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Synthesis of Aryl-1,2,4,5-tetrazinane-3-thiones, *in vitro* DNA binding studies, nuclease activity and its antimicrobial activity

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ABSTRACT

A series of small molecules with a tetrazine scaffold (**5–8**) was designed and synthesized as metal free DNA cleaving agent. These compounds (**5–8**) were characterized by using various spectroscopic (*via*; IR, ^{1}H, ^{13}C NMR and ESI-MS) and analytical methods. The interaction studies of (**5–8**) with CT DNA were carried out by using various biophysical techniques, which showed high binding affinity of **7** and **8** towards CT DNA. Gel electrophoresis pattern demonstrated that the compounds are efficient artificial nuclease and prefers minor groove binding site. The mechanistic pattern showed that compounds follow hydrolytic pathway for DNA cleavage. *In vitro* antibacterial screening of these tetrazine-thiones (**5–8**) gives remarkable results both against gram-positive and gram-negative strains. These compounds also possess moderate antifungal activity when compared with the standard drugs.

1. Introduction

Heterocyclic chemistry, as an applied science is an inexhaustible resource of novel compounds [1]. The majority of pharmaceutical products that mimic natural products with biological activity are heterocycles. Therefore, researchers are on a continuous pursuit to design and produce better pharmaceuticals, by following natural models. In the past decade, numerous small molecules possessing azine scaffold have been shown to exhibit a great variety of pharmacological effects. Various reports have been published on the application of such molecules, such as 5-lipoxygenase (5-LO) inhibitors [2], herbicides, bactericides, fungicides, antimicrobials [3], and gonadotropin-releasing hormone receptor (GnRH-R) antagonists [4]. Even for fused azine compounds not only antitumor and antimetastatic activities against a wide range of cancer cells but also kinase inhibiting activities could be observed [5]. In 2007, cancer accounted for about 7.9 million death cases (around 13% of all deaths). Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030 [6].

In the field of molecular biology and drug development, the cleaving agents of nucleic acid have attracted extensive attention due to their potential applications [7]. Metal complexes have been widely investigated as cleaving agents of nucleic acids and found to be reasonably efficient [8], but their use in pharmacy is restricted because of serious issues over the Lability and toxicity produced due to the free radical generation of some transition metal during the redox processes [9]. Göbel and co-workers have put forward the idea of metal-free cleavage agents. Although, the metal-free cleaving agents studies were mainly applied to active phosphodiesters, for example, nucleic acid mimic and RNA [10]. Under uncatalyzed physiological conditions, the phosphodiester bonds of DNA are extremely stable and the half life of DNA for hydrolysis is estimated to be about 200 million years [11]. Thus, developing new metal free molecules for DNA cleavage is an area of active research.

For the nucleic acid hydrolysis, the key functionality at the active site in natural nuclease *via*, staphylococcal nuclease (SNase) [12] and bovine pancreatic ribonuclease (RNase A) [13], is the positive charge group (guanidinium or ammonium), because of the binding ability to negative groups through hydrogen bonding and electrostatic interaction [14] in biological molecules with the phosphate of DNA or RNA [15,16]. It is reported in literature, that some of the compounds with positive charge groups (ammonium or guanidinium) as nuclease mimics for cleavage of phosphodiester, among those few of them were identified as efficient cleavers of RNA [9,17]. Furthermore, as ammonium or guanidinium group is positively charged, the corresponding compounds might also be with a reasonable aqueous solubility which could be helpful for cell infiltration at physiological conditions.

Herein, we report the synthesis of novel Aryl-1,2,4,5-tetrazinane-3-thiones (**5–8**) as metal free cleaving agents. The presence of —NH and —OH groups in the molecules can cooperatively participate in the interaction with DNA via hydrogen bonding.

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Furthermore, these compounds have also been screened for antibacterial and antifungal activity. It is indeed gratifying to note that these compounds (**5**–**8**) show significantly good antibacterial activity against both gram (+ve) as well as gram (−ve) and fungal strains, selected for the study.

2. Experimental

2.1. Materials and methods

Melting points were measured on a digital Kofler block apparatus and are uncorrected. IR spectra were recorded on Interspec 2020 FT-IR Spectrometer SpectroLab in KBr and only noteworthy absorptions are noted, its values are given in cm^{-1}. ^1H and ^{13}C NMR spectra were run in $CDCl_3$ on a Bruker Avance 400 MHz instrument with TMS as internal standard and its values are given in ppm (δ). Mass spectra were recorded on a JEOL D-300 mass spectrometer. Thin layer chromatography (TLC) plates with 0.5 mm layer of silica gel G and exposed to iodine vapors to check the purity as well as the progress of reaction. All the chemicals used were of analytical grade and were purchased from Sigma Aldrich, Merck and Otto-kemi Pvt. Ltd. The solvents were purified prior to use.

