2-Thiobarbituric Acid Test for Lipid Oxidation in Food: Synthesis and Spectroscopic Study of 2-Thiobarbituric Acid–Malonaldehyde Adduct

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ABSTRACT: Synthesis, purification, elemental analysis, and spectroscopic studies were undertaken to characterize the structure of the red adduct 2:1 thiobarbituric acid (TBA)-malonaldehyde involved in the evaluation of oxidative rancidity in fats and oils. Thin-layer chromatography, infrared and ultraviolet-visible absorption, ^1H (^1H NMR) and ^{13}C nuclear magnetic resonance (NMR) spectra were used. A yield of 93% was obtained in the synthesis. The results of elemental analysis agree with the formula for the chloro-monohydrated form, $C_{11}H_{11}N_4O_5S_2Cl$. Three characteristic absorption maxima at 532, 310, and 245 nm, respectively, were shown in acid aqueous medium (pH 2.9). The characteristic vibrations assigned to the -NH, -OH, -$C^{\alpha}H$ (exocyclic) and -C=S groups were confirmed in the infrared spectra. There was no evidence of thioenolization. ^1H NMR data at δ 5.10 (-CONH- group, H_2O and HCl molecules); δ 11.54 (-OH group of keto-enol tautomer, -NH group); and δ 176.4 (-CONH- group) also were observed. The experimental results obtained were consistent with the existence of two spectral equivalent tautomeric structures. The colored adduct was compared with other TBA–aldehyde compounds.

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KEY WORDS: IR, MDA, NMR, rancidity, spectroscopic data, synthesis, TBA–MDA adduct, thiobarbituric acid, TLC, UV-visible.

The 2-thiobarbituric acid (TBA) assay is used to evaluate autoxidative degradation of fats and oils. The TBA value or TBA number measures the degree of oxidation in edible fats (1) and is a significant parameter for the second autoxidation step (2). It can characterize the early steps of the autoxidation process in vegetable oils, lard, and cooking fats (2–4). However, use of the TBA assay is not advisable to monitor these processes in fried foods (5).

Early studies showed that the ultraviolet (UV)-visible spectrum obtained when oxidized milk fat was reacted with TBA reagent was similar to that of the red dye obtained when malonaldehyde (MDA) reacts with TBA (6). The TBA–MDA product formed in this reaction can be used to evaluate food rancidity (7), since MDA is an end-product in the autoxidation process (8). Other aldehydes, besides MDA, are also present in food (9,10), so it would be useful to compare the TBA–MDA adduct features with those of other TBA–aldehyde adducts that also could be formed in this reaction. When aliphatic and aromatic aldehydes are reacted with TBA, alkylidene-2-thiobarbituric acids and arylidene-2-thiobarbituric acids are obtained, respectively (11).

In this paper, synthesis, isolation, elemental analysis, and spectroscopic studies were developed to characterize the TBA–MDA adduct. At present it is well established that the characteristic red color that appears in the TBA assay in a weak acid medium (with a maximal wavelength at 532 nm) is due to the adduct formed between TBA and MDA. MDA is known to be released rapidly in the last steps of the autoxidation process of fatty foods and biological systems. The elucidation of the exact structure of the red adduct can help clarify important aspects related to the application and development of the reaction, adduct stability such as, type of chromophore involved, and the fact that the reaction occurs only in the presence of an excess of TBA reagent.

The features and properties of the red adduct obtained are compared with the features of other TBA–aldehyde pigments that are of interest in food research.

EXPERIMENTAL PROCEDURES

Synthesis and purification. Two procedures were used to prepare the TBA–MDA adduct. One of these methods was that proposed by Sinnhuber *et al.* (12). The other method is proposed by the authors and follows modified methods of Dox and Plaisance (13) and Kitamura and Suzuki (14).

