

EPR SPECTROSCOPY OF 5-YL RADICALS IN GAMMA-IRRADIATED SINGLE CRYSTALS OF 2-THIOTHYMININE

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Single crystals of 2-thiothymine (5-methyl-2-thiouracil) have been γ -irradiated at 300 K and studied using EPR spectroscopy. The 5-thymil (5-yl) radical, formed by a net hydrogen-atom addition to the C(6) of 2-thiothymine base, has been studied in detail. The hyperfine tensors of methyl and methylene proton couplings are given together with the g -tensor. 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 radicals in the single crystals of thymine and its derivatives. Although no large differences in the hyperfine couplings were found, calculated values of the g -tensor are appreciably larger in comparison to the other thymine-like systems, what is expected due to the presence of sulfur at C(2) in 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].

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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 induces 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 molecule 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-thymil (5-yl) and 7-thymil (7-yl) radicals, formed by the addition of hydrogen atom to C(6) and by the abstraction of hydrogen atom from 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-dihydro-thymine [21].

In forthcoming experiments in our laboratory, concerning studies of the hole transfer in systems related to nucleic acids, the single crystals of 2-thiothymine with the traces of copper will be used as a model of such transfer. In such experiments, Cu(II) ions, embedded in variable amounts in the 2-thiothymine lattice through previously mentioned planar complexes with the host molecules, serve as hole traps. Our preliminary experiments showed that the holes stabilized at Cu(II) give rise to an EPR signal well separated from the signals of other radicals present (5-yl and 7-yl radicals). They also revealed that the ratio of the amount of embedded Cu(II) (i.e. trapped holes) to the amount of the remaining radicals is at least ten times larger than the stoichiometric Cu(II)/2-thiothymine ratio, indicating significant hole transfer from 2-thiothymine to the sites of Cu(II)-2-thiothymine complexes and subsequent trapping by the latter. In order to learn more about the characteristics

of the "remaining radicals", in this paper we present a detailed EPR analysis of one of them (5-yl radical) and brief overview of another (7-yl radical).

2. Materials and methods

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 $a=426$ pm, $b=1451$ pm, $c=1026$ pm and $\beta = 92.27^\circ$ [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 $\langle a \rangle$, $\langle b \rangle$ and $\langle c \rangle$.

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

A) 5-yl radical

3.1. EPR spectra

Figure 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 of the system exhibit 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 by the solid line bars beneath the spectrum in Fig. 1. This group of resonances is easily recognized to be associated with the 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 the 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 of 2-thiothymine molecule (methyl protons) and the other two are the protons bound to C(6) (methylene protons).

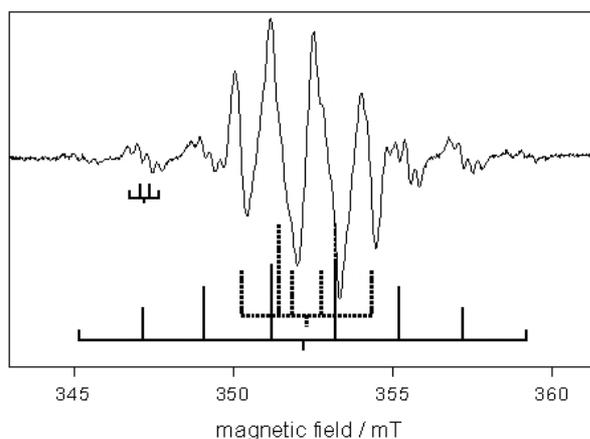


Fig. 1. EPR spectrum of γ -irradiated single crystal of 2-thiothymine. Spectrum was recorded at room temperature for magnetic field in plane ac ($H \perp b$), in the direction of the c crystallographic axis, with the microwave frequency of 9.881 GHz and the microwave power of 0.2 mW. The bars beneath the spectrum represent the position of 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).

Each of the eight resonance lines of 5-yl radical exhibits further hyperfine structure represented by the quartets with the line intensity ratio 1:2:2:1, as it is indicated by the minor solid line bars beneath 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. This superhyperfine structure of the resonance lines is probably raised by the coupling of the unpaired electron with N(1) and H(1) nuclei, indicating that small amount of the spin is located at the N(1)-H(1) region. Unfortunately, 1:2:2:1 quartet substructure is clearly resolved only in a few orientations of the crystal in external magnetic field (orientations in the plane ac ($H \perp b$), for the magnetic field being near c crystallographic axis), so the coupling tensors of this hyperfine interaction could not be precisely determined.