2.2. General method for the preparation of Aryl-1,2,4,5-tetrazinane -3-thiones

To a solution of acetophenone (**1–4**) (10.0 mmol) in ethanol (15 ml) was added thiocarbohydrazide (TCH) (10 mmol) in acetic acid (20 ml) and the reaction mixture was stirred at room temperature for 2–3 h. The yellowish/brownish colored precipitate so obtained was filtered out and washed thoroughly with water, cold ether and air dried. It was crystallized with chloroform ethanol to obtain the corresponding pure solid compound (**5–8**).

2.2.1. 6-Methyl-6-phenyl-1,2,4,5-tetrazinane-3-thione (*5*)

White colored solid; yield: 65%, m.p. 210–212 °C; Anal. Calc. for $C_9H_{12}N_4S$; C, 51.90; H, 5.81; N, 26.90; S, 15.39; found: C, 51.84; H, 5.86; N, 26.95; S, 15.45; IR (KBr) υ cm^{-1}: 3382 (N—H), 1635 (conjugated C=C), 1515 (C—N), 1455 (N—N), 1179 (C=S); for ^1H NMR (ppm, $CDCl_3$): 1.75 (s, 3H), 4.32 (br, 2H, N<u>H</u>—C—N<u>H</u>), 7.52–7.91 (m, $5H_{aromatic}$), 8.77 (s, 2H, —N<u>H</u>—CS—N<u>H</u>) and ^{13}C NMR ($CDCl_3$); 21.2 (CH_3), 74.7 (HN—C—NH), 126.2–149.0 (6C, $C_{aromatic}$), 178.1 (C=S). MS ES+: $[M]^{+\cdot}$ *m*/*z*: 208.

2.2.2. 6-(3-Hydroxyphenyl)-6-methyl-1,2,4,5-tetrazinane-3-thione (6)

Cream colored solid; yield: 65%, m.p. 255–257 °C; Anal. Calc. for $C_9H_{12}N_4OS$; C, 48.18; H, 5.40; N, 24.88; O, 7.15; S, 14.25; found: C, 48.20; H, 5.44; N, 24.94; O, 7.12; S, 14.35; IR (KBr) υ cm^{-1}: 3385 (N–H), 3549 (—OH aromatic), 1637 (conjugated C=C), 1460 (N—N), 1517 (C—N), 1185 (C=S); for ^1H NMR ($CDCl_3$-400 MHz ppm): 1.51 (s, 3H), 4.52 (br, 2H, N<u>H</u>—C—N<u>H</u>), 6.76–7.21 (m, $4H_{aromatic}$), 9.10 (s, 2H, —N<u>H</u>—CS—N<u>H</u>), 9.98 (s, 1H, Ph—OH) and ^{13}C NMR ($CDCl_3$); 22.1 (CH_3), 76.2 (HN—C—NH), 116.7–148.4 (6C, $C_{aromatic}$), 178.3 (C=S). MS ES+[M + Na]$^{+\cdot}$ *m*/*z*: 247.

2.2.3. 6-(4-Hydroxyphenyl)-6-methyl-1,2,4,5-tetrazinane-3-thione (*7*)

Yellow solid; yield: 70%, m.p. 253–255 °C; Anal. Calc. for $C_9H_{12}N_4OS$; C, 48.18; H, 5.40; N, 24.88; O, 7.15; S, 14.35; found: C, 48.22; H, 5.35; N, 24.92; O, 7.12; S, 14.40; IR (KBr) υ cm^{-1}: 3385 (N–H), 3549 (—OH aromatic),1639 (conjugated C=C), 1457 (N—N), 1520 (C—N), 1185 cm^{-1} (C=S); for ^1H NMR ($CDCl_3$): 1.65 (s, 3H), 4.45 (br, 2H, N<u>H</u>—C—N<u>H</u>), 6.79–7.45 (m, $4H_{aromatic}$), 9.14 (s, 2H, —N<u>H</u>—CS—N<u>H</u>), 9.85 (s, 1H, Ph—OH) and ^{13}C NMR ($CDCl_3$): 22.3 (CH_3), 76.8 (HN—C—NH), 117.6–159.2 (6C, $C_{aromatic}$), 178.3 (C=S); MS ES+: $[M + H]^{+\cdot}$ *m*/*z*: 225.

2.2.4. 6-(2,4-Dihydroxyphenyl)-6-methyl-1,2,4,5-tetrazinane-3-thione (*8*)

Yellow solid; yield: 75%, m.p. 260–270 °C; Anal. Calc. for $C_9H_{12}N_4O_2S$; C, 44.99; H, 5.03; N, 23.32; O, 13.32; S, 13.4; found: C, 45.03; H, 5.09; N, 23.37; O, 7.15; S, 14.39; IR (KBr) υ cm^{-1}: 3384 (N–H), 3549, 3555 (—OH aromatic),1635 (conjugated C=C), 1457 (N—N), 1520 (C—N), 1185 cm^{-1} (C=S); for ^1H NMR ($CDCl_3$): 1.58 (s, 3H), 4.46 (br, 2H, N<u>H</u>—C—N<u>H</u>), 6.51–7.45 (m, $3H_{aromatic}$), 8.96 (s, 2H, —N<u>H</u>—CS—N<u>H</u>), 9.10, 9.16 (s, 2H, Ph—OH) and ^{13}C NMR ($CDCl_3$);); 22.5 (CH_3), 76.9 (HN—C—NH), 125.3–158.1 (6C, $C_{aromatic}$), 178.7 (C=S).MS ES+: $[M + 2H]^{+\cdot}$ *m*/*z*:242.

