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ORIGINAL RESEARCH

Does dehydrocyclization of 4-benzoylthiosemicarbazides in acetic acid lead to *s*-triazoles or thiadiazoles?

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Abstract Ever since the recognition of strong pharmaceutical activities of triazoles and thiadiazoles, these scaffolds have been the subject of vigorous studies. One of the best strategies for synthesis of these azoles is dehydrocyclization of 1,4-disubstituted thiosemicarbazides, which leads to *s*-triazoles in alkaline media, whereas in strong acidic media 1,3,4-thiadiazoles are formed. However, the literature is riddled with contradictory communications regarding the nature of the products of such reactions under mild acidic conditions. As these compounds are not amenable to X-ray analysis, we have resorted to NMR and theoretical modelling to resolve this discrepancy. In this article, we present arguments indicating that dehydrocyclization of 4-benzoylthiosemicarbazides in glacial acetic acid leads to thiadiazole derivatives. These structural findings are augmented by studies of bioactivity of a few members of the studied class of compounds.

Keywords Thiadiazole · Triazole · NMR · DFT

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Introduction

The triazole and thiadiazole core structures represent pharmacophores that have been widely exploited in medicinal chemistry. The triazole- and thiadiazole-based compounds have been reported as antimicrobials [1–21], antivirals [22–24], antitumors [6, 10, 25–31], anticonvulsants [11, 32, 33] antidepressants [34–36], analgesic [8, 37–41] and anti-inflammatory [8, 9, 37, 38, 42–46] agents. An excellent strategy for the synthesis of these azoles is the dehydrocyclization of 1,4-disubstituted thiosemicarbazides. It is generally accepted that the cyclization of these compounds results in the formation of *s*-triazoles in an alkaline medium, whereas in an acidic medium 1,3,4-thiadiazoles are formed. Results from our laboratory, however, show that this is not generally true, and for some substituents, *s*-triazoles are obtained both under alkaline and acidic conditions. This leads to confusion regarding the actual structure of the bioactive compounds. For example, there is some disagreement in the literature regarding the identity of the product of 4-benzoylthiosemicarbazides dehydrocyclization in acetic acid. Some authors have reported the formation of *s*-triazoles [47, 48], while others claim to have obtained thiadiazoles [49–54] as illustrated in the Scheme 1. As this class of compounds exhibits significant antibacterial activity, it is important to unequivocally identify the structure of this bioactive compound. The uncertainty described above prompted us to study the

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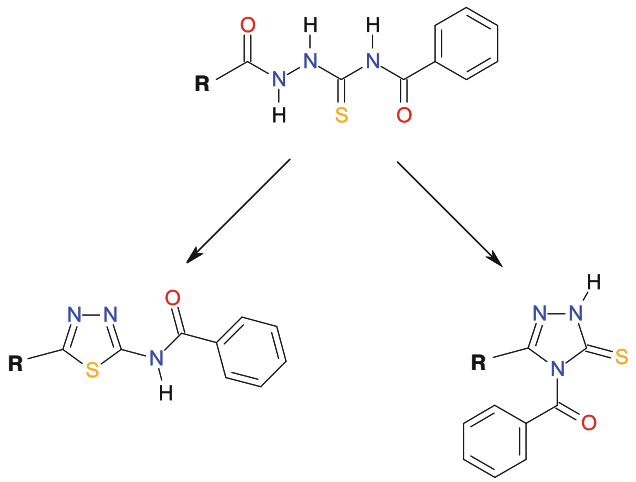

Scheme 1 Proposed [47–54] the direction of dehydrocyclization of 4-benzoylthiosemicarbazides in acetic acid

structure of the product of the 4-benzoylthiosemicarbazide dehydrocyclization (Scheme 1).

In this article, we present results of structural studies on dehydrocyclization of 4-benzoyl-1-(pyrrol-2-ylcarbonyl)-thiosemicarbazide (**1**, R = pyrrol-2-yl), which allowed us to confirm that the product of this reaction is 2-benzoylamino-5-(pyrrol-2-yl)-1,3,4-thiadiazole (**2**). Further studies compare bioactivity of its more complicated analogue, 2-benzoylamino-5-(4-methyl-imidazol-5-yl)-1,3,4-thiadiazole (**3**, R = 4-methyl-imidazol-5-yl), with its acyclic ('linear') precursor, 4-benzoyl-1-(4-methyl-imidazol-5-ylcarbonyl)-thiosemicarbazide (**4**), which shows excellent activity against Gram-positive bacteria, as well as inhibitory activity towards topoisomerase IV (topo IV) [55].