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

3.2. Evaluation of spectroscopic parameters

Because of the much larger total splitting the low-field (from 345 mT to 350 mT) and the high-field (354–359 mT), resonance lines of 5-yl radical are 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) and methylene (A)

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 \mathbf{B} and the term representing hyperfine interactions (summation goes over all coupling nuclei with spin $I \neq 0$) [23]

$$H = \beta g \mathbf{S} \cdot \mathbf{B} + \sum_i \mathbf{S} \cdot \mathbf{A}_i \cdot \mathbf{I}_i \quad (1)$$

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

Figure 2 shows the angular variations of the hyperfine couplings of methyl (a) and methylene (b) protons in 5-yl radical, in the three reference planes, for one of the two magnetically distinct sites in the unit cell. The circles (\circ) represent

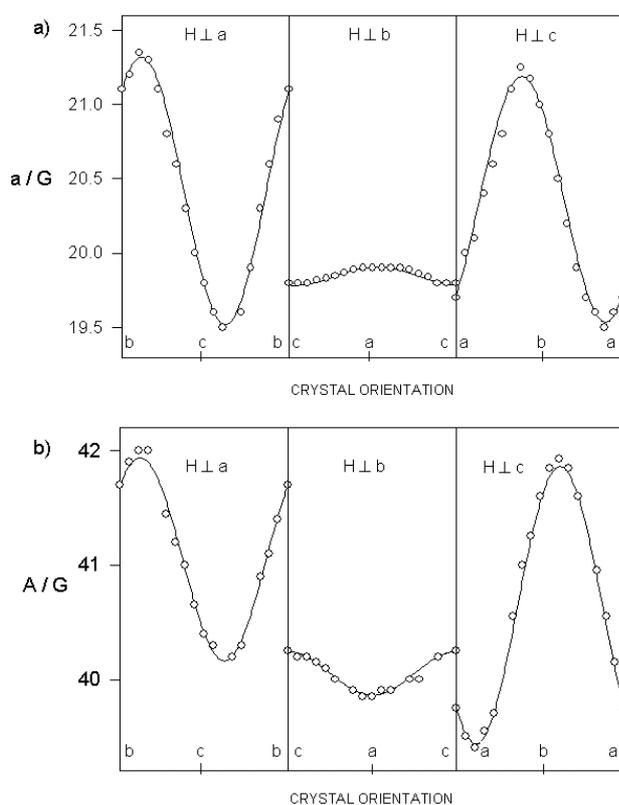


Fig. 2. Angular variations of the hyperfine couplings of methyl (a) and methylene (b) protons in 5-yl radical, in the three planes for one of the two magnetically distinct sets of data. The circles (\circ) represent the experimentally observed values and the full lines are the curves reproduced with the calculated tensor parameter values listed in Table 1.

experimentally observed values and the full lines are curves reproduced with the calculated tensors parameter values listed in Table 1. As can be seen, curves fit well the experimental data. Since the calculation (for the sake of simplicity) were performed for the reference system coinciding with the crystallographic one (and the difference between them is at most 2.27° in the β angle), some negligible discrepancies between values for the hyperfine splitting along a-axis for c-a-c and a-b-a plane occurred.

TABLE 1. Principal elements of the tensors.

Tensor	Principal values	Direction cosines		
		$\langle a \rangle$	$\langle b \rangle$	$\langle c \rangle$
A	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	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

As expected at room temperature, free rotation of the methyl group protons makes them magnetically equivalent with the equal hyperfine couplings, as was observed in 5-yl radicals in all thymine based systems [15–21]. Similarly, measurements of methylene proton couplings in all orientations, in each of the three crystal planes, revealed that these couplings in 2-thiothymine are equal (within the accuracy of the measurement), which is not the case for thymine [15–17], 1-methylthymine [18, 20] and 5,6-dihydro-thymine [21].