2.3. Binding studies

DNA binding experiments that include absorption spectral traces, luminescence experiments conformed to the standard methods [18,19] and practices previously adopted by our laboratory [20,21].

2.4. Nuclease activity

Cleavage experiments were performed with the help of Axygen electrophoresis supported by Genei power supply with a potential range of 50–500 V, visualized and photographed by Vilber-INFINITY gel documentation system. Cleavage experiments of supercoiled pBR322 DNA (300 ng) by **7** and **8** (10–50 μM) in (5 mM Tris–HCl/50 mM NaCl), buffer at pH 7.2 were carried out and the reaction followed by agarose gel electrophoresis. The samples were incubated for 1 h at 37 °C. A loading buffer containing 25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol was added and electrophoresis was carried out at 60 V for 1 h in Tris–HCl buffer using 1% agarose gel containing 1.0 μg/mL ethidium bromide. The reaction was also monitored upon addition of various radical inhibitors and/or activators such as DMSO, tert-butyl alcohol (TBA), sodium azide (NaN_3), superoxide dismutase (SOD), mercaptopropionic acid (MPA), glutathione (GSH), H_2O_2; groove binders-methyl green and DAPI.

2.5. Antimicrobial activity

2.5.1. Antibacterial studies

The newly prepared compounds were screened for their antibacterial activity against *Escherichia coli* (ATCC-25922), Methicillin resistant *Staphylococcus aureus* (MRSA + Ve), *Pseudomonas aeruginosa* (ATCC-27853), *Streptococcus pyogenes* and *Klebsiella pneumoniae* (Clinical isolate) bacterial strains by disk diffusion method [22,23]. A standard inoculums ($1–2 \times 10^7$ c.f.u./ml 0.5 McFarland standards) was introduced onto the surface of sterile agar plates, and a sterile glass spreader was used for even distribution of the inoculums. The disks measuring 6 mm in diameter were prepared from Whatman No. 1 filter paper and sterilized by dry heat at 140 °C for 1 h. The sterile disks previously soaked in a known concentration of the test compounds were placed in nutrient agar medium. Solvent and growth controls were kept. Ciprofloxacin was used as positive control whereas DMSO was used as negative control. The plates were inverted and incubated for 24 h at 37 °C. The susceptibility was assessed on the basis of diameter of zone of inhibition against gram-positive and gram-negative strains of bacteria. Inhibition zones were measured and compared with the controls.

Minimum inhibitory concentrations (MICs) were determined by broth dilution technique. The nutrient broth, which contained logarithmic serially two fold diluted amount of test compound and

controls were inoculated with approximately 5×10^5 c.f.u./ml of actively dividing bacteria cells. The cultures were incubated for 24 h at 37 °C and the growth was monitored visually and spectrophotometrically. To obtain the minimum bacterial concentration (MBC), 0.1 ml volume was taken from each tube and spread on agar plates. The number of c.f.u. was counted after 18–24 h of incubation at 35 °C. MBC was defined as the lowest drug concentration at which 99.9% of the inoculums were killed.

2.5.2. Antifungal studies

Antifungal activity was also done by disk diffusion method. For assaying antifungal activity *Candida albicans*, *Aspergillus fumigatus*, *Penicillium marneffei* and *Trichophyton mentagrophytes* (recultured) in DMSO by agar diffusion method [22–24]. Sabourands agar media was prepared by dissolving peptone (1 g), D-glucose (4 g) and agar (2 g) in distilled water (100 ml) and adjusting pH to 5.7. Normal saline was used to make a suspension of spore of fungal strain for lawning. A loopful of particular fungal strain was transferred to 3 ml saline to get a suspension of corresponding species. Twenty milliliters of agar media was poured into each Petri dish. Excess of suspension was decanted and the plates were dried by placing in an incubator at 37 °C for 1 h. Using an agar punch, wells were made and each well was labeled. A control was also prepared in triplicate and maintained at 37 °C for 3–4 days. The fungal activity of each compound was compared with Greseofulvin as standard drug. The cultures were incubated for 48 h at 35 °C and the growth was monitored. To obtain the minimum fungicidal concentration (MFC), 0.1 ml volume was taken from each tube and spread on agar plates. The number of c.f.u. was counted after 48 h of incubation at 35 °C. MFC was defined as the lowest drug concentration at which 99.9% of the inoculums were killed. The minimum inhibitory concentration and minimum fungicidal concentration are given in Table 4.