The method by Sinnhuber et al. (12) consisted of reacting 0.00625 mole of 1,1,3,3-tetramethoxypropane (TMP) (Sigma, St. Louis, MO) with 0.0125 mole of TBA (Merck®, Darmstadt, Germany) in 12% hydrochloric acid medium (90 min at 100°C, reflux heating). The proposed method consisted of reacting 1.04 mL of TMP (Sigma) with 90 mL aqueous neutral solution containing 2% wt/vol of TBA (Merck®), in 4 M hydrochloric acid medium (30 min at 50–60°C, with magnetic stirring) (15). The adduct obtained by either of these methods was purified and recrystallized by refluxing 40 min with 200 mL 0.6 M hydrochloric acid, then cooled to 60°C

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TABLE 1
Elemental Analyses Data for the Thiobarbituric Acid–Malonaldehyde (TBA-MDA) Adduct

	%C	%H	%N	%S
Calculated for $C_{11}H_{11}N_4O_5S_2Cl$	36.35	2.86	14.83	17.44
Prepared by method of Sinnhuber *et al.* (12)	36.35	2.86	14.82	17.44
Prepared by the procedure proposed	35.84	2.90	15.07	17.73

and filtered on a sintered glass funnel, and washed successively with 100 mL 0.6 M hydrochloric acid, 25 mL ethanol, and 100 mL diethyl ether. The dark purple needles formed did not melt even when held at 350°C for 1 h.

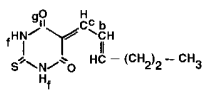
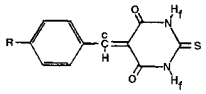
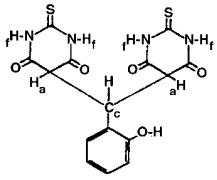
Characterization of TBA–MDA adduct. Elemental analyses (Table 1) were carried out by using a Perkin Elmer (Norwalk, CT) autoanalyzer that determined the percentages of carbon, hydrogen, nitrogen, and sulfur in the organic matrix, by a combustion process (1000°C) followed by a gas-chromatography determination. Thin-layer chromatography (TLC) (TLC Spreader-Quickfit, London, United Kingdom) was used, consisting of Silica gel GF_{254} as an adsorbent, *n*-butanol/ethanol/acetic acid/water (2:2:1:5, by vol) as the developing solvent system, and with a developing time of 3 h. The infrared spectrum (IR, KBr) was registered on a Bomen-Michelson 100 spectrophotometer (Quebec, Canada). The UV-visible spectra were recorded on a Spectronic 3000 Milton Roy spectrophotometer (Milton Roy Company®, Rochester, NY). The ^1H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra of the adduct in deuterated dimethylsulfoxide (DMSO-d_6), and tetramethylsilane (TMS) as an internal standard were measured on a Brucker WP spectrometer (Wissembourg, France) by using 200 MHz and 50 MHz fields, respectively. The mass spectrum could not be obtained with the instrumentation available. Two replicates were accomplished for each procedure to confirm the results obtained.

RESULTS AND DISCUSSION

TBA reacts easily with aldehydes and ketones due to its high reactivity at the C-5 location in the molecule. When aldehydes react with TBA through a Knoevenagel condensation (16), alkylidene or arylidene derivatives of TBA are formed. This reaction follows the Perkin condensation mechanism (17). With aliphatic, aromatic, and heterocyclic aldehydes, compounds of structure 1 or 2 (Table 2) are obtained. Both 1 and 2 have the same structure. With dialdehydes, a structure similar to that presented in Figure 1 is formed. In some cases, as with salicylaldehyde, a bis-derivative (structure 3, in Table 2), the 5-(salicylidene)bis-2-thiobarbituric acid, is achieved (18).

In the TBA–MDA adduct synthesis, the same yield (93%) was obtained by the two procedures used. Table 1 lists the results of elemental analyses; they are compared with the calculated formula, for the chloro-monohydrated form

TABLE 2
^1H Nuclear Magnetic Resonance (NMR) Data and Other Characteristics for TBA–Aldehyde Compounds

