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Heterocyclic substituted thiosemicarbazones and their Cu(II) complexes: Synthesis, characterization and studies of substituent effects on coordination and DNA binding

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Abstract

By reacting thiosemicarbazides substituted on the aminic nitrogen with 5-formyluracil, several new 5-formyluracil thiosemicarbazones (H_3ut) derivatives were synthesised and characterized. These ligands, treated with copper chloride and nitrate, afforded two different kinds of compounds. In the complexes derived from copper chloride the metal atom is pentacoordinated, being surrounded by the neutral ligand binding through SNO donor atoms and by two chlorines, while the nitrate derivatives consist of monocations and nitrate anions. The copper coordination (4 + 2) involves the SNO ligand atoms, two water oxygens and an oxygen atom of a monodentate nitrate group. On varying the substituents on the thiosemicarbazidic moiety, remarkable modifications of the coordination geometry are not observed for the complexes with the same counterion. For all the compounds, interactions with DNA (calf thymus) were studied using UV–Vis spectroscopy; the nuclease activity was verified on plasmid DNA pBR 322 by electrophoresis.

Keywords: Thiosemicarbazones; Copper(II) complexes; DNA interactions

1. Introduction

Heterocyclic aromatic thiosemicarbazones show a wide range of biological properties [1–18]. Among the heterocyclic compounds, fluorouracil shows by itself a remarkable antitumor activity and is in use in medical practice [19]; 5-fluorouracil (5FU) is an antimetabolite fluoropyrimidine analog of the nucleoside pyrimidine with antineoplastic activity. Fluorouracil and its metabolites possess a number of different mechanisms of action. In vivo it is converted into the active metabolite 5-fluoroxyuridine monophosphate (F-UMP); replacing uracil, F-UMP incorporates into RNA and inhibits RNA processing, thereby inhibiting cell growth. Another among its active metabolites, 5-fluoro-2′-deoxyuridine-5′-*O*-monophosphate (F-dUMP), inhibits thymidylate synthase, resulting in the depletion of thymidine triphosphate (TTP), a necessary constituent of DNA. Other fluorouracil metabolites incorporate into both RNA and DNA; incorporation into RNA results in major effects on both RNA processing and functions. The study of prodrugs of 5-fluorouracil shows that also its derivatives have a high potential antitumor effect [20] and their study is important in order to overcome the limits and side effects of the parent drug.

On this basis, it is supposed that other modified uracil derivatives are molecules with potential biological properties. In particular in recent years we have already worked on 5-formyluracil thiosemicarbazone, and we have found that its complexes show appreciable biological activity [21–23]. In these compounds in fact a cooperative effect between the thiosemicarbazide and uracil moieties seems to improve the properties of the individual molecules.

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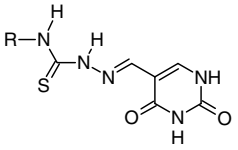


R	Compound	
CH_3-CH_2-	Et-H_3ut	(**1**)
CH_2=CH-CH_2-	Allyl-H_3ut	(**2**)
	Ph-H_3ut	(**3**)
	MePh-H_3ut	(**4**)

Scheme 1. Chemical drawing of the R-H_3ut ligands.

Moreover, uracil provides oxygens as additional potential coordinating sites to the thiosemicarbazidic chain.

The present paper deals with the syntheses and characterization of four 5-formyluracil thiosemicarbazones (H_3ut) with substituents on the aminic nitrogen of the thiosemicarbazide chain. The introduction of the different groups: ethyl-, allyl-, phenyl-, methylphenyl-groups (Scheme 1) is made in order to vary the hydrophilic–lipophilic character of the compounds with the aim of increasing the absorption of the drugs in cells.

Substitutions on the terminal N position can also affect the coordination and biological properties [5,6,10,11,24–29]. Moreover, as small molecules, they could react at specific sites along a DNA strand through a series of weak interactions [30]. Many anticancer drugs exert their antitumor effects through binding to DNA in one way or another, thereby blocking the replication of DNA and inhibiting the growth of tumor cells. The understanding of DNA binding and cleavage is the basis for the design of new, efficient antitumor drugs, their effectiveness depending on the binding mode and affinity towards DNA [31,32]. The biological action mechanism of thiosemicarbazone complexes is not clear or well defined so far. One of the proposed pathways is DNA oxidative damage induced by the copper ion [1]. In this view the presence of a moiety with a potential high DNA affinity could act as a Trojan horse in order to anchor the active metal complex to the target site. Copper complexes are also synthesized and identified by means of different spectroscopic and analytical techniques.

To quantify the DNA binding capability in aqueous solution, a spectroscopic investigation with calf thymus DNA has been carried out. Nuclease activity has been verified on plasmid DNA pBR 322 by electrophoresis.