3. Results and discussion

3.1. Characterization

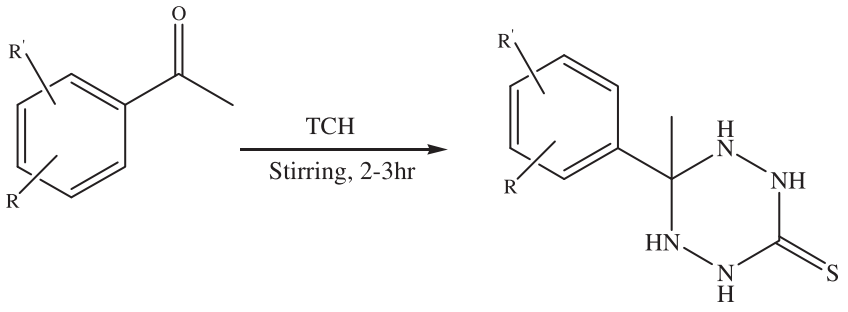
All the four compounds (Scheme 1) were prepared by stirring acetophenone/substituted acetophenones and thiocarbohydrazide (TCH) in the mixture of ethanol and acetic acid. The structures of these compounds were characterized by spectroscopic (IR, ^{1}H NMR, ^{13}C NMR, ESI MS) and analytical methods. IR spectrum of the compounds (**5–8**) exhibited important diagnostic bands at 3382–3385 cm^{-1} and 1179–1185 cm^{-1} attributed to N—H and C=S stretching frequencies respectively. Moreover the absorption band at 3500–3549 cm^{-1} along with aromatic overtone was ascribed to the aromatic phenols. The lack of band for carbonyl group in the IR spectra of all the synthesized compounds and appearance of weak bands at 1455–1460 cm^{-1} (N—N) gave a clear signal for the formation of 1,2,4,5-tetrazine-3-thiones [25].

The ^{1}H NMR spectra of the title compounds displayed signal associated with the methyl protons attached to benzyl carbon as a sharp singlet's integrating for three protons at around 1.75–1.51 ppm. A broad singlet's integrating for two protons each observed at ~4.32–4.52 ppm was assigned to NH—C—NH. The downfield singlet of two protons at 9.10–8.77 ppm was attributed to NH—CS—NH and is due to the influence of C=S groups. The phenolic —OH in case of (**6–8**) was resonating as in independent singlets at 9.98 ppm, 9.85 ppm and 9.10, 9.16 ppm respectively. Aromatic protons were appeared as a multiplet in each case at around 7.52–6.51 ppm. ^{13}C NMR signals are in good agreement with proposed structure of the prepared compounds and the characteristic signal exhibited at 178.1–178.7 ppm due to C=S. The mass spectral data of these compounds showed molecular ion peaks $M^{+\cdot}$ at *m*/*z* 208 for compound (**5**), *m*/*z* 225 $(M + H)^+$ for compound (**6**), at *m*/*z* 247 $(M + Na)^+$ for compound (**7**), and at *m*/*z* 242 $(M + 2H)^+$ for compound (**8**).

3.2. Binding studies

3.2.1. Absorption titration

The interaction of Aryl-1,2,4,5-tetrazinane-3-thiones (**5–8**) with CT DNA was monitored by spectrophotometric titrations in 5 mM Tris–HCl/50 mM NaCl, buffer of pH = 7.4 at 25 °C. The UV spectra of the compounds in methanol showed a significant absorption bands at 205, 260 nm (**5**), 205, 297 nm (**6**), 205, 307 nm (**7**), 206, 254 and 327 nm (**8**), these bands are attributed to π–π^* transitions. The absorption spectra of compounds (**5–8**), in presence and in the absence of CT-DNA are shown in Fig. 1a–d. On increasing the concentration of CT DNA, there was concomitant increase in the absorption intensity – 'hyperchromism' up to 25–40% with a blue shift of 2–4 nm. This strong "hyperchromic" effect with a moderate red shift is suggestive of the compounds possessing a higher propensity



R	R'		R	R	
H	H	(**1**)	H	H	(**5**)
3-OH	H	(**2**)	3-OH	H	(**6**)
4-OH	H	(**3**)	4-OH	H	(**7**)
2-OH	4-OH	(**4**)	2-OH	4-OH	(**8**)

Scheme 1. Synthesis of novel Aryl-1,2,4,5-tetrazinane-3-thiones.