Experimental

Chemistry

All commercial reactants and solvents with the highest purity were purchased from either Sigma-Aldrich or Lancaster and used without further purification. Melting points were determined on a Fischer–Johns block and were uncorrected. Elemental analysis was determined by an AMZ-CHX elemental analyser (are within ±0.4 % of the theoretical values). IR spectra (ν, cm^{-1}) were recorded in KBr using a Specord IR-75 spectrophotometer. ^{1}H-NMR spectra (δ, ppm) of (**1**) and (**3**) were recorded on a Bruker Avance 300 in DMSO-d_6 with TMS as internal standard. Analytical thin layer chromatography (TLC) was performed with Merc $60F_{254}$ silica gel plates and visualized by UV irradiation (254 nm).

*NMR spectra of 2-benzoylamino-5-(pyrrol-2-yl)-1,3,4-thiadiazole (**2**)*

^{1}H, ^{13}C and ^{15}N NMR spectra were recorded at 300 K in DMSO-d_6 on Bruker Avance II Plus spectrometer at 700.21, 176.09 and 70.96 MHz, respectively. ^{1}H and ^{13}C chemical shifts were calibrated on solvent signals at 2.49 and 39.7 ppm, respectively. Liquid ammonia was the reference compound for ^{15}N chemical shifts. The signal assignments were based on analyses of ^{1}H and ^{13}C 1D NMR, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC and HMBC, and ^{1}H-^{15}N HSQC spectra. As presented in the discussion based on the obtained spectra, the compound was identified as 2-benzoylamino-5-(pyrrol-2-yl)-1,3,4-thiadiazole (**2**) with the following signal assignments:

^{1}H NMR δ (DMSO-d_6) 6.21 (dt, 1 H, J 2.4 and 3.6 Hz, C′-4), 6.73 (m, 1H, C′-3), 6.98 (m, 1 H, C′-5), 7.56 (t, 2H, J 7.77 Hz, $Ph_{3,5}$), 7.66 (t, 1 H, Ph_4), 8.12 (dd, 2 H, J 8.5, 1.3 Hz, $Ph_{2,6}$), 11.97 (br s, 1 H, N-2), 12.99 (br s, 1 H, N-1); and ^{13}C NMR δ (DMSO-d_6) 109.9 (C-4 pyrrol), 111.4 (C-3 pyrrol), 122.1 (C-5 pyrrol), 122.3, 128.5 ($Ph_{2,6}$), 128.8 ($Ph_{3,5}$), 131.7 (Ph_1), 133.1 (Ph_4), 155.7 (C-2 pyrrol or thiadiazol), 157.2 (C-2 pyrrol or thiadiazol), 165.1 (carbonyl).

*Synthesis of 4-benzoyl-1-(pyrrol-2-ylcarbonyl)-thiosemicarbazide (**1**)*

The compound (**1**) was obtained as described previously for the synthesis of (**4**) [55].

Yield: (2.68 g, 93 %). Mp: 203-5 °C. IR (ν, cm^{-1}) 3298 (NH), 1672 (C=O), 1621, 1489, 886, 753, 707 (Ar–H). ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 6.15–6.18 (m, 1H, CH), 6.97–6.99 (m, 2H, 2 × CH), 7.51–7.57 (m, 2H, 2 × CH), 7.64–7.70 (m, 1H, CH), 7.97–8.03 (m, 2H, 2 × CH), 10.63, 11.71, 12.33 (3s, 3H, 4 × NH). Anal. Calc. for $C_{13}H_{12}N_4O_2S$: C, 54.15; H, 4.19; N, 19.43. Found: C, 54.55; H, 3.87; N, 19.30.