2. Experimental

2.1. Materials and physical measurements

5-Formyluracil (Aldrich, 98%), 4-ethylthiosemicarbazide (Janssen), 4-allylthiosemicarbazide (Janssen), 4-(3-methyl)-phenylthiosemicarbazide (Janssen), 4-phenylthiosemicarbazide (Janssen), $Cu(NO_3)_2 \cdot 3H_2O$ (Carlo-Erba) and $CuCl_2 \cdot 2H_2O$ (Carlo-Erba) were commercially available and were used without further purification. C, H, N and S analyses were obtained with a Carlo-Erba 1108 instrument. IR spectra were recorded using KBr pellets in the 4000–400 cm^{-1} range and using CsI pellets in the 650–150 cm^{-1} range on a Nicolet 5PC FTIR spectrophotometer. Calf thymus (CT) DNA was obtained from Serva and was used as received. DNA samples were dissolved in 50 mM NaCl/5 mM Tris, pH 7.2. A stock solution of CT-DNA was prepared by dissolving the nucleic acid in this buffer in order to obtain a *ca.* 10^{-5} M in base-pair, [bp] solution that gave ratios of UV absorbance at 260 and 280 nm, A_{260}/A_{280}, of *ca.* 1.8, indicating that the CT-DNA was sufficiently protein free. The concentration of the nucleic acid solutions was determined by UV absorbance at 260 nm after 1:100 dilution of the stock solution. The extinction coefficient ε_{260} was taken as 13100 $M^{-1}\,cm^{-1}$ [33]. Stock solutions were stored at 4 °C and used after no more than 4 days. TRIS and TRIS–acetate–EDTA 10× buffers and agarose were purchased from Sigma. pBR322 DNA plasmid (Roche), DirectLoad Wide-Range DNA marker, ethidium bromide and gel loading solution type I 6× concentrate were from Sigma. Chemical ionization-mass (*m*/*z*, 70 eV) fragmentation patterns were obtained, using samples dissolved in methanol, with a Finnegan 1020 6c mass spectrometer equipped with a quadrupole mass selector MATSSQ 710. Melting points were determined with a Gallenkamp instrument. UV measurements were performed on a Perkin–Elmer UV/Vis Lambda 25 spectrometer with quartz cuvettes. Thermogravimetric analyses were performed on a Perkin–Elmer TGA 7 from 30 °C to 450 °C with a 20 °C/min gradient in N_2 atmosphere. Conductivity measurements were performed on a Mayo International Srl digital conductimeter with samples dissolved in absolute EtOH. NMR spectra were recorded on a Bruker AC300 Spectrometer.

2.2. Synthesis of the ligands

5-Formyluracil thiosemicarbazones (**1**), (**3**) and (**4**) (R-H_3ut, Scheme 1) were synthesized by dissolving ca. 0.80 mmol of aldehyde in 20 mL of water at reflux temperature and adding dropwise an equimolar amount of thiosemicarbazide previously dissolved by refluxing in 20 mL of the same solvent. To the 5-formyluracil solution a couple of drops of HCl were added as a catalyst. The mixture was left under magnetic stirring at reflux temperature for 3 h. All compounds afforded shining yellow powders with cooling, which were filtered on Buchner funnel, washed several times with H_2O and allowed to dry in air. The same method has already been used to synthesize ligand **2** [18,34].

2.2.1. Et-H_3ut (*1*)

Yield: 129 mg (67%). *Anal.* Calc. for $C_8H_{11}N_5O_2S$: C, 39.83; H, 4.59; N, 29.03; S, 13.29. Found: C, 40.15; H, 4.46; N, 28.47; S, 13.43%. Main IR peaks (KBr, cm^{-1}): ν(N–H) 3434 m and 3169 w; ν(C–$H_{aliphatic}$) 2973 w;

ν(C=O) 1711 vs and 1675 vs; ν(C=N) 1631 s; ν(C=S) 772 m. UV–Vis (DMF, nm, M^{-1} cm^{-1}) 270 (18900), 334 (21000). MS (*m*/*z*, %) MH^+ (242, 85%); ^{1}H NMR ($CDCl_3$, ppm): 11.55 (CSN*H*, 1H, br s), 9.95 (N3H_{ur}, 1H, s), 9.55 (N1H_{ur}, 1H, s), 8.51 (CH_{ur}, 1H, s), 8.03 (CH_2N*H*, 1H, t), 7.86 (C*H* = N, 1H, s), 3.05 (CH_2, 2H, m), 1.14 (CH_3, 3H, t). ^{13}C NMR ($CDCl_3$, ppm): 178.3 (*C*S), 175.9 (*C*H_2N) 164.0 (*C*4$_{ur}$), 151.4 (*C*2$_{ur}$), 146.5 (*C*6$_{ur}$), 113.7 (*C*5$_{ur}$), 132.2 (*C*HN), 38.1 (*C*H_3). M.p. 185 °C.