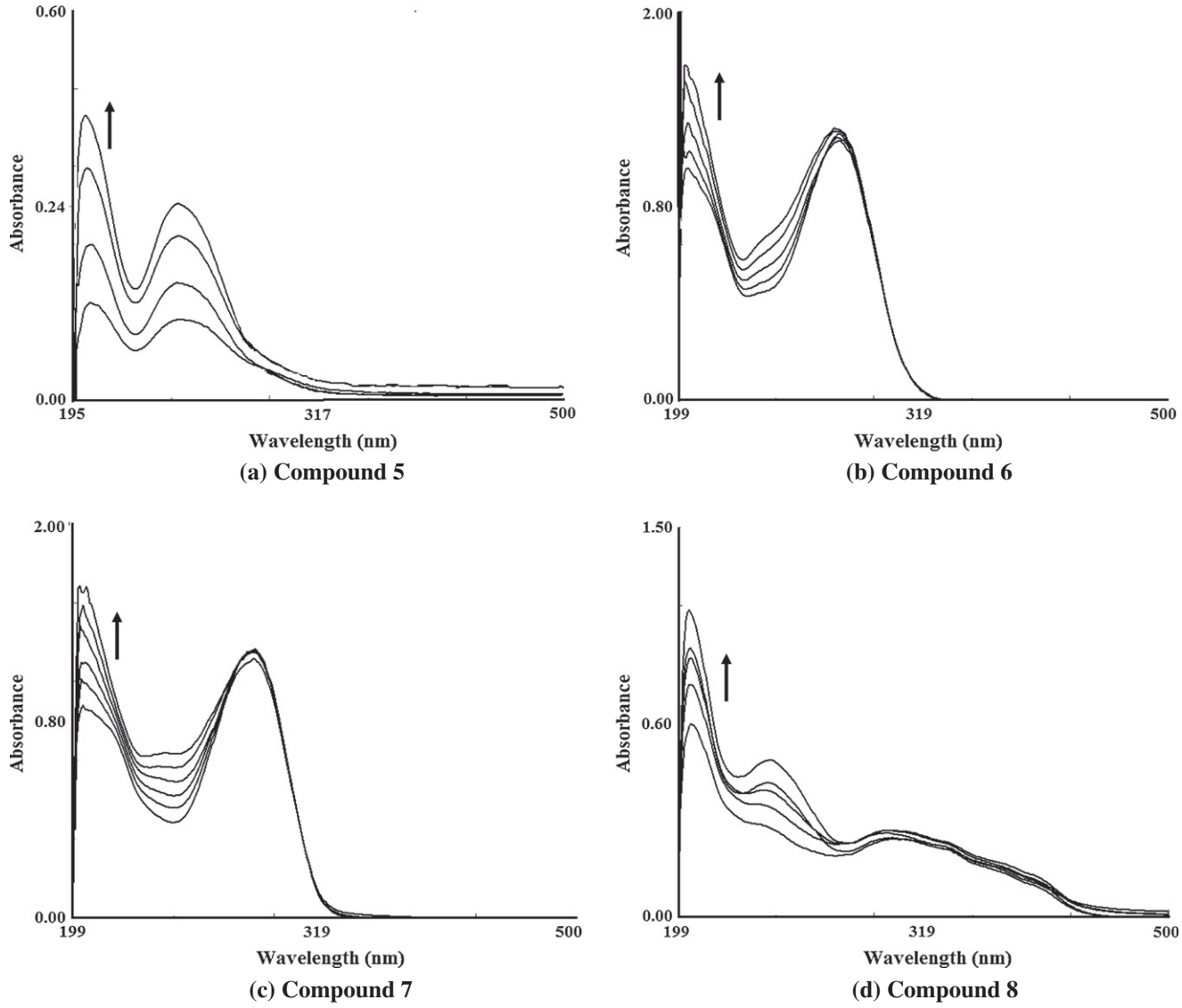


Fig. 1. Variation of UV–vis absorption for tetrazines (a) compound **5**, (b) compound **6**, (c) compound **7** and (d) compound **8**, with increase in the concentration of CT DNA in buffer 5 mM Tris –HCl/50 mM NaCl, pH = 7.2 at room temperature.

for DNA binding via non-intercalative binding *via*, electrostatic and/or hydrogen bonding. Nevertheless, DNA double helix possesses many hydrogen bonding sites positioned on the edges of the DNA bases, it is quite amenable that the —NH groups and —OH groups of the compounds participate in hydrogen bonding with the DNA base pairs [26]. Thus, these compounds bind and electrophilically activate the anionic phosphodiester through hydrogen bonding and electrostatic interaction, whereas hydroxyl groups working as nucleophilic group in the intermolecular transphosphorylation [27].

To study quantitatively, the binding ability of **5**–**8** compounds with CT DNA, the intrinsic binding constant K_b values were determined. The binding constant K_b value follows the order **8** ($6.72 \times 10^4\ M^{-1}$) > **7** ($5.40 \times 10^4\ M^{-1}$) > **6** ($1.99 \times 10^4\ M^{-1}$) > **5** ($0.76 \times 10^3\ M^{-1}$). From the results of the binding constants, it was concluded that the compound **8** revealed a stronger binding affinity for DNA double helix. The relative difference in the K_b values can be associated with the number and the position of —OH groups in the compounds, which facilitates the hydrogen bonding and provides better binding affinity.

