EPR study of the free radicals in the single crystals of 2-thiothymine γ-irradiated at 300 K

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ABSTRACT

EPR spectroscopy has been used for the study of the free radicals formed in the single crystals of 2-thiothymine (5-methyl-2-thiouracil) γ-irradiated at room temperature (300 K). The radicals formed by a net hydrogen-atom addition to C(6) (5-thymyl or 5-yl radicals) and by a net hydrogen abstraction from the methyl group (7-thymyl or 7-yl radicals) has been studied in detail. The hyperfine tensors of proton couplings in these radicals are given together with their g-tensors. It has been shown that the calculated hyperfine proton couplings and relative orientation of the coupling tensors along the pyrimidine ring are similar to those observed earlier in 5-yl and 7-yl radicals in the single crystals of thymine and its derivatives. Although no large differences in hyperfine couplings were found, calculated values of the g-tensor elements of the 5-yl radical are appreciable larger, what is expected due to the presence of sulfur at C(2) in 2-thiothymine. Also, the observed superhyperfine 1:2:2:1 splitting of the resonance lines of the 5-yl radical indicated that the presence of sulfur in 2-thiothymine slightly affects the spin distribution in this radical insomuch that appreciable amount of spin is located on one of N–H fragments of 2-thiothymine.

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1. Introduction

In comparison to the natural bases thioanalogs of the nucleic-acid bases absorb light at longer wavelengths, and thus could be selectively photoactivated into the electronic triplet state with high affinity for crosslinking to other nucleic-acid bases and amino acid residues, which make them excellent intrinsic photolabels for probing the structure and organization of nucleic acids and proteins in nucleoprotein complexes [1–3]. Thioanalogs of the nucleic-acid bases are also known to be good traps of the radiation energy. In a number of studies, it has been shown that these substances, imbedded in small quantities in ordered structures of the nucleic-acid bases, represent preferred sites for localization of the migrating electron/holes [4–10].

In the single crystals of nucleic acids and their constituents, as well as in the single crystals of 2-thiocytosine [4], 6-thioguanine [7] and 2-thiouracil [11], γ-irradiated at low temperature (77 K), only radicals of the π type were observed. Also, radiation-induced sulfur-centered free radicals, stabilized on the base thioanalogs molecules, are always of the cationic origin. Irradiated thioanalog of thymine, 2-thiothymine (5-methyl-2-thiouracil), exhibits some different properties from regular bases and other thio-nucleobases. Only in the single crystals of 2-thiothymine ionizing radiation in-

duces two types of sulfur-centered radicals, with the characteristics quite unusual in comparison to the free radicals formed in similar systems. One of the free radicals associated with sulfur atom of 2-thiothymine is the cation radical of σ-electron configuration, formed by a loss of an electron from 2-thiothymine and subsequent deprotonation at N(3) [12]. Sulfur-centered σ radical was the first observed radical of σ-electron configuration in all nucleic-acid bases or their thioanalogs in ordered structure that is not associated with three-electron bond formed by the interaction with electron-donating group, like Cl⁻ in some hydrochlorinated matrices [5,7]. The other sulfur-centered radical is of π-electron configuration and anionic origin, primarily formed by the capture of an electron at S(2) and subsequently neutralized by the formation of the N′(3)–S(2) hydrogen bonding between two adjacent 2-thiothymine molecules, with the H′(3) participating in protonation of an anion [13].

Also, 2-thiothymine has great affinity for Cu(II) ions. In irradiated single crystals of 2-thiothymine containing traces of copper, stable paramagnetic complexes of Cu(II) ions with the molecules of 2-thiothymine were observed. It has been shown that the structure of these complexes is planar, formed by two pairs of sulfur and nitrogen atoms from two adjacent 2-thiothymine molecules in the crystal lattice [14].

Upon thermal annealing of the single crystals of 2-thiothymine, from 77 K to room temperature, sulfur-centered radicals decay and transform into well-known 5-thymyl (5-yl) and 7-thymyl (7-yl)

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radicals, formed by the addition of hydrogen atom to C(6) and by the abstraction of hydrogen atom from the methyl group of 2-thiothymine molecule, respectively. These types of radicals are always present in irradiated single crystals of thymine [15–17], thymidine [18,19], 1-methyl-thymine [18,20] and 5,6-dyhidro-thymine [21].