2.2.2. Allyl-H_3ut (**2**)

Yield: 135 mg (65%). *Anal.* Calc. for $C_9H_{11}N_5O_2S$: C, 42.68; H, 4.38; N, 27.65; S, 12.66. Found: C, 42.31; H, 4.48; N, 27.54; S, 12.92%. Main IR peaks (KBr, cm^{-1}): ν(N–H) 3163 m; ν(C–H$_{aromatic}$) 3014; ν(C=O) 1713 vs and 1672 vs; ν(C=N) 1630 m; ν(C=S) 793 m. UV–Vis (DMF, nm, $M^{-1}cm^{-1}$) 270 (20800), 335 (23300). MS (*m*/*z*, %) MH^+ (254, 72%); ^{1}H NMR ($CDCl_3$, ppm): 11.61 (CSN*H*, 1H, br s), 10.03 (N3H_{ur}, 1H, s), 9.58 (N1H_{ur}, 1H, s), 8.56 (CH_{ur}, 1H, s), 8.10 (CH_2N*H*, 1H, t), 7.90 (C*H* = N, 1H, s), 5.88 (C*H* gem, 1H, m), 5.12 (CH_2 *cis* + *trans*, 2H, m), 4.22 (CH_2NH, 2H, m). ^{13}C NMR ($CDCl_3$, ppm): 179.1 (*C*S), 164.5 (*C*4$_{ur}$), 152.1 (*C*2$_{ur}$), 147.5 (*C*6$_{ur}$), 135.5 ($CH_2 = CHCH_2$), 115.1 ($CH_2 = CHCH_2$), 113.9 (*C*5$_{ur}$), 134.2 (*C*HN), 45.3 (*C*H_2N). M.p. 192 °C.

2.2.3. Ph-H_3ut (**3**)

Yield: 162 mg (68%). *Anal.* Calc. for $C_{12}H_{11}N_5O_2S$: C, 49.82; H, 3.83; N, 24.21; S, 11.08. Found: C, 50.02; H, 4.03; N, 24.33; S, 10.98%. Main IR peaks (KBr, cm^{-1}): ν(N–H) 3391 s and 3204 w; ν(C–H$_{aromatic}$) 3045; ν(C=O) 1726 mw and 1668 vs; ν(C=N) 1621 m; ν(C=S) 784 m. UV–Vis (DMF, nm, M^{-1} cm^{-1}) 270 (23900), 351 (26010). MS (*m*/*z*, %) MH^+ (290, 78%); ^{1}H NMR ($CDCl_3$, ppm): 11.60 (CSN*H*, 1H, br s), 10.10 (N3H_{ur}, 1H, s), 9.52 (N1H_{ur}, 1H, s), 8.54 (CH_{ur}, 1H, s), 8.10 (Ph-N*H*, 1H, s), 7.85 (C*H* = N, 1H, s), 7.54–7.00 (CH_{ar}, 5H, m). ^{13}C NMR ($CDCl_3$, ppm): 178.8 (*C*S), 165.1 (*C*4$_{ur}$), 151.8 (*C*2$_{ur}$), 146.4 (*C*6$_{ur}$), 114.2 (*C*5$_{ur}$), 126.7–124.8 (*C*$_{ar}$), 134.2 (*C*HN). M.p. 201 °C.

2.2.4. MePh-H_3ut · H_2O (**4**)

Yield: 195 mg (74%). *Anal.* Calc. for $C_{13}H_{15}N_5O_3S$: C, 48.59; H, 4.70; N, 21.79; S, 9.98. Found: C, 48.49; H, 4.68; N, 21.61; S, 10.19%. Main IR peaks (KBr, cm^{-1}): ν(O–H) 3429 bs; ν(N–H) 3251 m and 3200 w; ν(C–H$_{aromatic}$) 3039; ν(C–H$_{aliphatic}$) 2999 w; ν(C=O) 1718 s and 1676 vs; ν(C=N) 1632 m; ν(C=S) 773 m. UV–Vis (DMF, nm, M^{-1} cm^{-1}) 272 (21800), 359 (24300). MS (*m*/*z*, %) $MH^+ - H_2O$ (304, 85%); ^{1}H NMR ($CDCl_3$, ppm): 11.58 (CSN*H*, 1H, br s), 10.10 (N3H_{ur}, 1H, s), 9.54 (N1H_{ur}, 1H, s), 8.54 (CH_{ur}, 1H, s), 8.08 (Ph-N*H*, 1H, s), 7.84 (C*H* = N, 1H, s), 7.55–7.00 (CH_{ar}, 4H, m), 2.31 (CH_3, 3H, s). ^{13}C NMR ($CDCl_3$, ppm): 178.6 (*C*S), 165.4 (*C*4$_{ur}$), 152.0 (*C*2$_{ur}$), 146.5 (*C*6$_{ur}$), 114.2 (*C*5$_{ur}$), 128.0–124.2 (*C*$_{ar}$), 134.2 (*C*HN), 17.9 (*C*H_3). M.p. 210 °C.