3.2.2. Fluorescence studies

3.2.2.1. Steady-state emission titration. The emission spectra of the compound (**5**–**8**) in the absence and in presence of increasing amounts of CT-DNA are depicted in Fig. 2a–d. In the absence of CT-DNA, all four compounds emits moderate luminescence in Tris–HCl buffer at 25 °C with a maxima appearing at 290–340 nm when excited at 260–327 nm. However, the subsequent addition of CT-DNA from 0 to 0.33×10^{-4} M causes gradual enhancement in the fluorescence intensity of the compounds with no apparent change in the shape and position of the emission bands. This implies that the compounds strongly interact with CT-DNA probably due to the inaccessibility of the solvent water molecules to reach the hydrophobic environment inside the DNA helix, and the mobility of the compounds is restricted at the binding site ultimately leading to decrease in vibrational mode of relaxation. However, this enhancement is much less as compared to classical intercalators [28,29]. The binding constant (K) estimated for compounds **5**–**8** by Scatchard equation were $7.92 \times 10^3\ M^{-1}$, $1.31 \times 10^4\ M^{-1}$, $1.99 \times 10^4\ M^{-1}$, and $3.5 \times 10^4\ M^{-1}$ respectively.

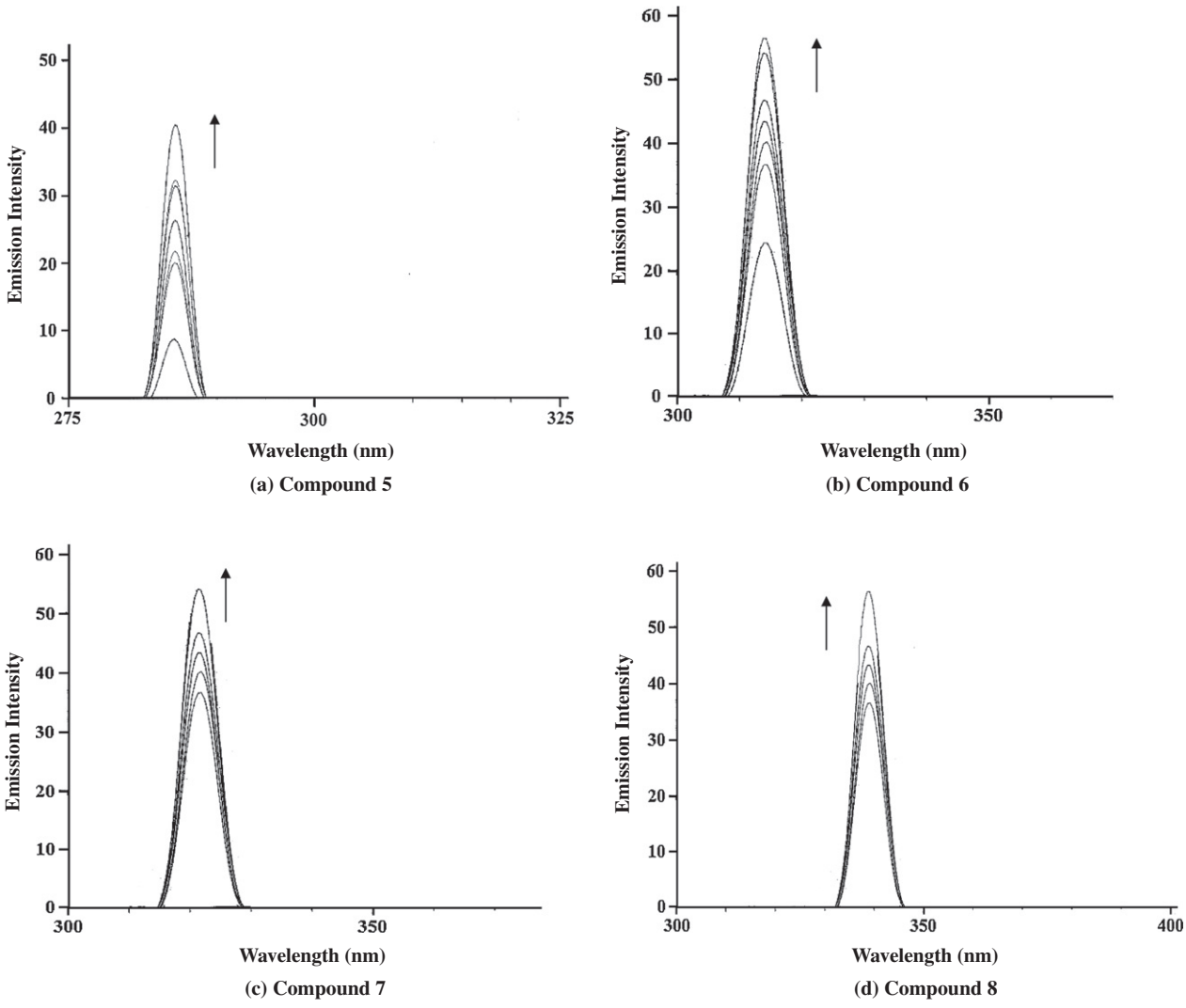


Fig. 2. Emission spectra of tetrazines (a) compound **5**, (b) compound **6**, (c) compound **7** and (d) compound **8**, in the absence and in presence of CT DNA in buffer 5 mM Tris–HCl/ 50 mM NaCl, pH = 7.2 at 25 °C.