*Synthesis of 2-benzoylamino-5-(pyrrol-2-yl/4-methyl-imidazol-5-yl)-1,3,4-thiadiazole (**2**) and (**3**)*

A solution of (**1**) or (**4**) (0.01 mol) in glacial acetic acid (30 mL) was refluxed, and progress of the reaction was monitored by thin layer chromatography. After 5 h, the reaction was completed, and the reaction mixture was kept for 12 h at room temperature. The precipitate was filtered, dried and crystallized from ethanol.

*2-Benzoylamino-5-(pyrrol-2-yl)-1,3,4-thiadiazole (**2**)*

Yield: (2.16 g, 80 %). Mp: 276–8 °C. IR (ν, cm^{-1}) 3458 (NH), 3002, 1581, 1537, 1468, 905, 735, 696 (Ar–H), 1671 (C=O), 1600 (C=N). Anal. Calc. for $C_{13}H_{10}N_4OS$: C,

57.76; H, 3.73; N, 20.73. Found: C, 58.10; H, 4.11; N, 20.54.

*2-Benzoylamino-5-(4-methyl-imidazol-5-yl)-1,3,4-thiadiazole (**3**)*

Yield: (1.17 g, 41 %). Mp: 256–8 °C. IR (ν, cm^{-1}) 3229 (NH), 1666 (C=O), 1610, 1579, 1511, 1488, 766, 711 (Ar–H), 2924, 2853, 1451, 1353 (Aliph.), 1622 (C=N). ^{1}H-NMR (300 MHz, DMSO-d_6) δ_H 2.56 (s, 3H, CH_3), 6.54–7.69 (m, 3H, 3 × CH), 7.70 (s, 1H, CH), 8.11–8.14 (m, 2H, 2 × CH), 12.45 (s, 1H, NH), 12.91 (s, 1H, NH). Anal. Calc. for $C_{13}H_{11}N_5OS$: C, 54.72; H, 3.89; N, 24.55. Found: C, 54.47; H, 3.74; N, 24.85.

Antimicrobial assay

Microorganisms used in this study were as follows: *S. aureus* ATCC 25923, *S. aureus* ATCC 6538, *S. epidermidis* ATCC 12228, *M. luteus* ATCC 10240, *B. subtilis* ATCC 6633, *B. cereus* ATCC 10876 and *B. cereus* ATCC 11778 (as representative examples of Gram-positive bacteria); and *E. coli* ATCC 25922, *E. coli* ATCC 10538, *E. coli* NCTC 8196, *P. vulgaris* NCTC 4635, *K. pneumoniae* ATCC 13883, *P. aeruginosa* ATCC 15442, *P. aeruginosa* NCTC 6749, *P. aeruginosa* ATCC 27853, *P. aeruginosa* ATCC 9027, *P. mirabilis* ATCC 12453 and *B. bronchiseptica* ATCC 4617 (as representative examples of Gram-negative bacteria).

Preliminary antibacterial potencies of (**1**) and (**3**) against a panel of Gram-positive and Gram-negative bacteria were screened on the basis of growth inhibition zones (GIZ) by means of the agar well diffusion method. Then, the MICs were determined by the agar dilution method (for (**1**)) or the broth microdilution technique (for (**3**)). The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the compound, preventing growth of the tested microorganism. In both the methods, the recommended Mueller–Hinton medium was used—agar and broth, respectively [56, 57].

The disc diffusion method

The disc diffusion method was used to determine preliminary activity of (**1**). For this method, sterile filter paper discs (9-mm diameter) were dripped with the compound solution to load 400 μg of one compound per disc. Dry discs were placed on the surface of Mueller–Hinton II agar medium. The diameter of the growth inhibition zone was read after 18 h of incubation at 35 °C.

The agar well diffusion method

The agar well diffusion method was used to determine preliminary activity of (**3**). For this method, sterile swabs were used to spread the microbial suspensions (0.5 McFarland inoculum diluted 1:100 in Mueller–Hinton broth) onto the medium surface, and then the compound solution at a concentration of 5,000 μg/mL was introduced into wells (80 μL per well separately). The wells (8-mm diameter) were made on the agar with a sterile cork borer. The plates were preincubated at room temperature for 1.5 h, to allow the diffusion of solution into the medium, and were then incubated at 37 °C for 18 h.