The angles between the principal values of the calculated tensors and 2-thiothymine ring normal, C(5)–C(7) and C(6)–H(6) bond directions are given in Table 2. The eigenvector of the maximum principal value of methyl proton coupling (a_{\max}) is within the C(5)–C(7) bond direction (with the deviation of 6.6°). Eigenvector of the maximal principal value of methylene proton coupling (A_{\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 methyl and methylene proton 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°). Figure 3 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 of its analogs [15–21].

TABLE 2. The angles between the principal values of the tensors and crystallographic directions.

Eigenvalues	Ring normal, n	C(5)–C(7)	C(6)–H(6)
A_{\max}	91.9°	-	1.0°
A_{med}	89.2°	-	89.3°
A_{\min}	2.7°	-	89.8°
a_{\max}	84.9°	6.6°	-
a_{med}	90.6°	86.1°	-
a_{\min}	4.7°	94.0°	-
g_{\max}	99.2°	-	8.4°
g_{med}	91.7°	-	66.5°
g_{\min}	2.8°	-	91.4°

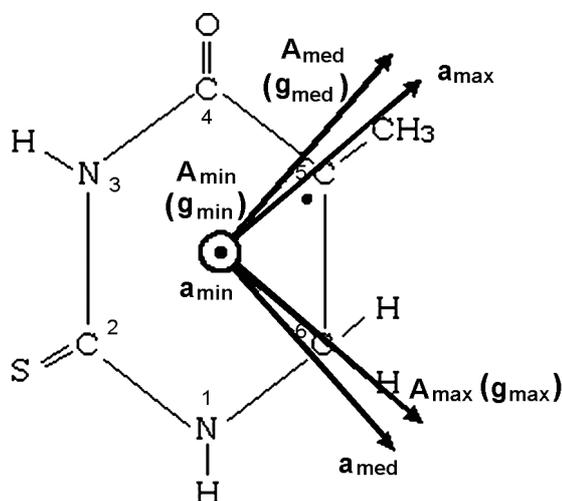


Fig. 3. Chemical structure of the radical and the relative orientations of the tensor elements with respect to the 2-thiothymine molecule.

B) 7-yl radical

Resonance lines of 5-yl radical are superimposed with more intensive resonances in the middle of the spectrum depicted in Fig. 1. This group of resonances is represented by the sextet with the intensity ratio 1 : 1 : 2 : 2 : 1 as it is depicted with dashed line bars under the spectrum and it was easily found to be associated with 7-yl radical, previously observed in irradiated single crystals of thymine and all of its derivatives [17, 18]. 7-yl radical is formed by the abstraction of hydrogen atom from 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).

Because of the overlapping with the most intensive resonance lines of 5-yl radical, the resonance lines of 7-yl radical were not fully resolved in all crystal orientations, in each of the three reference planes, and the proton couplings could not be precisely determined. Careful analysis of a few partly resolved EPR spectra, recorded in the orientations in the plane bc ($H \perp c$) for the magnetic field being near a-axis, showed that the methylene proton coupling is about 2.5 mT and the coupling of the proton bound to C(6) is about 1.5 mT. Approximate values of these couplings are similar to those observed in irradiated single crystals of thymine [15–17] and thymidine [17–19]. As expected, couplings are quite isotropic and their values do not vary much with the rotation of the sample in the three crystallographic planes.

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-dihydro-thymine using EPR or ENDOR spectroscopy. The review of methyl and methylene proton couplings in 5-yl radicals in these systems is given in Table 3.

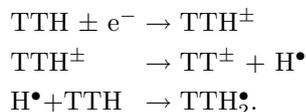
Maximal principal values of methyl proton couplings are similar in all thymine derivatives (variation from 2.1 to 2.2 mT). Regarding methylene proton couplings, the quite large differences between them (up to 1 mT) were found in the single crystals of thymine monohydrate, anhydrous thymine, 1-methyl-thymine and 5,6-dihydro-thymine. On the contrary, in irradiated single crystals of thymidine and 2-thiothymine methylene, proton couplings were found to be equal or the differences between them is very small (0.1 mT). Much more similarities found in the molecular conformation of the unit cell in the crystal structure of 2-thiothymine [22] and thymidine [24] could be indicative for the observed behaviour of methylene proton couplings in these systems. Still, whether those structural differences are indeed responsible for the observed spectral features remains speculative.