2.3. Synthesis of the Cu(II) complexes

The Cu(II) complexes were synthesised following this general procedure: *ca.* 0.30 mmol of the ligands were dissolved in EtOH (40 mL) with gentle heating. An equimolar amount of $Cu(NO_3) \cdot 3H_2O$ or $CuCl_2 \cdot 2H_2O$ was dissolved in the minimum quantity of the same solvent and added dropwise to the pale yellow ligand solutions. All the resulting mixtures slowly became light green coloured and were left under magnetic stirring at reflux temperature for 1 h. By slow solvent evaporation dark green powders were afforded and characterized.

2.3.1. [Cu(Et-H_3ut)$NO_3(OH_2)_2$]$NO_3 \cdot 2H_2O$ (**5**)

Anal. Calc. for $C_8H_{19}CuN_7O_{12}S$: C, 19.18; H, 3.82; N, 19.57; S, 6.40. Found: C, 19.21; H, 3.78; N, 19.48; S, 6.26%. Main IR peaks (KBr, cm^{-1}): ν(O–H) 3583 m; ν(N–H) 3479 m and 3237 m; ν(C–H$_{aromatic}$) 3056; ν(C–H$_{aliphatic}$) 2919 w; ν(C=O) 1731 s and 1643 vs; ν(C=N) 1613 s; ν(NO_3^-) 1385 vs and 1304 s; ν(C=S) 775 m. Main Far IR peaks (CsI, cm^{-1}): ν(Cu–N) 450 m; ν(Cu–O) 417 w; ν(Cu–S) 348 m. UV–Vis (DMF, nm, M^{-1} cm^{-1}) 257 (11700), 286 (13800), 335 (22000), 648 (165). Λ (Ω^{-1} cm^2 mol^{-1}) 50. TGA (°C, % weight loss) 105, 7; 195, 8; 230, 12; 295, 10; 340, 15.6.

2.3.2. [Cu(Et-H_3ut)Cl_2] · $4H_2O$ (**6**)

Anal. Calc. for $C_8H_{19}Cl_2CuN_5O_6S$: C, 21.46; H, 4.28; N, 15.64; S, 7.16. Found: C, 21.85; H, 3.99; N, 15.73; S, 7.31%. Main IR peaks (KBr, cm^{-1}): ν(O–H) 3424 m; ν(N–H) 3344 m sh and 3248 m; ν(C–H$_{aromatic}$) 3050; ν(C–H$_{aliphatic}$) 2938 w; ν(C=O) 1730 s and 1652 vs; ν(C=S) 758 m. Main Far IR peaks (CsI, cm^{-1}): ν(Cu–N) 451 m; ν(Cu–S) 352 m; ν(Cu–Cl) 315 w and 303 s. UV–Vis (DMF, nm, M^{-1} cm^{-1}) 253 (9200), 285 (11700), 335 (20300), 645 (129), 960 (25). Λ (Ω^{-1} cm^2 mol^{-1}) 32. TGA (°C, % weight loss) 220, 12; 260, 11; 290, 20.

2.3.3. [Cu(Allyl-H_3ut)$NO_3(OH_2)_2$]$NO_3 \cdot 3H_2O$ (**7**)

Anal. Calc. for $C_9H_{21}CuN_7O_{13}S$: C, 20.36; H, 3.99; N, 18.47; S, 6.04. Found: C, 20.10; H, 4.17; N, 18.58; S, 6.42%. Main IR peaks (KBr, cm^{-1}): ν(O–H) 3422 m; ν(N–H) 3185 m; ν(C–H$_{aromatic}$) 3024; ν(C–H$_{aliphatic}$) 2948 w; ν(C=O) 1730 s and 1650 s; ν(C=N) 1600 m; ν(NO_3^-) 1385 vs and 1304 s; ν(C=S) 792 w. Main Far IR peaks (CsI, cm^{-1}): ν(Cu–N) 452 m; ν(Cu–O) 418 w; ν(Cu–S) 350 m. UV–Vis (DMF, nm, M^{-1} cm^{-1}) 250 (8950), 279 (12050), 339 (18300), 649 (108). Λ (Ω^{-1} cm^2 mol^{-1}) 54. TGA (°C, % weight loss) 102, 10; 198, 7; 308, 14.