	Data obtained (δ, ppm)[a]	Data from Nair and Turner (20) (δ, ppm)[a]	Data from Kosugi *et al.* (28) (δ, ppm)	Data from Guillén-Sans and Guzmán-Chozas (30) (δ, ppm) 1[b]	2[c]	3[d]	Assignment
Structure							
Type of aldehyde	Aliphatic (MDA)	Aliphatic (MDA)	Aliphatic (2-hexenal)	Aromatic (anisaldehyde)	Aromatic (*p*-dimethylamino-benzaldehyde)	Aromatic (salicylaldehyde)	
	—	—	—	—	—	3.42 (*dd*)[e]	a
Signals	8.54 (*t*)[f]	8.56 (*t*)	7.85 (*dd*)	—	—	—	b
	7.69 (*d*)[g]	7.72 (*d*)	8.06 (*d*)	8.27 (*s*)	8.15 (*s*)	1.05 (*t*)	c
	5.10 (*s*)[h]	6.11 (*s*)	8.86 (*s*); 8.95 (*s*)	—	—	5.10 (*s*); 5.50 (*s*)	f
	11.54 (*s*)	—	—	12.23 (*s*); 12.32 (*s*)	11.95 (*s*); 12.05 (*s*)	11.90 (*s*); 12.28 (*s*)	f
	11.54 (*s*)	11.52 (*s*)	8.86 (*s*); 8.95 (*s*)	—	—	—	g
Field (MHz)	200	360	400	80	80	80	
Solvent	DMSO-d_6	DMSO-d_6	Cl_3CD	DMSO-d_6	DMSO-d_6	DMSO-d_6	
TBA–aldehyde ratio	2:1	2:1	1:1	1:1	1:1	2:1	
Color	Dark violet	Dark red-violet	Colorless	Yellow	Rough-red	White	

[a]See structures A and B (Fig. 1) for assignments, [b]5-(Anisylidene)-2-thiobarbituric acid, R: -OCH_3, [c]5-(*p*-dimethylaminobenzylidene)-2-thiobarbituric acid, R: -$N(CH_3)_2$, [d]5-(salicylidene)-bis-2-thiobarbituric acid, [e]double doublet, [f]triplet, [g]doublet, [h]singlet. See Table 1 for other abbreviation. DMSO, dimethylsulfoxide.

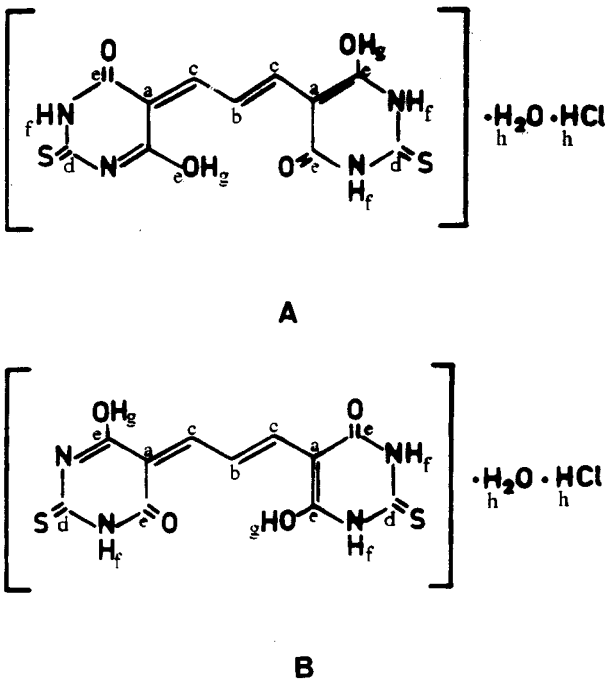


FIG. 1. The two tautomeric structures (A and B) for the red 2:1 thiobarbituric acid–malonaldehyde adduct. Small letters (a, b, ... g, h) refer to the different carbon atoms (a, b, c, d, e) and hydrogen atoms (f, g, h) of the molecule.

$C_{11}H_{11}N_4O_5S_2Cl$. By applying TLC, a sole pink spot was obtained, that was confirmed by UV light (254 nm), with an R_f (average) ± S_{n-1} = 0.92 ± 0.03 (n = size sample = 5), slightly higher than that reported in the literature (19).

In aqueous medium (pH = 2.9) the adduct showed three characteristic absorption maxima at λ_{max} = 532 nm (ε = molar absorptivity = $1.46.10^5$ L·mole^{-1}·cm^{-1}), 310 nm (ε = $1.14.10^4$ L·mole^{-1}·cm^{-1}), and 245 nm (ε = $2.23.10^4$ L·mole^{-1}·cm^{-1}); the pigment was quite stable in this medium. These results agree with those offered in the literature (12,20). From these results it could be inferred that the system is highly conjugated.