2. Experimental

Single crystals of 2-thiothymine were grown from saturated aqueous solution of 2-thiothymine powder (purchased from Aldrich) by slow, controlled cooling from about 45 °C to about 30 °C. The obtained anhydrous crystals were of monoclinic structure, with the space group $P2_1/n$ and the unit cell dimension a = 426, b = 1451, c = 1026 pm and β = 92.27° [22]. There are four molecules in the unit cell, two of them magnetically distinct. For the evaluation of the spectroscopic tensors the spectra were recorded in three mutually perpendicular planes. Since the reference system deviated from the crystallographic system by only 2.27° (which is within the experimental error in the alignment of the crystal in the external magnetic field), three mutually perpendicular planes were defined by the axes <a>, <b> and <c>.

The crystals were irradiated with ^{60}Co gamma rays at room temperature (300 K) to the total dose of 65 kGy. The EPR measurements were done at the same temperature with a Bruker Elexsys E500 X-band spectrometer. After irradiation, crystals were mounted on the sample holders with any of the crystallographic axes perpendicular to the external magnetic field. The spectra were recorded as the crystals were rotated about the pre-selected axis in a step-wise manner, at 5° or 10° intervals.

3. Results

3.1. 5-yl radical

3.1.1. EPR spectra

Fig. 1 presents the EPR spectrum of the single crystal of 2-thiothymine, γ-irradiated and recorded at room temperature, for the magnetic field in the direction of the c crystallographic axis. For this orientation all paramagnetic species in the lattice are magnetically equivalent and raise the same EPR spectrum. The EPR spectra

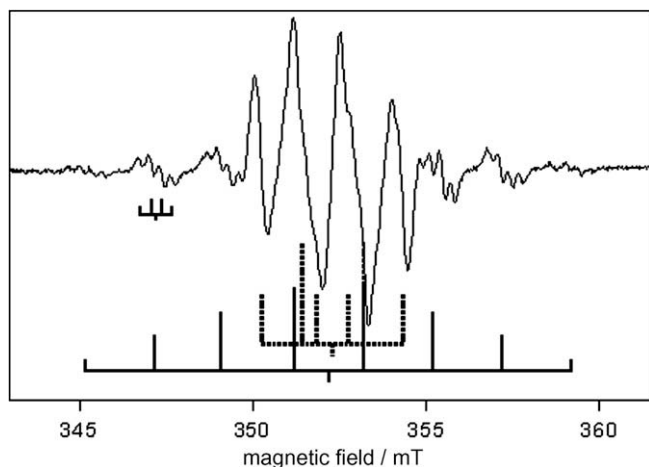**Fig. 1.** EPR spectrum of γ-irradiated single crystal of 2-thiothymine. Spectrum was recorded at room temperature for magnetic field in plane ac (H⊥b), in the direction of the c crystallographic axis, with the microwave frequency of 9.881 GHz and the microwave power of 0.2 mW. The bars beneath the spectrum represent the position of the resonance lines of 5-yl radical (solid lines) and 7-yl radical (dashed lines). Each of the eight resonance lines of 5-yl radical is further split into 1:2:2:1 quartets (minor solid lines).

of the system exhibits the pattern composed of two groups of lines. One of them is represented by an eight-line resonance pattern with the intensity ratio 1:3:5:7:7:5:3:1 and total splitting of about 14 mT, as it is depicted with the solid line bars beneath the spectrum in Fig. 1. This group of resonances is easily recognized to be associated with very well-known 5-yl radical, formed by a net hydrogen-atom addition to C(6). After addition, the 5-yl radical is formed with the most of the spin density located in p-orbital of C(5) [15]. The observed octet structure is brought about by the coupling of the unpaired electron with five β-protons – three of them are magnetically equivalent protons of the methyl group (methyl protons) and the other two are the protons bound to C(6) (methylene protons).

Each of the eight resonance lines of 5-yl radical exhibits further hyperfine substructure represented by the quartets with the line intensity ratio 1:2:2:1, as it is indicated with the minor solid line bars under the spectrum in Fig. 1. Quartets with this line intensity ratio are characteristic for the coupling of the unpaired electron with one nitrogen nucleus and proton. Unfortunately, 1:2:2:1 quartet substructure was clearly resolved only in a few orientations of the crystal in external magnetic field (orientations for the magnetic field being perpendicular or nearly perpendicular to the ring plane, for which the nitrogen hyperfine splitting in organic radicals is dominant), so the coupling tensors of this hyperfine interaction could not be precisely determined. Without calculated hyperfine tensors values and the direction cosines the assignment of this small coupling to the interaction of the unpaired electron with N(1)–H or N(3)–H was impossible.