2.3.4. [Cu(Allyl-H_3ut)Cl_2] · $3H_2O$ (**8**)

Anal. Calc. for $C_9H_{17}Cl_2CuN_5O_5S$: C, 24.47; H, 3.88; N, 15.85; S, 7.26. Found: C, 24.18; H, 4.12; N, 15.65; S, 7.17%. Main IR peaks (KBr, cm^{-1}): ν(O–H) 3433 m; ν(N–H) 3250 m; ν(C–H$_{aromatic}$) 3047; ν(C–H$_{aliphatic}$) 2931 w; ν(C=O) 1729 s and 1652 vs; ν(C=N) 1604 m; ν(C=S) 754 m. Main Far IR peaks (CsI, cm^{-1}): ν(Cu–N) 450 m; ν(Cu–S) 355 m;

ν(Cu–Cl) 316 w and 303 s. UV–Vis (DMF, nm, $M^{-1}\,cm^{-1}$) 252 (9500), 278 (11 620), 335 (21 000), 644 (131), 652 (330). Λ ($\Omega^{-1}\,cm^2\,mol^{-1}$) 22. TGA (°C, % weight loss) 103, 12; 258, 8.5; 288, 9.

2.3.5. *[Cu(Ph-H$_3$ut)(NO$_3$)(OH$_2$)$_2$]NO$_3$ (**9**)*

Anal. Calc. for $C_{12}H_{15}CuN_7O_{10}S$: C, 28.10; H, 2.95; N, 19.12; S, 6.25. Found: C, 28.15; H, 2.63; N, 18.87; S, 6.55%. Main IR peaks (KBr, cm^{-1}): ν(O–H) 3475 m; ν(N–H) 3294 m and 3217 m; ν(C–$H_{aromatic}$) 3024; ν(C–$H_{aliphatic}$) 2939 m; ν(C=O) 1739 s and 1639 s; ν(C=N) 1606 m; ν(NO_3^-) 1385 vs; ν(C=S) 769 w. Main Far IR peaks (CsI, cm^{-1}): ν(Cu–N) 452 m; ν(Cu–O) 417 w; ν(Cu–S) 352 m. UV–Vis (DMF, nm, $M^{-1}\,cm^{-1}$) 265 (13 850), 356 (14 200), 630 (110). Λ ($\Omega^{-1}\,cm^2\,mol^{-1}$) 40. TGA (°C, % weight loss) 189, 8; 230, 15.

2.3.6. *[Cu(Ph-H$_3$ut)Cl$_2$] · H$_2$O (**10**)*

Anal. Calc. for $C_{12}H_{13}Cl_2CuN_5O_3S$: C, 32.62; H, 2.96; N, 15.85; S, 7.26. Found: C, 33.06; H, 2.64; N, 15.57; S, 7.47%. Main IR peaks (KBr, cm^{-1}): ν(O–H) 3429 m; ν(N–H) 3309 w and 3200 m; ν(C–$H_{aromatic}$) 3062 m; ν(C–$H_{aliphatic}$) 2935 w; ν(C=O) 1739 s and 1670 vs; ν(C=N) 1597 m; ν(C=S) 762 m. Main Far IR peaks (CsI, cm^{-1}): ν(Cu–N) 448 m; ν(Cu–S) 349 m; ν(Cu–Cl) 318 w and 303 s. UV–Vis (DMF, nm, $M^{-1}\,cm^{-1}$) 273 (12 920), 360 (14 130), 661 (135). Λ ($\Omega^{-1}\,cm^2\,mol^{-1}$) 24. TGA (°C, % weight loss) 105, 4; 255, 8.5; 292, 9.

2.3.7. *[Cu(MePh-H$_3$ut)NO$_3$(OH$_2$)$_2$]NO$_3$ · 3H$_2$O (**11**)*

Anal. Calc. for $C_{13}H_{15}CuN_7O_{13}S$: C, 26.88; H, 3.99; N, 16.88; S, 5.52. Found: C, 27.11; H, 3.87; N, 17.05; S, 5.47%. Main IR peaks (KBr, cm^{-1}): ν(O–H) 3446 m; ν(N–H) 3290 w and 3095 m; ν(C–$H_{aromatic}$) 3032; ν(C–$H_{aliphatic}$) 2947 vw; ν(C=O) 1737 ms and 1642 ms; ν(C=N) 1607 m; ν(NO_3^-) 1385 vs; ν(C=S) 789 w. Main Far IR peaks (CsI, cm^{-1}): ν(Cu–N) 450 m; ν(Cu–O) 419 w; ν(Cu–S) 350 m. UV–Vis (DMF, nm, $M^{-1}\,cm^{-1}$) 264 (13 100), 360 (14 050), 611 (195). Λ ($\Omega^{-1}\,cm^2\,mol^{-1}$) 45. TGA (°C, % weight loss) 105, 10; M.p. (°C) 164.