The IR spectrum exhibited the characteristic bands of the TBA–MDA adduct. Thus, the amide -NH stretching vibrations at 3134 cm^{-1} (broad peak), 3066 cm^{-1}, and 2925 cm^{-1} can be observed (21,22). Also, broad peaks of stretching and bending vibrations of the -OH groups appeared at 3445 and 1368 cm^{-1}, respectively (20). The -C$^{\alpha}$H (exocyclic) group, characteristic of the 5-alkylidene-2-thiobarbituric acids (23), absorbs at 1217 cm^{-1}. The amide I band (carbonyl stretching) and thioamide (-C=S stretching) appeared at 1635 and 1132 cm^{-1}, respectively (24). The -NH bending vibration, at 1500 cm^{-1}, overlapped the -CN stretching of the S=C-NH- group. There was no evidence of thioenolization, because the existence of a strong band at approximately 2500 cm^{-1} (characteristic of a -SH group), and the simultaneous absence of the -C=S stretching band were not observed.

Table 2 lists the most representative assignments from the ^{1}H NMR spectrum of the isolated TBA–MDA adduct. A singlet at δ 5.10 was assigned to the two protons of the -CONH- group (25), in addition to the two protons of the water molecule (monohydrate) and the acid hydrogen of hydrochloric acid (chlorhydrate) (26). A singlet appeared at δ 11.54 that can be assigned to the -OH group of the keto-enol tautomer of the heterocyclic part of the adduct molecule (two hydrogen atoms) and of the hydrogen atom from the remaining -NH group. These assignments agree with the integrations corresponding to five hydrogens (singlet at δ 5.10) and to three hydrogens (singlet at δ 11.54). The enolic hydrogens appeared at low field (δ 11.54) because of the positive character of the hydrogen bond (27).

The signals at δ 7.69 (doublet) assignable to the two vinyl protons (hydrogen$_{(c)}$ atoms in the tautomers A and B, Fig. 1) of the MDA moiety, and at δ 8.54 (triplet) assignable to the vinyl proton of the MDA structure were detected. For the colorless compound 1:1 TBA-2-hexenal a doublet appeared at δ 8.06, that Kosugi *et al.* (28) assigned to the hydrogen of the exocyclic carbon (Table 2), which was equivalent to the c carbons of the TBA–MDA adduct (Fig. 1). As can be observed, the resonance of the -SH group at δ 1.3–1.7 was not apparent (29). This fact confirms the conclusions inferred from the IR spectra.

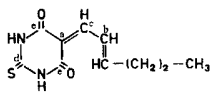
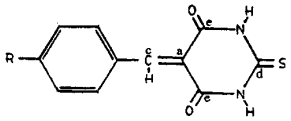
When ^{1}H NMR data from the synthesized TBA–MDA adduct were compared with data obtained from the condensation products of TBA with aromatic aldehydes (Table 2), such as anisaldehyde, *p*-dimethylaminobenzaldehyde, and salicylaldehyde (aldehydes from essential oils of fruits and spices), the -NH signals also appeared as singlets into the δ 11.90–12.32 range (30). These signals are not equivalent because of the nonsymmetrical molecular structures (31). 5-(Salicylidene)bis-2-thiobarbituric acid exhibits a structure similar to the TBA–MDA adduct in which two singlets appear at δ *ca.* 5, and two other singlets appear at δ *ca.* 12, these are assignable to the -NH groups (32).

In Table 2, ^{1}H NMR data and other features for the TBA–aldehyde compounds are summarized. The signal for the vinyl proton of the c carbons (alpha carbon, C$^{\alpha}$, or exocyclic carbon) appeared at δ approximately 8 for compounds 1 and 2 in Table 2. This signal is dependent on the type of solvent used (23,31,32). The keto-form was predominant in the arylidene-2-thiobarbituric acids; this was confirmed by the absence of characteristic signals of the enolic -COH group in the IR and ^{1}H NMR spectra (30,33–35).