Saturation of the resonance lines of the 5-yl radical occurs at the microwave power of only 1.5 mW, and the intensities of the lines remained unchanged for months.

3.1.2. Evaluation of spectroscopic parameters

Because of the much larger total splitting, the low-field (from 345 to 350 mT) and the high-field (354–359 mT) resonance lines of the 5-yl radical were not overlapped with the intensive resonances in the middle of the spectrum (350–354 mT), and methyl and methylene proton splittings were easily analyzed for each crystal orientation.

The couplings of the unpaired electron with methyl ($A_{C(7)}$) and methylene ($A_{C(6)}$) protons were determined in a first order perturbation procedure by solving the spin Hamiltonian which included Zeeman energy of the unpaired electron with external magnetic field B and the term representing hyperfine interactions (summation goes over all coupling nuclei with spin $I \neq 0$) [23]

$$H = \beta g \vec{S} \cdot \vec{B} + \sum_i \vec{S} \cdot \vec{A}_i \cdot \vec{I}_i \tag{1}$$

Also, correction of the hyperfine splittings caused by the anisotropy of the g-factor has been taken into account.

The calculated tensors parameter values of the 5-yl radical are listed in Table 1. As expected at room temperature, free rotation of the methyl group protons makes them magnetically equivalent with the equal hyperfine couplings ($A_{C(7)}$), as was observed in the 5-yl radicals in all thymine-based systems [15–21]. Similarly, measurements of methylene proton couplings ($A_{C(6)}$) in all orientations, in each of the three crystal planes, revealed that these couplings in 2-thiothymine have roughly the same magnitude (within the resolution of the EPR measurement), which is not the case for thymine [15–17], 1-methyl-thymine [18,20] and 5,6-dyhidro-thymine [21].

The angles between the directions of the principal values of the calculated tensors and 2-thiothymine ring normal, C(5)–C(7) and C(6)–H(6) one can easily calculate from the data given in Table 1. The eigenvector of the maximum principal value of methyl proton coupling ($A_{C(7)max}$) are within C(5)–C(7) bond direction (with the deviation of 6.6°). Eigenvector of the maximal principal value of

Table 1
Magnetic parameters of the 5-yl radical in γ-irradiated 2-thiothymine

Tensor	Principal values	Direction cosines		
		<a>	<b>	<c>
$A_{C(6)}$	4.16 mT	0.297	0.771	0.564
	4.08 mT	0.565	−0.620	0.545
	3.94 mT	0.812	0.103	−0.577
$A_{C(7)}$	2.10 mT	−0.354	0.842	−0.408
	2.04 mT	0.474	0.537	0.698
	1.92 mT	0.807	0.054	−0.589
g	2.0057	0.236	0.698	0.676
	2.0045	−0.395	0.880	−0.264
	2.0033	0.793	0.093	−0.602
Thiothymine ring normal, n		0.767	0.122	−0.630
C(5)–C(7) direction		−0.377	0.871	−0.317
C(6)–H(6) direction		0.311	0.763	0.567

Table 2
Magnetic parameters of the 7-yl radical in γ-irradiated 2-thiothymine

Tensor	Principal values	Direction cosines		
		<a>	<b>	<c>
$A_{C(6)}$	1.54 mT	−0.328	0.794	0.577
	1.08 mT	−0.815	−0.047	0.578
	0.53 mT	−0.328	0.899	−0.290
$A_{C(7)}$	2.43 mT	−0.302	0.917	−0.261
	1.57 mT	−0.811	−0.051	0.583
	0.70 mT	0.211	0.804	0.556
$A_{C(7)}$	2.30 mT	−0.298	0.902	−0.312
	1.50 mT	−0.796	0.040	0.604
	0.69 mT	−0.223	−0.799	−0.559
g	2.0077	0.316	−0.914	0.253
	2.0069	−0.719	−0.057	0.693
	2.0061	0.538	0.696	0.475
Thiothymine ring normal, n		0.767	0.122	−0.630
C(5)–C(7) direction		−0.377	0.871	−0.317
C(6)–H(6) direction		0.311	0.763	0.567

methylene proton coupling ($A_{C(6)max}$) deviates only 1.0° from C(6)–H(6) bond direction. The eigenvectors of minimal principal value of both couplings are perpendicular to the ring plane (deviations of 4.7° and 2.7° for $A_{C(7)}$ and $A_{C(6)}$ couplings, respectively). g-tensor maximal principal value is towards C(6)–H(6) bond (deviation 8.4°), and the minimal value is perpendicular to the ring plane (deviation 2.8°). Fig. 2a shows the orientation of the eigenvectors of the principal values of these tensors within the 2-thiothymine molecule. The orientation is similar to those observed earlier in irradiated single crystals of thymine and all thymine-like systems [15–21].