The agar dilution method

The agar dilution method was used to determine MICs for (**1**). For the determination, the compound was dissolved in DMSO. Concentrations of the agent tested in solid medium ranged from 6.25 to 400 μg/mL. The final inoculum of all organisms studied was 10^4 colony-forming units per mL (CFU/mL). Minimal inhibitory concentrations were read after 18 h (for bacteria) of incubation at 35 °C. Ciprofloxacin (5 μg per disc) was used as the control antimicrobials. The MIC results were repeated three times and are illustrated in Table 1.

The broth microdilution method

The broth microdilution method was used to determine MICs for (**3**). This method was performed in 96-well microplates with the Mueller–Hinton broth containing from 1.95 to 1,000 μg/mL of (**1**). 20 μL of each bacterial 0.5 McFarland suspension was added to the Mueller–Hinton broth per each well; total volume was 200 μL. After incubation (37 °C for 18 h), the optical density (OD_{600}) measurements were determined for bacterial cultures in the presence and in the absence of tested compounds. Cefuroxim at concentrations of 0.06 to 500 μg/mL was used as control antimicrobials. The MIC results were repeated three times and are illustrated in Table 1.

Inhibition of bacterial type IIA topoisomerases

Supercoiling assay

The assays were performed using *S. aureus* Gyrase Supercoiling Assay Kits (Inspiralis). In brief, supercoiled pBR322 plasmid DNA (0.5 μg) was incubated with 1 unit of gyrase, in the dedicated supercoiling assay buffer supplied by the manufacturer, in the presence of varying concentrations of the test compounds. Reactions were carried out at 37 °C for 1 h and then terminated by the addition of equal volume of 2× STOP Buffer (40 % sucrose, 100 mM Tris–Cl pH 7.5, 1 mM EDTA, and 0.5 mg/ml bromophenol blue) and chloroform/iso-amyl alcohol. Samples were vortexed, centrifuged and run through a 15 cm 1 % agarose gel

Table 1 In vitro antimicrobial activities of (**1**), (**3**),(**4**) and reference antibiotics expressed as the growth inhibition zone (GIZ, mm) and minimal inhibitory concentration (MIC, μg/mL)

Compound	*S. aureus*		*S. epidermidis*		*M. luteus*		*B. subtilis*		*B. cereus*	
	GIZ	MIC	GIZ	MIC	GIZ	MIC	GIZ	MIC	GIZ	MIC
(**1**)	$18^{a,b}$	$50^{a,b}$	20^{c}	50^{c}	15^{d}	25^{d}	20^{e}	25^{e}	14^{f}	25^{f}
(**3**)	50^{a}	125^{a}	19^{c}	62.5^{c}	44^{d}	31.25^{d}	50^{e}	62.5^{e}	8^{g}	$>1{,}000^{g}$
	8^{a}	500^{b}								
(**4**)	34^{a}	500^{a}	24^{c}	3.91^{c}	50^{d}	0.98^{d}	21^{e}	7.81^{e}	8^{g}	62.5^{g}
	18^{b}	$1{,}000^{b}$								
Cefuroxim		0.49^{a}		0.98^{c}		0.98^{d}		0.98^{e}		31.25^{g}
		0.98^{b}								
Ciprofloxacin		0.5^{a}		0.5^{c}		2^{d}		$<0.125^{e}$		1^{f}
		0.5^{b}								

Strains ATCC: [a]25923, [b]6538, [c]12228, [d]10240, [e]6633, [f]11778, [g]10876

in TAE buffer (40 mM Tris–acetate, 2 mM EDTA) for 3 h at 50 V. Gels were stained with ethidium bromide and visualized under UV light.