Table 3 lists characteristic assignments in the ^{13}C NMR spectrum of the TBA–MDA adduct compared with assignments and properties of other TBA–aldehyde adducts. Quaternary carbons from the heterocyclic ring involved in the exocyclic double bond (assignment a, Table 3) are symmetrical, equivalent carbons absorbing at δ 101.3. A broad peak at δ 161.8 assignable to the thioamide -C=S (assignment d, Table 3) group is observed. Tertiary carbons from the MDA moiety appeared at δ 117.5 and δ 157.4 (assignments b and c, respectively, in Table 3). The ^{13}C NMR absorption at δ 176.4 is assigned to the e carbon (Table 3) of the -NHCO- amide group.

The 11 carbons of the TBA–MDA molecule offer only five ^{13}C NMR signals (symmetrical molecule); three of them cor-

TABLE 3
^{13}C NMR Data and Other Characteristics for TBA–Aldehyde Compounds

	Data obtained (δ, ppm)[a]	Data from Nair and Turner (20) (δ, ppm)[a]	Data from Kosugi *et al.* (28) (δ, ppm)	Data from Guillén-Sans and Guzmán-Chozas (30) (δ, ppm) 1[b]	2[c]	Assignment
Structure						
Type of aldehyde	Aliphatic (MDA)	Aliphatic (MDA)	Aliphatic (2-hexenal)	Aromatic (anisaldehyde)	Aromatic (*p*-dimethylamino benzaldehyde)	
Signals	101.3	101.3	115.9	115.5	109.1	a
	117.5	117.5	156.5	—	—	b
	157.4	157.4	160.5	156.0	154.4	c
	161.8	161.9	179.6	178.2	177.1	d
	176.4	176.3	161.2; 161.9	159.8; 164.0	160.1; 162.6	e
Field (MHz)	50	90.56	100	90	90	
Solvent	DMSO-d_6	DMSO-d_6	THF-d_8	DMSO-d_6	DMSO-d_6	
TBA–aldehyde ratio	2:1	2:1	1:1	1:1	1:1	
Color	Dark violet	Dark red-violet	Colorless	Yellow	Rough-red	

[a]See structures A and B (Fig. 1) for assignments, [b]5-(Anisylidene)-2-thiobarbituric acid, R: -OCH_3, [c]5-(*p*-Dimethylaminobenzylidene)-2-thiobarbituric acid, R:-$N(CH_3)_2$. See Tables 1 and 2 for abbreviations.

respond to distinct quaternary carbons (assignments a, d, e, Table 3), and the other two correspond to tertiary carbons (assignments b and c, Table 3). These assignments are consistent with the distinction of two tautomers (36). Signals for alkylidene-2-thiobarbituric acids, that are presented as data from Kosugi *et al.* (28) column in Table 3 resemble those obtained for the arylidene-2-thiobarbituric acids. Both compound types exhibit a 1:1 TBA–aldehyde stoichiometry, taking into account that other authors (28) employed a different solvent. As can be observed, from the chemical shifts from the 2:1 TBA–MDA adduct and the arylidene-2-thiobarbituric acid only those belonging to exocyclic carbon (assignment c, Table 3) are similar. This behavior seems to indicate that the stoichiometry of these compounds has considerable influences on the ^{13}C NMR signals.

The experimental results obtained are consistent with the existence of two (A and B) spectral equivalent tautomeric structures (Fig. 1). The strong polarization of the exocyclic carbon–carbon double bond can be deduced from the ^{13}C NMR chemical shifts. The resonances for the -C=O carbons of the heterocyclic part of the arylidene-2-thiobarbituric acid molecules differ by about 2 ppm and could be explained in accordance with diverse authors (37–39) either by the different dishielding effect or by a possible steric hindrance occurrence (39). For the 2:1 TBA–MDA adduct only one signal was obtained in this case, due to its symmetry.