3.2. 7-yl radical

3.2.1. EPR spectra
Resonance lines of the 5-yl radical are superimposed with more intensive resonances in the middle of the spectrum depicted in Fig. 1. Since the saturation of these intensive resonance lines were observed at three times higher microwave power (4.5 mW) than those applied for the 5-yl radical (1.5 mW), by applying sufficiently high microwave power (3 mW) it was quite easy to fully resolve EPR spectra of this radical species.

EPR spectra of the radical are represented by the sextet with the intensity ratio 1:1:2:2:1:1, as it is depicted with dashed line bars under the spectrum in Fig. 1. Such six-resonance line EPR spectrum is characteristic of three inequivalent and nonexchangeable α-proton interactions and it was unequivocally assigned to 7-yl radical, previously observed in all thymine derivatives studied so far [17,18,24–26]. 7-yl radical is formed by a net hydrogen abstraction from the methyl group of 2-thiothymine, which causes that all of spin density in this radical is located only on two atoms – C(6) and C(7). Finally, characteristic sextet structure with the line intensity ratio 1:1:2:2:1:1 is raised by the coupling of the unpaired electron with two remaining methyl protons and a proton at C(6).

3.2.2. Evaluation of spectroscopic parameters
Careful analysis with the same procedure performed as in the case of the 5-yl radical yielded the evaluated magnetic parameters of the 7-yl radical listed in Table 2. As can be seen, the calculated g-tensor and $A_{C(7)}$ hyperfine coupling tensor are mutually coaxial within the accuracy of the measurement. Directions between eigenvectors of the principal values of the calculated tensors with the respect to the 2-thiothymine molecule are depicted in Fig. 2b. Deviations between the directions of the derived tensors values and the relevant crystallographic directions are not exceeding 6° and could be easily calculated from the data in Table 2. Somewhat larger discrepancy between calculated directions of the smallest principal values of the g-tensor and C(6)–H direction may come from the experimental error, but the difference probably also indicates a slight reorientation of some of the bonds after the methyl hydrogen atom has been removed.

4. Discussion

The well-known spectral features of 5-yl and 7-yl radicals were recognized in the EPR spectra of the single crystals of 2-thiothymine γ-irradiated at room temperature. These radicals were easily found in all thymine constituents and were analyzed in detail in the irradiated single crystals of thymine, thymidine, 1-methyl-thymine and 5,6-dyhydro-thymine using EPR or ENDOR spectroscopy. The review of methyl and methylene proton couplings in the 5-yl radicals in these systems is given in Table 3.

As a consequence of the freely rotation of the methyl group protons at the room temperature, the equal hyperfine couplings were observed in the 5-yl radicals in all thymine-based systems so far [15–21]. Also, the magnitudes of the maximal principal values of methyl proton couplings were found the same in all thymine derivatives (variation from 2.1 to 2.2 mT). Unlike that, the quite large differences between methylene proton couplings (up to 1 mT) were found in the single crystals of thymine monohydrate, anhydrous thymine, 1-methyl-thymine and 5,6-dyhydro-thymine. On the other hand, the differences between methylene proton couplings in irradiated single crystals of thymidine were found to be very small (0.1 mT). EPR measurements of methylene proton couplings in 2-thiothymine, in all orientations, in each of the three crystal planes, revealed that these couplings have approximately the same magnitude. Still, from the observed EPR data the author
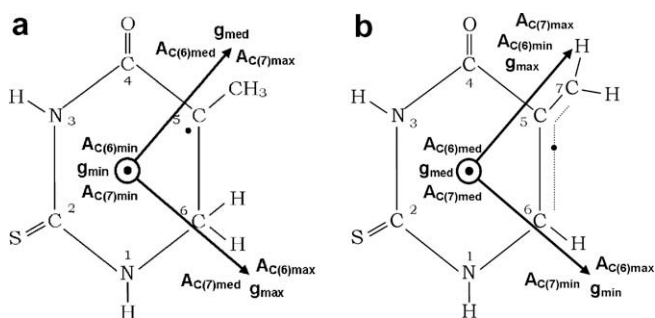

Fig. 2. Chemical structure of 5-yl (a) and 7-yl (b) radical and the relative orientations of the tensor elements with respect to the 2-thiothymine molecule.