TABLE 3. Methyl and methylene proton couplings in 5-yl radicals in thymine and its analogs.

System	Methyl proton couplings (mT)	Methylene proton couplings (mT)		Ref.
Thymine-monohydrate	$A_{\parallel} = 2.16$ $A_{\perp} = 1.93$	H H'	$A_{\parallel} = 3.58$ $A_{\perp} = 3.28$ $A_{\parallel} = 4.44$ $A_{\perp} = 4.06$	[15]
thymine (anhydrous)	$A_{\max} = 2.18$ $A_{\text{med}} = 1.92$ $A_{\min} = 1.90$	H H'	$A_{\max} = 3.46$ $A_{\text{med}} = 3.09$ $A_{\min} = 3.04$ $A_{\max} = 4.78$ $A_{\text{med}} = 4.44$ $A_{\min} = 4.36$	[17]
1-methyl-thymine	1.99	H H'	3.42 4.08	[17]
thymidine	2.05	H and H'	4.05	[19]
thymidine	$A_{\max} = 2.18$ $A_{\text{med}} = 1.92$ $A_{\min} = 1.90$	H H'	$A_{\max} = 4.22$ $A_{\text{med}} = 3.84$ $A_{\min} = 3.78$ $A_{\max} = 4.32$ $A_{\text{med}} = 4.09$ $A_{\min} = 4.01$	[18]
5,6-dihydro-thymine	$A_{\parallel} = 2.20$ $A_{\perp} = 1.95$	H H'	3.90 3.65	[21]
2-thiothymine	$A_{\max} = 2.10$ $A_{\text{med}} = 2.04$ $A_{\min} = 1.92$	H and H'	$A_{\max} = 4.16$ $A_{\text{med}} = 4.08$ $A_{\min} = 3.94$	this article

Although the single crystals of 2-thiothymine were grown from aqueous solution, no H₂O molecules were found in its crystal structure [22]. It means that the source of H atoms which add to C(6) must be the thymine molecules themselves, indicating that the 2-thiothymine base is not only good acceptor but also a good donor of H atoms. It is probable that the earlier proposed reaction scheme for the damage of

nucleic acids and their constituents [25] accounts for the present observation of 5-yl radicals:



Firstly, 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^\bullet .

5. Summary

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-like systems for formation 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 compared to the other thymine analogs. Although no differences in electronic structure or conformation of the 5-yl radical in all thymine derivatives were observed, the presence of the sulfur in 2-thiothymine, due to its large value of spin-orbit coupling, obviously increases the value of the measured g -factor. The calculated values of the g -tensor ($g_{\max} = 2.0057$) are appreciably larger than those found in thymine ($g_{\max} = 2.0044$) [15] and thymidine ($g_{\max} = 2.0043$) [19]. Much larger g -factor, together with the observed equal methylene proton couplings, are the only two observed differences in the features of the 5-yl radical in 2-thiothymine which make them distinct from the same radicals observed in regular base of thymine and most of thymine based compounds.

Acknowledgements

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EPR SPEKTROSKOPIJA 5-IL RADIKALA U KRISTALIMA 2-TIOTIMINA
OZRAČENIM GAMA ZRAČENJEM

U ozračenim kristalima 2-tiotimina, pri sobnoj se temperaturi opažaju EPR spektri 5-il i 7-il radikala, nastalih u hvatom vodikova atoma na ugljikov C(6) atom, odnosno njegovim otcjepljenjem od metilne skupine molekule 2-tiotimina. EPR spektroskopska analiza pokazala je da su vrijednosti protonskih cijepanja u 5-il radikalima, kao i orijentacija svojstvenih vrijednosti tenzora protonskih hiperfinskih cijepanja vrlo slični onima opaženim u ozračenim kristalima timina i njemu sličnim sistemima. Nasuprot tomu, zbog prisutnosti sumporovog atoma u molekuli 2-tiotimina, opazili smo značajno veće vrijednosti g -tenzora u odnosu na vrijednosti u timinu i njegovim derivatima.