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REFERENCES

1. Schmidt, H., Thiobarbituric Acid Number as Measure of The Oxidation of Edible Fats, *Fette Seifen Anstrichm. 61*:127–133 (1959).
2. Vicario, I., R. Guillén-Sans, and M. Guzmán-Chozas, Optimisation du Dosage du TBA de L′Huile d′Olive avec Extraction Unique, *Rev. Franç. Corps Gras 35*:443–446 (1988).
3. Pokorny, J., H. Valentová, and J. Davidek, Modified Determination of 2-Thiobarbituric Acid Value in Fats and Oils, *Die Nahrung 29*:31–38 (1985).
4. Guillén-Sans, R., and M. Guzmán-Chozas, Oxidative Alterations of Edible Vegetable Fats and Oils Measure by the Thiobarbituric Acid Assay: Applied Methodologies, *Rev. Franç. Corps Gras 40*:49–52 (1993).
5. Kim, D.H., and Y.S. Maeng, Relationship Between Rancidity Development and Changes of Physico-Chemical Characteristics of Commercial Deep-Fat Frying Oils During Thermal Oxidation, *Nonglim Noagip 24*:101–112 (1984).
6. Patton, S., and G.W. Kurtz, A Note on the Thiobarbituric Acid Test for Milk Lipid Oxidation, *J. Dairy Sci. 34*:669–674 (1951).
7. Tarladgis, B.G., B.M. Watts, M.T. Younathan, and L.R. Dugan, Jr., A Distillation Method for the Quantitative Determination of Malonaldehyde in Rancid Foods, *J. Am. Oil Chem. Soc. 37*:44–48 (1960).
8. Frankel, E.N., Recent Advances in Lipid Oxidation, *J. Sci. Food Agric. 54*:495–551 (1991).
9. Guillén-Sans, R., and M. Guzmán-Chozas, Aldehydes in Foods and Its Relation with The TBA Test for Rancidity, *Fat Sci. Technol. 7*:285–286 (1995).
10. Guzmán-Chozas, M., I.M. Vicario, and R. Guillén-Sans, Spectrophotometric Profiles of Off-Flavor Aldehydes by Using Their Reaction with 2-Thiobarbituric Acid, *J. Agric. Food Chem. 45*: 2452–2457 (1997).
11. Guillén-Sans, R., and M. Guzmán-Chozas, Thiobarbituric Acid