Table 3
Methyl and methylene proton couplings in the 5-yl radicals in thymine and its analogs

System	$A_{C(7)}$/mT	$A_{C(6)}$/mT		Reference
Thymine-monohydrate	$A_\parallel$ = 2.16	H	$A_\parallel$ = 3.58	[15]
	$A_\perp$ = 1.93		$A_\perp$ = 3.28	
		H′	$A_\parallel$ = 4.44	
			$A_\perp$ = 4.06	
Thymine (anhydrous)	A_{max} = 2.18	H	A_{max} = 3.46	[17]
	A_{med} = 1.92		A_{med} = 3.09	
	A_{min} = 1.90		A_{min} = 3.04	
		H′	A_{max} = 4.78	
			A_{med} = 4.44	
			A_{min} = 4.36	
1-Methyl-thymine	1.99	H 3.42		[18]
		H′ 4.08		
Thymidine	2.05	H and H′ 4.05		[19]
Thymidine	A_{max} = 2.18	H	A_{max} = 4.22	[18]
	A_{med} = 1.92		A_{med} = 3.84	
	A_{min} = 1.90		A_{min} = 3.78	
		H′	A_{max} = 4.32	
			A_{med} = 4.09	
			A_{min} = 4.01	
5,6-Dihydro-thymine	$A_\parallel$ = 2.20	H 3.90		[21]
	$A_\perp$ = 1.95	H′ 3.65		
2-Thiothymine	A_{max} = 2.10	H and H′	A_{max} = 4.16	This article
	A_{med} = 2.04		A_{med} = 4.08	
	A_{min} = 1.92		A_{min} = 3.94	

can only speculate on the magnetic equivalence between two methylene protons and suggest to address this issue by using the more precise ENDOR spectroscopy.

Much more similarities found in the molecular conformation of the unit cell in the crystal structure of 2-thiothymine [22] and thymidine [27] could be indicative for the observed specific behavior of methylene proton couplings in these systems. Still, whether those structural differences are indeed responsible for the observed spectral features remains speculative.

The substitution of the oxygen with sulfur at C(2) in 2-thiothymine does not change a tendency of thymine-like systems for formation hydrogen addition 5-yl radical, but obviously affects the magnitude of the g-tensor elements. Due to its large value of spin-orbit coupling, sulfur increases the values of the calculated g-tensor, g_{max} = 2.0057, which is appreciable larger than those found in thymine (g_{max} = 2.0044) [15] and thymidine (g_{max} = 2.0043) [19].

Except methyl and methylene proton couplings, no other couplings of observable magnitude could be associated with the 5-yl radical in thymine, thymidine or other thymine-based systems studied so far. Unlike that, EPR spectra of γ-irradiated single crystals of 2-thiothymine exhibit 1:2:2:1 hyperfine substructure of the resonance lines, clearly resolved only in a few orientations of the crystal in external magnetic field. This EPR observable interaction of the unpaired electron with one of the N–H fragments of 2-thiothymine molecule indicates that certain amount of spin is found on one of nitrogen atom of 2-thiothymine. To the best author's knowledge, such N–H coupling has not been reported in thymine and thymine derivatives studies so far.

Although the single crystals of 2-thiothymine were grown from aqueous solution, no H_2O molecules were found in its crystal structure [22]. It means that the source of hydrogen atoms which add to C(6) must be the thymine molecules themselves (the abstracted hydrogen atoms from the methyl group in the 7-yl radicals), indicating that the 2-thiothymine base is not only good acceptor but also a good donor of hydrogen atoms. The exact mechanism of formation of the 5-yl radicals is still a matter of investigations

[17,18,26,28]. Although some novel investigations [17,18] proposed mechanisms which involve protonation at C(5) followed by electron addition or direct hydrogen addition to C(5) or C(6), it is most probable that the earlier proposed reaction scheme for the damage of nucleic acids and their constituents accounts for the present observation [29]

$$TTH \pm e^- \rightarrow TTH^\pm$$
$$TTH^\pm \rightarrow TT^\pm + H^\cdot$$
$$H^\cdot + TTH \rightarrow TTH_2^\cdot.$$
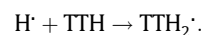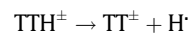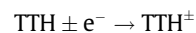
First, 2-thiothymine base, TTH, is ionized. The ion-radicals are unstable and transformed, releasing a hydrogen atom, H, which adds to another 2-thiothymine base, finally forming the hydrogen addition 5-yl radical, $TTH_2^\cdot$.