Decatenation assay

The assay was performed using *S. aureus* topoisomerase IV decatenation kits (Inspiralis). Interlinked kDNA substrate (0.5 μg) was incubated with 1 unit of topoisomerase IV (Inspiralis), in the dedicated decatenation assay buffer supplied by the manufacturer, in the presence of varying concentrations of the test compounds. Reactions were carried out at 37 °C for 1 h and then terminated by the addition of equal volume of 2× STOP Buffer (40 % sucrose, 100 mM Tris–Cl pH 7.5, 1 mM EDTA and 0.5 mg/ml bromophenol blue) and chloroform/iso-amyl alcohol. Samples were vortexed, centrifuged and run through a 15 cm 1 % agarose gel in TAE buffer for 1.5 h at 80 V. Gels were stained with ethidium bromide and visualized under UV light. The concentrations of the inhibitor that prevented 50 % of the kinetoplast DNA from being converted into decatenated minicircles (IC_{50} values) were determined by plotting the results obtained from the densytometric analyses of the gel images by means of Quantity One software (BioRad).

Theoretical calculations

Structures of alternative products of dehydrocyclization of (**1**) (*s*-triazole and thiadiazole) were optimized by means of the B3PW91 functional expressed in the 6-31+G(d,p) basis set [58, 59], as implemented in Gaussian09 [60]. Continuum solvent model IEFPCM with parameters for DMSO was used in all calculations. Theoretical NMR shifts were then obtained for the optimized structures by means of the B3LYP functional [61] with 6-311+G(2d,p) basis set. Default thresholds were used in all calculations. It has been shown that reoptimization by means of the B3LYP functional led to same conclusions.

Discussion

Recently, a series of products of dehydrocyclization of 4-benzoylthiosemicarbazides in glacial acetic acid were obtained in our laboratory with the aim of comparing their bioactivity with those of their acyclic 4-benzoylthiosemicarbazide precursors. All the compounds were characterized by ^{1}H NMR spectra, which clearly confirmed the process of aromatization of the 'linear' substrates. However, based on the broad resonance of the NH group at about 13 ppm, we could not confirm whether the *s*-triazole or thiadiazole derivative was formed. Typically, the ^{1}H NMR spectra of *s*-triazoles display a sharp NH resonance signal at about 14 ppm, while for thiadiazoles, a broad signal in the range 10–12 ppm is observed. The NH signals at about 13 ppm, for products of aromatization of 4-benzoylthiosemicarbazides in glacial acetic acid, are halfway between these typical values, and thus, are ascribed in the literature either to *s*-triazole or thiadiazole moieties. Unfortunately, structures of these compounds cannot be confirmed based on X-ray analysis because none of them are prone to crystallization. Thus, in order to establish the actual structure, 2D NMR experiments (as detailed in the "Experimental" section) were carried out based on the example of dehydrocyclization of (**1**).

^{1}H-^{15}N HSQC spectrum shows two correlations of NH protons to attached nitrogen atoms: 12.99–130.5 and 11.97–155.5 ppm. In the COSY spectrum (Fig. 1a), the signal at 11.97 ppm shows correlations with other protons and is the entering point for assignment of protons at 6.21, 6.73 and 6.98 ppm as protons of the pyrrole ring. Thus, the second spin system in the COSY spectra at 7.56, 7.66 and 8.12 ppm corresponds to the phenyl ring protons. Signal at 8.12 ppm was assigned as *ortho* protons in phenyl ring. The second NH atom is connected with carbonyl carbon in the benzamide fragment (left) or to thiocarbonyl group within triazole ring. In HMBC spectrum (Fig. 1b), signal at 12.99 ppm correlates with the carbon atom at 165.1 ppm. Correlation with the same atom was detected for ortho