Condensation Reaction. Spectral and Stability Features of The Chromogens Obtained with Flavor Aldehydes, *Belg. J. Food Chem. Biotechnol. 43*:147–149 (1988).
12. Sinnhuber, R.O., T.C. Yu, and Yu T. Chang, Characterization of the Red Pigment Formed in the 2-Thiobarbituric Acid Determination of Oxidative Rancidity, *Food Res. 23*:626–633 (1958).
13. Dox, A.W., and G.P. Plaisance, The Condensation of 2-Thiobarbituric Acids with Aromatic Aldehydes, *J. Am. Chem. Soc. 38*:2164–2166 (1916).
14. Kitamura, R., and S. Suzuki, Action of Hydrogen Peroxide upon Organosulfur Compounds. VIII. Thiobarbituric Acid and Its Condensation Products with Aldehydes, *J. Pharm. Soc. Japan 57*:659–670 (1937).
15. Guillén-Sans, R., F.J. Bautista, and M. Guzmán-Chozas, Synthesis, Properties and Preliminary Analytical Evaluation of 5-Anisylidene-2-Thiobarbituric Acid, *Boll. Chim. Farm. 124*:115–123 (1985).
16. Knoenevenagel, E., Condensationen Zwischen Malonester un Aldehyden unter dem Einfluss von Ammoniak und Organischen Aminen, *Chem. Ber. 31*:2585–2595 (1898).
17. Sykes, P., *Mecanismos De Reacción En Química Orgánica*, 4th edn., edited by Martínez Roca, S.A., Barcelona, 1973, pp. 234–235.
18. Guillén-Sans, R., and M. Guzmán-Chozas, Spectrophotometric Assay of 5-(Salicylidene)bis-2-Thiobarbituric Acid, *Arch. Pharm. (Weinheim) 321*:65–68 (1988).
19. Siu, G.M., and H.H. Draper, A Survey of the Malonaldehyde Content of Retail Meats and Fish, *J. Food Sci. 43*:1147–1149 (1978).
20. Nair, V., and G.A. Turner, The Thiobarbituric Acid Test for Lipid Peroxidation: Structure of the Adduct with Malonaldehyde, *Lipids 19*:804–805 (1984).
21. Goenechea, S., Infrared Spectroscopic Investigation of Barbiturates, *Z. Anal. Chem. 218*:416–426 (1966).
22. Bult, A., and H.B. Klasen, Ag(I) Complexes of 5,5-Disubstituted Barbituric Acid Derivatives, *Pharm. Weekblad 110*:533–538 (1975).
23. D'Yachkov, A.I., B.A. Ivin, N.A. Smorygo, and E.G. Sochilin, Studies of Pyrimidines. Condensation of 2-Thiobarbituric Acid with Benzaldehydes. Composition and Structure of Reaction Products in Some Solvents, *Zh. Org. Khim. 12*:1115–1122 (1976).
24. Sucharda-Sobczyk, A., and J. Bojarski, Infrared Spectra Near 1700 cm^{-1} of *N*-Acylderivatives of Barbituric Acid, *Rocz. Chem. 44*:2333–2339 (1970).
25. Pretsch, E., T. Clerc, J. Seibl, and W. Simon, *Tablas para la Elucidación Estructural de Compuestos Orgánicos por Métodos Espectroscópicos,* 1st edn., edited by Alhambra, S.A., Madrid, 1980, p. 133.
26. Simon, W., and T. Clerc, Elucidación Estructural de Compuestos Orgánicos por Métodos Espectroscópicos, Vol. I, 1st edn., edited by Alhambra, S.A., Madrid, 1980, pp. 64–86.
27. Pasto, D.J., and C.R. Johnson, *Determinación de Estructuras Orgánicas*, 1st edn., edited by Reverté, S.A., Barcelona, 1974, pp. 177–242.
28. Kosugi, H., T. Kato, and K. Kikugawa, Formation of Yellow, Orange, and Red Pigments in the Reaction of Alk-2-Enals with 2-Thiobarbituric Acid, *Anal. Biochem. 165*:456–464 (1987).
29. Mohacsi, E., Characteristic Nuclear Magnetic Resonance Spectral Positions for Hydrogen in Organic Structures, *J. Chem. Educ. 41*:38 (1964).
30. Guillén-Sans, R., and M. Guzmán-Chozas, Spectroscopy Data of Some Arylidenethiobarbituric Acids, *Pharmazie 43*:415–417 (1988).
31. Ivin, B.A., I.M. D'Yachkov, N.A. Vishnyakov, N.A. Smorygo, and E.G. Sochilin, Pyrimidines. XXI. Structure and Solvolysis of 5-Arylidenebarbituric Acids, *Zh. Org. Khim. 11*:1337–1342 (1975).
32. Bednar, R., E. Haslinger, U. Herzig, O.E. Polansky, and P. Wolschann, Organic Lewis Acids. NMR Spectra of Some Substituted Benzylidenebarbituric Acids, *Monatsh. Chem. 107*:1115–1125 (1976).
33. Gavrilin, G.F., V.E. Chistyakov, and G.A. Kononenko, Tautomerism of 5,5-Disubstituted Barbituric Acids, *Zh. Obshch. Khim. 40*:669–672 (1970).
34. Neville, G.A., H.W. Avdovich, and A.W. By, Physicochemical Properties of Some New 1,5- and 5,5-Alkyl-Substituted Barbituric Acids, *Can. J. Chem. 48*:2274–2279 (1970).
35. Shugar, D., and K. Szczepaniak, Tautomerism of Pyrimidines and Purines in the Gas Phase and in Low-Temperature Matrices, and Some Biological Implications, *Int. J. Quantum Chem. 20*:573–585 (1981).
36. Levy, G.C., and G.L. Nelson, *Resonancia Magnética Nuclear de Carbono 13*, 1st edn., edited by Bellaterra, S.A., Barcelona, 1976, pp. 125–145.
37. Whiteley, M.A., and H. Mountain, Studies in the Barbituric Acid Series (II). 1,3-Diphenyl-2-Thiobarbituric Acid and Some Colored Derivatives, *Proc. Chem. Soc. 26*:121 (1909).
38. Robinson, C.N., and C.C. Irving, Carbon-13 NMR Chemical Shift-Substituent Effect Correlations in *p*-Substituted 5-Benzylidenebarbituric Acids and 2-Benzylidene-1,3-Indanediones, *J. Heterocyclic Chem. 16*:921–923 (1979).
39. Carroll, I.F., and C.G. Moreland, Carbon-13 Nuclear Magnetic Resonance Spectra of 5-Alkyl-5-(1-Methylbutyl)Barbituric Acids, *J. Chem. Soc., Perkin II*:374–376 (1974).

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