2.3.8. *[Cu(MePh-H$_3$ut)Cl$_2$] · 3H$_2$O(**12**)*

Anal. Calc. for $C_{13}H_{19}Cl_2CuN_5O_5S$: C, 31.75; H, 3.89; N, 14.24; S, 6.52. Found: C, 32.05; H, 3.75; N, 14.37; S, 6.29%. Main IR peaks (KBr, cm^{-1}): ν(O–H) 3436 br; ν(N–H) 3255 m; ν(C–$H_{aromatic}$) 3057 m; ν(C–$H_{aliphatic}$) 2927 w; ν(C=O) 1723 s and 1650 vs; ν(C=S) 770 w. Main Far IR peaks (CsI, cm^{-1}): ν(Cu–N) 465 w; ν(Cu–S) 351 m; ν(Cu–Cl) 318 w and 303 m. UV–Vis (DMF, nm, $M^{-1}\,cm^{-1}$) 272 (1050), 373 (12 500), 640 (250). Λ ($\Omega^{-1}\,cm^2 mol^{-1}$) 22. TGA (°C, % weight loss) 105–130, 11; 240, 8; 295, 9.

2.4. DNA interaction studies

Binding constants for the interaction of the studied compounds with nucleic acid were determined as described in [35]. These studies were carried out on the ligands and their nitrate complexes because with their ionic character they are expected to show more interactions towards DNA than the neutral chloro complexes. The intrinsic binding constant K_b for the interaction of the studied compounds with CT-DNA was calculated by absorption spectral titration data using the following equation: $1/\Delta\varepsilon_{ap} = 1/(\Delta\varepsilon K_b D) + 1/\Delta\varepsilon$ where $\Delta\varepsilon_{ap} = |\varepsilon_A - \varepsilon_f|$, $\Delta\varepsilon = |\varepsilon_B - \varepsilon_f|$, $D = [DNA]$, and ε_A, ε_B and ε_f are the apparent, bound and free extinction coefficients of the compound, respectively. K_b is given by the ratio of the slope to intercept from the plot of $[DNA]/(\varepsilon_A - \varepsilon_f)$ versus [DNA] and it is expressed in M^{-1}. The previous equation, originally used to calculate the binding constants for hydrophobic derivatives, is now broadly used to investigate a wide variety of metal complexes containing phenanthroline and its derivatives and it has been adopted to obtain binding constant values from metal complexes with different ligands [23,30,36–39]. Fixed amounts of the ligands and of the complexes were dissolved in DMSO because the high solubility of the compounds in this solvent allowed us to prepare concentrated solutions and therefore to utilize reduced volumes (5 μL) for titrations. It was also verified that the DMSO percentage (0.7%) added to the DNA solution did not interfere with the nucleic acid; in fact, the 260 nm absorption band is not subjected to modifications in intensity and position. Concentrated solutions of NaCl, Tris–HCl (pH 7.2) buffer and DNA were prepared. Calculated amounts of the described stock solutions were taken to final concentration values of 50 mM NaCl, 5 mM Tris–HCl and increasing amounts of DNA over a range of DNA concentrations from 10^{-5} to 10^{-3} M. These solutions were then added to the 5 μL solution of the considered compounds in order to maintain the final volume of the solutions as fixed to 700 μL. The compounds were titrated at room temperature. The changes in absorbance of DNA of an intraligand (IL) band upon each addition were monitored at the maximum wavelengths 334, 335, 351 and 359 nm for **1**, **2**, **3** and **4**, respectively for the ligands, and at the maximum wavelengths 336, 339, 356 and 360 nm for complexes **5**, **7**, **9** and **11**, respectively.

2.5. Nuclease activity

The DNA cleavage was carried out on double stranded plasmid DNA pBR 322 by electrophoresis. Buffers were prepared using sterile distilled water. Solutions composed of 1.6 μL of plasmid (40 μg/mL), 4 μL of the studied compounds dissolved in DMSO (0.1 mM) and 8.4 μL Tris buffer solution (10 mM) were incubated for 1 h at 37 °C. This was followed by addition of a gel loading solution, and each sample was loaded directly into different wells on a 1.5% agarose gel for analysis by electrophoresis at 75 V for 3 h. The gel was stained with ethidium bromide and photographed under UV light in a transilluminator.

3. Results and discussion

3.1. Synthesis and spectroscopy

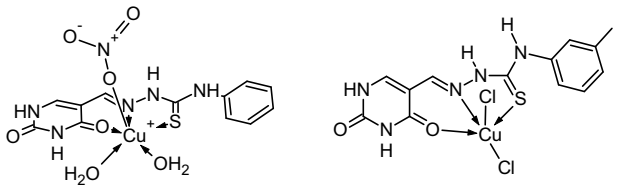
The ligands were synthesised in water in good yields. The products absorbed water giving rise to a gel-like texture and therefore it was necessary to pay attention to the drying step on the Buchner funnel and in air.