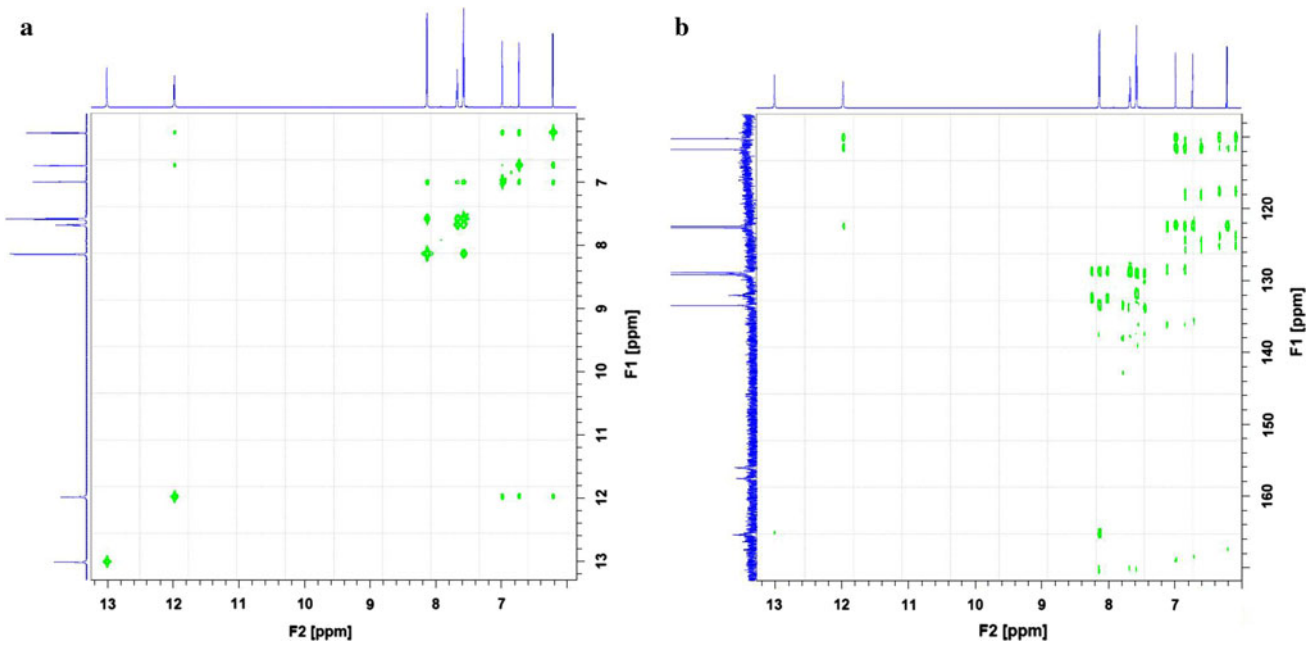

Fig. 1 **a** ^{1}H-^{1}H COSY and **b** ^{1}H-^{13}C HMBC NMR spectra used in elucidation of the structure of the product of dehydrocyclization of (**1**)

phenyl protons at 8.12 ppm. From this, it can be concluded that the product of the dehydrocyclization of (**1**) is the thiadiazole (**2**).

The assignment was augmented by the results of theoretical modeling of ^{13}C and ^{15}N spectra. The three signals with the highest chemical shifts and two signals with the lowest chemical shifts in theoretical ^{13}C spectra of both alternative products clearly support the thiadiazole structure as evidenced by the data illustrated in Table 2. Also the difference between NH signals in ^{15}N spectra calculated for triazole (40.1 ppm) and thiadiazole (20.8 ppm), when compared with the experimental value of 24.5 ppm, supports this conclusion. The unambiguous assignment of some carbon signals, which is experimentally impossible due to lack of long-range couplings, was achieved in the theoretical manner. The selected DFT functional, B3PW91 [62–64], has been shown previously to be adequate for calculating properties of compounds of this class [65–68].

Table 2 Comparison of selected ^{13}C NMR signals (ppm), predicted theoretically at the IEFPCM(DMSO)/B3LYP/6-311+G(2d,p)//IEFPCM(DMSO)/B3PW91/6-31+G(d,p) for alternative structures with the experimental values

Thiadiazole	Experiment	Triazole
166.3	164.9	170.6
160.8	157.0	168.6
159.1	155.6	152.1
111.7	111.2	118.6
111.6	109.7	112.2

Figure 2 shows all ^{13}C signals and relates them to the corresponding carbon atoms.

