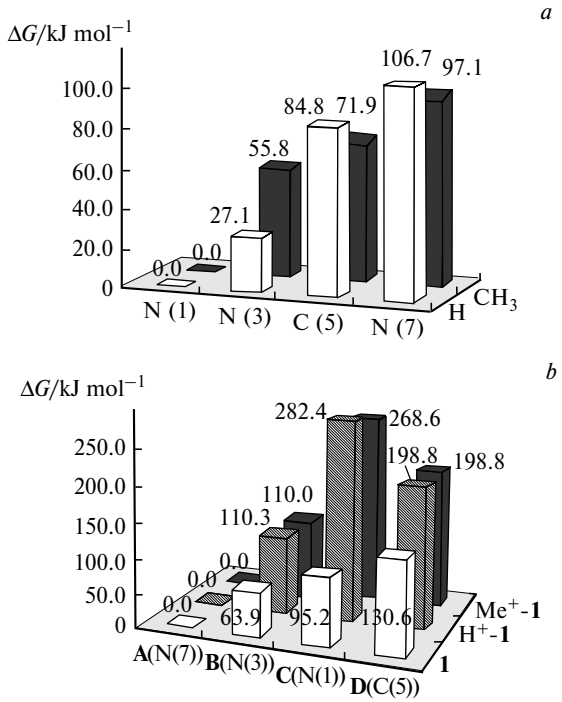


Fig. 5. Relative free energies (RB3LYP/6-31G(d)//RHF/6-31G) for the compound **1** and products of its protonation (methylation) at different positions (*a*) and for different tautomers (*b*).

Within the framework of this research it is firmly established that in thiocytosines protonation (methylation) occurs at N(1), although hypothetically there are several more possible atoms. Having such experimental material in hands, it is interesting to discuss the ability of the theory to forecast the observed picture.

There are examples when the analysis of the affinity to the proton or of the full energy of corresponding tautomer forms allowed the description of a tautomeric equilibrium.[20] These quantities changes concordantly, hence for the investigated structures only corresponding free energies (ΔG, RB3LYP/6-31G(d)) were calculated. In Fig. 5 differences in the free energies of forms protonated (methylated) at different positions are presented for compound **1**.

As seen, in a full agreement with experiment, the computations predict a considerable preferability of the N(1)-protonated (methylated) form.

Furthermore, within the framework of the same approach the evaluation of the stability of various tautomers of uncharged structure **1** (see Fig. 5, *b*) was conducted. It turned out that the tautomer **A** is be far more stable than the others both in a neutral molecule (see Fig. 5, *b*), and for charged structure, which is in a full agreement with experiment.

Thus, the thermodynamic approach adequately describes the picture observed experimentally. Moreover, taking into consideration the efficiency of such an approach also for other protonated pyrimidines,[21] this methodology apparently has a good predictability.

The following conclusions were drawn due to the present study. The efficiency of the use of 2D $^{1}H—^{15}N$ HSQC/HMBC NMR experiments for the determination of the protonation (methylation) position and tautomeric composition of nitrogen-containing heterocycles has been demonstrated.

For the studied compounds it has been established that the addition of H (Me) occurs at the atom N(1). The tautomer with the amine form of a substituent at the position 4 is the predominated one.

Ab initio calculations of CS (GIAO B3LYP/6-31G(d)//HF/6-31G) well reproduce the experimental effects. The analysis of experimental ^{15}N CS is a reliable tool for the determination of the protonation (methylation) position and tautomer structure.

The stability of various protonated (methylated) and tautomer species is governed by a thermodynamic mechanism.

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