3.2.2.2. Ethidium bromide displacement assay. To further investigate the mode and extent of binding of compounds (**5**–**8**), ethidium bromide displacement assay was carried out [30,31]. The molecular fluorophore EthBr emits intense fluorescence in presence of CT DNA due to its strong intercalation between the adjacent DNA base pairs. Addition of second molecule, which binds to DNA more strongly than EthBr, would quench the DNA-induced EthBr by either replacing the EthBr and/or by accepting the excited-state electron of the EthBr through a photoelectron transfer mechanism [32]. The extent of emission quenching of the EthBr bound to DNA would reflect the extent of DNA binding affinity of compounds. On addition of **5–8,** to CT DNA pretreated with EthBr ([DNA]/[EthBr] = 1) a decrease in emission intensity was observed. The emission intensity of EthBr-DNA in the absence and in presence of **5–8,** is depicted in Fig. 3a–d. As there is no complete quenching of emission intensity of the EthBr-DNA adduct induced by all the compounds, thus the intercalative mode of binding is ruled out. The non-replacement based quenching has been suggested with DNA-mediated electron transfer from the excited ethidium bromide to acceptor compounds. Furthermore, quantitatively quenching extent i.e.; K_{sv} was evaluated for compounds **5**–**8** by following the Stern–Volmer equation and were found to be $1.9 \times 10^2\ M^{-1}$, $2.56 \times 10^3\ M^{-1}$, $3.68 \times 10^3\ M^{-1}$, $5.56 \times 10^4\ M^{-1}$, respectively, the values obtained follows the order **8** > **7** > **6** > **5**. These results are consistent with the findings obtained from absorption spectral studies.

3.3. Nuclease activity

The DNA cleaving ability of compound **7** and **8** was studied by agarose gel electrophoresis using supercoiled pBR322 plasmid DNA as a substrate [33,34]. The activity of **7** and **8** was assessed by the conversion of DNA from Form I to Form II or Form III. A concentration-dependent DNA cleavage by **7** and **8** was performed. With the increase of concentration of **7** and **8**, DNA was converted from Form I (supercoiled form) to Form II (nicked circular form), however no conversion to form III (Linear form) was observed in both the compounds. At a 30 μM concentration, **7** exhibited efficient nuclease activity whereas **8** showed significantly good activity at 20 μM. At still higher concentrations there was complete conversion of SC form into NC form as depicted in Figs. 4 and 5.

To predict the mechanism of pBR322 plasmid DNA cleavage by **7** and **8**, comparative reactions were carried out in the presence of various radical inhibitors or trappers [35,36] such as singlet oxygen scavenger sodium azide (NaN_3), hydroxyl radical scavengers via; dimethylsulfoxide (DMSO) t-butyl alcohol (TBA) and superoxide