We have recently documented for the first time inhibitory properties of 4-benzoylthiosemicarbazides against bacterial topoisomerase IV [55]. Among tested compounds 4-benzoyl-1-(indol-2-ylcarbonyl)-thiosemicarbazide (**5**, R = indol-2-yl) was found to be the best inhibitor of topo IV with IC_{50} = 14 μM. A decrease in this inhibitory activity (IC_{50} = 90 μM) was observed when indole moiety was replaced with imidazole in (**4**). 1,4-Dibenzoylthiosemicarbazide (**6**, R = phenyl), with a considerably different structure from both (**4**) and (**5**), was inactive as inhibitor of both DNA gyrase and topoisomerase IV. Based on the DFT and docking studies it was proposed that two factors: (i) the structure of the thiosemicarbazide and (ii) substituent at N1 nitrogen atom are the key functionalities required for inhibition of topo IV rather than H-bond interaction between NH–NH–C(=S)–NH core and the enzyme. In order to expand these initial findings with further details on the structure–activity relationship for this chemical class, (**3**), the cyclic product of (**4**), was synthesized and its biological activity evaluated. It was found that (**3**) exhibits no inhibitory activity against topo IV. Based on these findings, we can conclude that both substituents as well as the NH–NH–C(S)–NH core are important for interactions of thiosemicarbazide-based compounds with topo IV.

Interestingly, inhibitory activities of 4-benzoyltiosemicarbazides and their cyclic derivatives do not parallel their antibacterial activities. The comparison of these activities for compounds (**1**) and (**3**) with the corresponding literature data on (**4**) is given in Table 1 in the Experimental section.

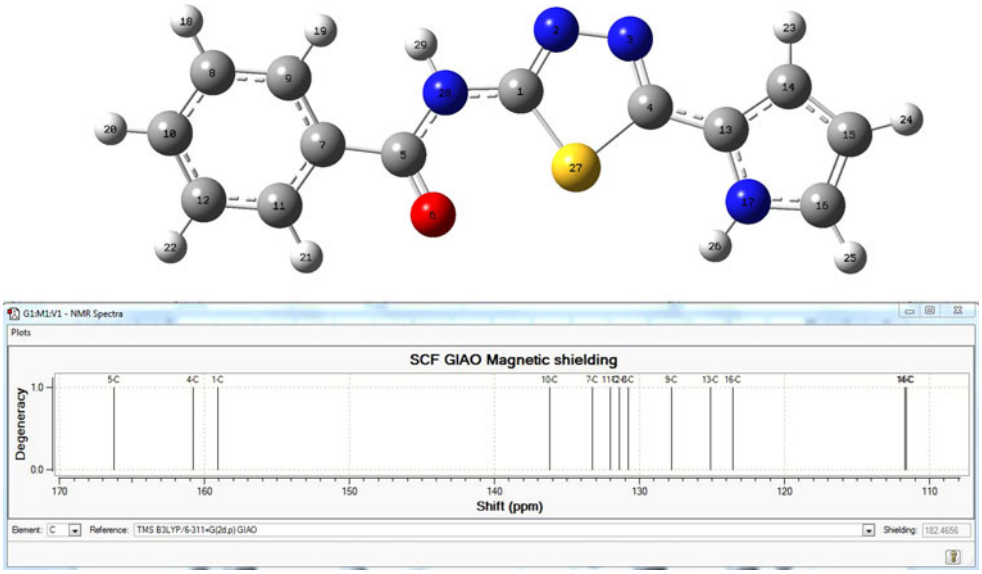

Fig. 2 ^{13}C NMR spectrum predicted theoretically for (**2**). Atom numbering at the *top*

As can be seen, the replacement of the imidazole with pyrrole ring, exemplified by (**1**), resulted in increase in antibacterial activity against *S. aureus* ATCC 25923 and 6538 as compared to (**4**). The same trend was observed when antibacterial potency of thiosemicarbazide (**4**) with its cyclic derivative (**3**) was compared. Compound (**1**) was more active against *B. cereus* ATCC 10876 than (**4**) and control antibiotic cefuroxim. This implies that 4-benzoyltiosemicarbazides and their cyclic derivatives participate in at least two different mechanisms of antibacterial activity: one is connected with inhibition of topo IV, while the nature of the other cannot be elucidated from the limited data collected thus far. Evidently, broader structure–activity relationship analysis is necessary. Systematic search among this chemical class is in progress in our laboratory.

Conclusion

In this contribution, arguments indicating that dehydrocyclization of 4-benzoylthiosemicarbazides in glacial acetic acid leads to thiadiazole derivatives were presented. These structural findings were augmented with studies on bioactivities of a few members of the studied class of compounds. They further evidence that thiosemicarbazide derivatives have at least two different targets corresponding to their antibacterial activities.

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