The copper complexes were synthesised by the direct reaction of the inorganic salt solutions with the ligand solutions. Under the experimental conditions used both monovalent anions are always present and therefore no ligand deprotonation is observed. The synthesis of the complexes is reproducible and the thiosemicarbazones coordinate as tridentate ligands in their neutral form (H_3ut). The elemental analyses are in agreement with the general formula $M(R\text{-}H_3ut)X_2 \cdot nH_2O$, where X is the counterion. Conductivity measurements show that complexes bearing nitrate as counterions behave as 1:1 electrolyte type compounds and therefore are monocationic [40], while complexes bearing chlorine as counterions behave as neutral molecular complexes. Thermogravimetric analyses show that two water molecules are directly bound to the metal center in the nitrate derivatives. Therefore, as a consequence of the inorganic anionic nature, two types of stoichiometries are exhibited, namely $[Cu(R\text{-}H_3ut)NO_3(OH_2)_2]NO_3$ and $[Cu(R\text{-}H_3ut)Cl_2]$ (see Scheme 2).

These observations agree with previous results [23].

3.2. IR and electronic spectra

The infrared spectral data of the ligands and their metal complexes are reported in Section 2 with their tentative assignments. It is interesting to note that the uracyl moiety owns two carboxyl groups but only the one whose ν(C=O) is assigned at *ca.* 1670 cm^{-1} is involved in coordination. Upon metal binding this carboxyl stretching is shifted downwards by 20–30 cm^{-1} while ν(C=O) not directly linked to the metal is shifted to higher frequencies because of inductive effects arising from the metal coordination. On coordination of the azomethine nitrogen, ν(C=N) shifts to lower wavenumbers by 15–25 cm^{-1}, as the band shifts from *ca.* 1630 cm^{-1} in the uncomplexed thiosemicarbazone spectra to *ca.* 1600 cm^{-1} in the spectra of the complexes. Coordination of the azomethine nitrogen is confirmed with the presence of bands at *ca.* 450 cm^{-1} (at 465 cm^{-1} for complex **12**) assignable to ν(Cu–N) for these complexes. The decrease in the stretching frequency of the C=S bond from *ca.* 770–790 cm^{-1} in the thiosemicarbazones upon complexation indicates coordination *via* the sulfur atom. The presence of a new band in the 345–355 cm^{-1} range assignable to ν(Cu–S), is another indication of the involvement of sulfur coordination. The presence of an absorption at *ca.* 420 cm^{-1} in the spectra of the complexes is assignable to ν(Cu–O). The IR spectra of the chloro complexes also display bands at *ca.* 315 and 300 cm^{-1}, characteristic of coordinated ν(Cu–Cl).



Scheme 2. Hypothesized structure for complex **9** (left) and **10** (right) deduced from spectroscopic data.

The electronic spectra of the complexes in DMF solution are presented in Section 2. Each thiosemicarbazone and its copper(II) complexes have ring $\pi \rightarrow \pi^*$ bands in the range 260–312 nm and $n \rightarrow \pi^*$ bands in the range 312–357 nm. The ligand field strength of the sulfur atom has been found to vary more than that of any other donor atom, with variations of up to 50% being known [40]. This is probably due to the variation in π-bonding effects according to whether the sulfur atom has two or three lone pairs, as in thioketones (>C=S) or mercaptide ions (≡C–S^-), which allows ample scope for M-S π-bonding. The complex spectra have bands in the range 330-357 nm which must be due to L → M transfer from sulfur to copper [41].

3.3. Absorption spectral features of DNA binding

The binding constants obtained for ligands **1**, **2**, **3** and **4** are 2.1×10^3, 1.0×10^3, 2.9×10^2 and 5.6×10^3, respectively. The binding constants obtained for complexes **5**, **7**, **9** and **11** are 2.5×10^2, 5.9×10^2, 2.3×10^1 and 2.3×10^3, respectively. In Fig. 1 the plot of $[DNA]/(\varepsilon_A - \varepsilon_f)$ versus [DNA] for absorption titration of the studied complexes is reported, useful for obtaining K_b by the ratio of the slope to intercept.

Our experimental K_b values are lower than those observed for classical intercalators (ethidium-DNA, 1.4×10^6 in 25 mM Tris–HCl/40 mM NaCl buffer, pH 7.8 [42]; 3.0×10^6 in 5 mM Tris–HCl/50 mM NaCl buffer, pH 7.2 [this work]), indicating that the compounds bind DNA with less affinity, as already noticed for copper(II) complexes with macrocyclic ligands [36]. It is interesting to note that the metal complexes reported in this work bind to DNA with less affinity than copper complexes with aliphatic thiosemicarbazones [37], complexes synthesised with formyluracil thiosemicarbazones bearing less bulky substituents on the side chain [23] and copper complexes with thiosemicarbazide [43]. Since upon complexation no significant increase of K_b was observed, the constant values can probably be related to the role played by the ligand in the binding to DNA. The terminal NH group seems to play an important function in the binding process: as the access to the NH becomes more difficult with the presence of bulky substituents, the association to the nucleic acid becomes weaker. Compounds **4** and **11**, having the bulkier methylphenyl substituent, show a major affinity towards the nucleic acid with respect to the phenyl analogues **3** and **9** (K_b 5.6×10^3 (**4**) versus 2.9×10^2 (**3**) and K_b