Magnetic parameters of the 7-yl radicals observed in thymine derivatives studied so far by EPR or ENDOR are listed in Table 4. As can seen, hyperfine coupling tensors of the 7-yl radicals are similar in all thymine-based systems. The data presented in this article agree well with the previous findings [16,17,24–26], indicating that substitution of oxygen with sulfur at C(2) in 2-thiothymine does not affect EPR features of the 7-yl radical and the values of the evaluated tensors. The calculated large values of the g-tensor elements (g_{max} = 2.0077) of the 7-yl radical in 2-thiothymine is expected since the large values of the g-tensor were evaluated in irradiated single crystals of thymine monohydrate (g_{max} = 2.0078) [16].

From studies of frozen thymine solutions it has been shown that the 7-yl radical may act as the successor for initially formed cations [30]. Consequently, in 2-thiothymine, 7-yl radical may be formed both by deprotonation of the methyl group at C(7) of a pristine oxidized molecule and by direct hydrogen abstraction from the same position [31].

Table 4
$A_{C(7)}$ and $A_{C(6)}$ proton couplings in the 7-yl radicals in thymine and its analogs

System	$A_{C(7)}$/mT	$A_{C(6)}$/mT		Reference
Thymine-monohydrate	–	H and H′	A_{max} = 2.24	[16]
			A_{med} = 1.56	
			A_{min} = 0.81	
Thymine (anhydrous)	A_{max} = 1.55	H	A_{max} = 2.54	[17]
	A_{med} = 1.11		A_{med} = 1.58	
	A_{min} = 0.54		A_{min} = 0.80	
		H′	A_{max} = 2.38	
			A_{med} = 1.54	
			A_{min} = 0.78	
Thymine (anhydrous)	A_{max} = 1.55	H	A_{max} = 2.44	[26]
	A_{med} = 1.09		A_{med} = 1.44	
	A_{min} = 0.52		A_{min} = 0.70	
		H′	A_{max} = 2.28	
			A_{med} = 1.44	
			A_{min} = 0.69	
Thymidine	A_{max} = 1.51	H	A_{max} = 2.39	[24]
	A_{med} = 1.06		A_{med} = 1.52	
	A_{min} = 0.51		A_{min} = 0.78	
		H′	A_{max} = 2.35	
			A_{med} = 1.53	
			A_{min} = 0.75	
Thymidine	A_{max} = 1.49	H	A_{max} = 2.40	[18]
	A_{med} = 1.06		A_{med} = 1.53	
	A_{min} = 0.50		A_{min} = 0.79	
		H′	A_{max} = 2.33	
			A_{med} = 1.53	
			A_{min} = 0.73	
2-Thiothymine	A_{max} = 1.54	H	A_{max} = 2.43	This article
	A_{med} = 1.08		A_{med} = 1.57	
	A_{min} = 0.53		A_{min} = 0.70	
		H′	A_{max} = 2.30	
			A_{med} = 1.50	
			A_{min} = 0.69	

5. Conclusions

This work gives another proof that the formation of 5-yl and 7-yl radicals is a property of thymine base regardless of its environment. The substitution of the oxygen atom with sulfur at C(2) in the molecular structure of 2-thiothymine does not change a tendency of thymine-based systems for the formation of 5-yl and 7-yl radicals and does not affect the values of the coupling tensors and the relative orientation of the eigenvectors of their principal values. Although no drastic differences in electronic structure or conformation of the 5-yl radical in all thymine derivatives were observed so far, properties of these radicals in 2-thiothymine is slightly different. The presence of the sulfur in 2-thiothymine obviously increases the value of the measured g-factor, which is significantly larger than those found in thymine and thymidine. Also, appreciable amount of spin found on one of nitrogen nuclei and related observed weakly superhyperfine 1:2:2:1 splitting of the resonance lines is the second property of the 5-yl radicals in 2-thiothymine, which makes them distinguishable from the same radicals in similar systems.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molstruc.2008.06.027.

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