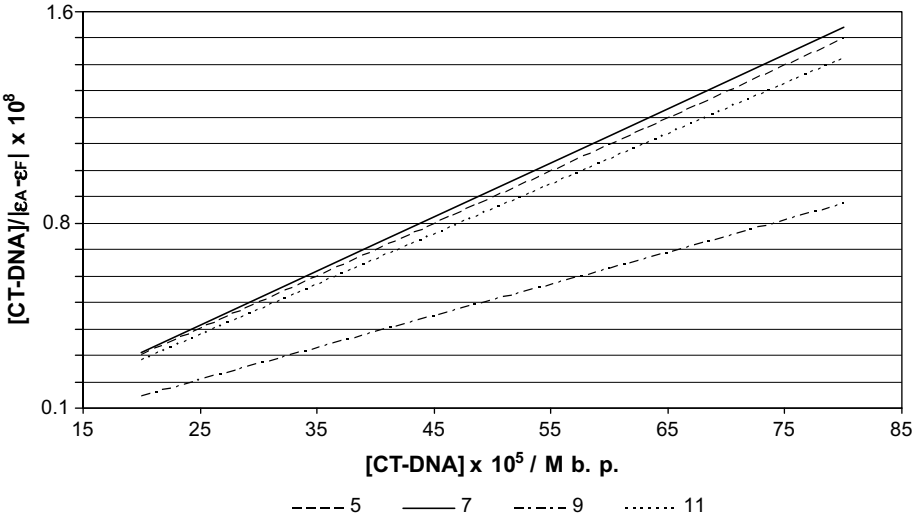


Fig. 1. Typical plots of $[DNA]/(\varepsilon_A - \varepsilon_f)$ vs [CT-DNA] for absorption titration with CT-DNA in 50 mM NaCl/5 mM Tris buffer, pH 7.2 at 25 °C.

2.3×10^3 (**11**) versus 2.3×10^1 (**9**)). This effect can probably be ascribed to the major deviation from planarity of the overall thiosemicarbazonic system due to the methyl substituent on the aromatic ring, as observed in N terminal substituted thiosemicarbazones [28] that decreases the steric hindrance on increasing the accessibility to the aminic group. The weak interactions between our compounds and the nucleic acid are also confirmed by a negligible hypochromism and bathochromism in the electronic absorption spectra of DNA bound to different compounds. These phenomena are generally attributed to intercalation, involving a strong stacking interaction between the aromatic chromophores and the base pairs of DNA. The ratio r ($r = [CT\text{-}DNA]/[complex]$) varied between 0 and 15 in 5 mM Tris–HCl buffer (pH 7.2), 50 mM NaCl at 25 °C. Our results are in agreement with the fact that the glycosidic bond of a formyluracil residue derivative in DNA is about 3 orders of magnitude more labile than that of the parent pyrimidine, thymine and is assignable to the electron-withdrawing effect of the 5-formyl substituent [44]. Additionally, the electron-withdrawing substituent destabilizes the *N*-glycosidic bond, rendering oligonucleotides containing formyluracil, highly susceptible to hydrolysis. The thiosemicarbazidic chain in the five position of the uracil moiety could additionally form potentially irreversible covalent cross-links with an amino group of DNA-binding proteins, resulting in locking the binding instead of blocking binding [44]. The presence of substituents on the terminal nitrogen seems to weaken the capability of bonding towards DNA.

3.4. Nuclease activity

In addition to the studies carried out with CT-DNA, a gel electrophoresis experiment using pBR322, a circular plasmid DNA, was performed with all our compounds. From the results (data not shown) it was verified that at millimolar concentrations for all tested compounds the supercoiled was the predominant form of DNA followed by a slight amount of the open circular form, indicating that there is no significant breaking of plasmid-DNA due to oxidative stress or phosphodiester hydrolysis.

4. Conclusions

As hypothesized in the introduction, the presence of a substituent on the thiosemicarbazidic chain strongly affects the DNA interactions of thiosemicarbazones and their metal complexes. The variation of the hydrophilic/lipophilic character due to the different alkylic or aromatic groups could vary the uptake by the cells but this feature has no consequence on the direct effects of the compounds towards DNA; on the contrary, with respect to the unsubstituted analogous compounds, the free NH_2 thiosemicarbazone moiety seems to play an important role in DNA binding. The presence of the substituents, on the other hand, has no consequence on the coordination mode that, on the contrary, seems to be more influenced by the counterion.

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