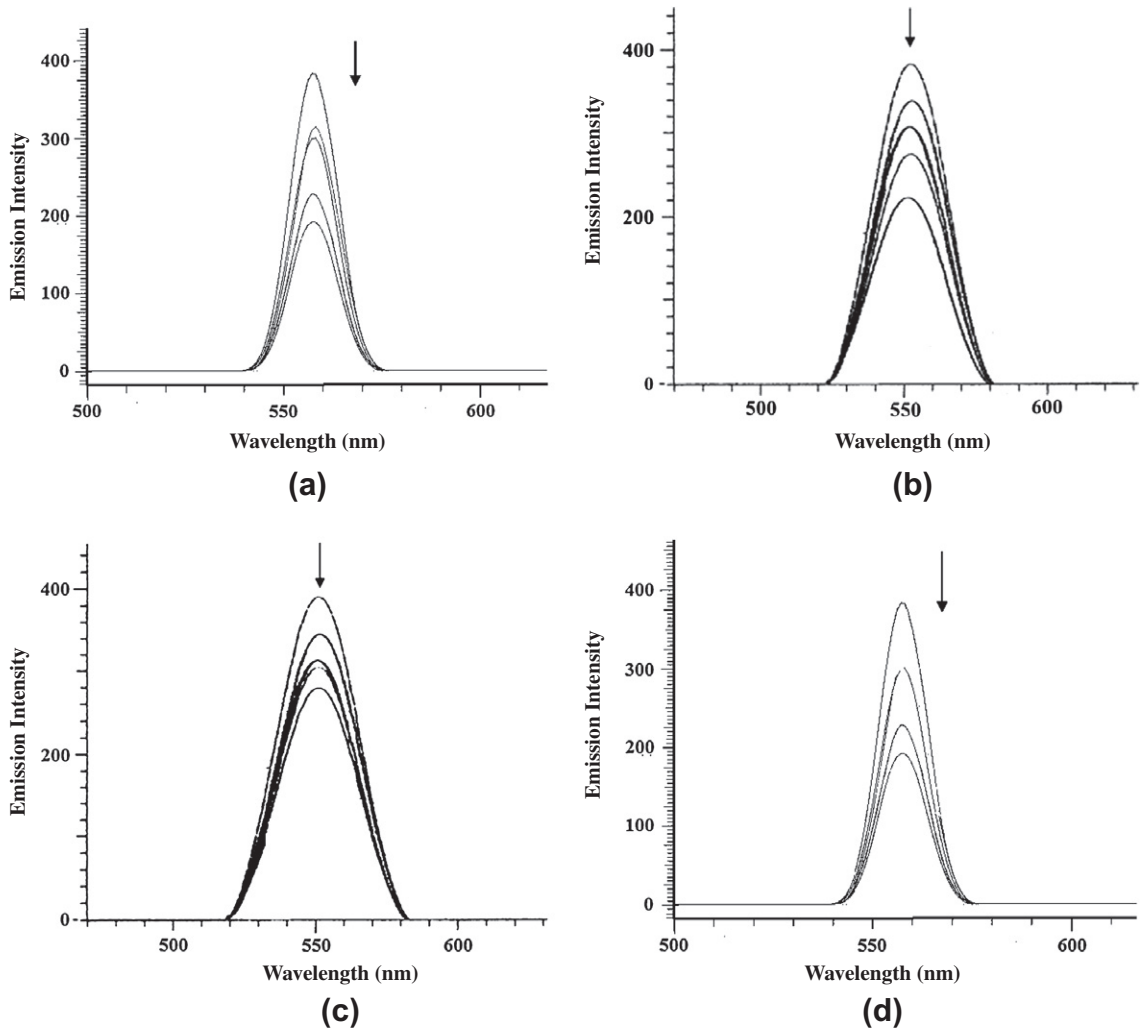


Fig. 3. Quenching spectra of ethidium bromide bound DNA, with increasing concentration of tetrazines (a) compound **5**, (b) compound **6**, (c) compound **7** and (d) compound **8**, in buffer 5 mM Tris–HCl/ 50 mM NaCl, pH = 7.2 at room temperature.[Complex] (6.60–33.30) μM.

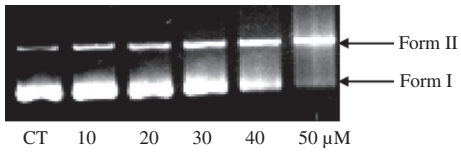


Fig. 4. Agarose gel electrophoresis patterns of pBR322 plasmid DNA (300 ng) cleaved by tetrazine (**7**) (10–50 μM), after 1 h incubation time (concentration dependent) Lane 1: DNA control; Lane 2: 10 μM **7** + DNA; Lane 3: 20 μM **7** + DNA; Lane 4: 30 μM **7** + DNA. Lane 5: 40 μM **7** + DNA; Lane 6: 50 μM **7** + DNA, in buffer (5 mM Tris –HCl/50 mM NaCl, pH = 7.2 at 25 °C).

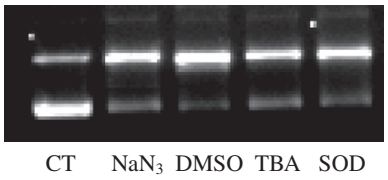


Fig. 6. Agarose gel electrophoresis pattern for the cleavage of pBR322 supercoiled DNA (300 ng) by **7** (30 μM), in presence of different radical scavengers. Lane 1: DNA control; Lane 2: **7** + NaN_3 + DNA. Lane 3: **7** + DMSO + DNA; Lane 4: **7** + t-butyl alcohol + DNA; Lane 5: **7** + SOD + DNA, in buffer (5 mM Tris –HCl/ 50 mM NaCl, pH = 7.2 at 25 °C).

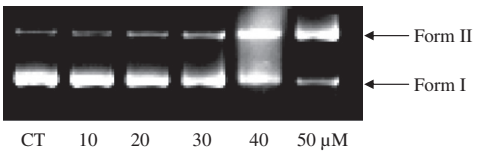


Fig. 5. Agarose gel electrophoresis patterns of pBR322 plasmid DNA (300 ng) cleaved by (**8**) (10–50 μM), after 1 h incubation time (concentration dependent) Lane 1: DNA control; Lane 2: 10 μM **8** + DNA; Lane 3: 20 μM **8** + DNA; Lane 4: 30 μM **8** + DNA. Lane 5: 40 μM **8** + DNA; Lane 6: 50 μM **8** + DNA, in buffer (5 mM Tris –HCl/50 mM NaCl, pH = 7.2 at 25 °C).

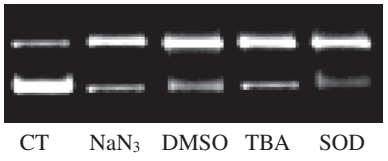


Fig. 7. Agarose gel electrophoresis pattern for the cleavage of pBR322 supercoiled DNA (300 ng) by compound **8** (30 μM) in presence of different radical scavengers.) Lane 1: DNA control; Lane 2: **8** + NaN_3 + DNA. Lane 3: **8** + DMSO + DNA; Lane 4: **8** + t-butyl alcohol + DNA; Lane 5: **8** + SOD + DNA, in buffer (5 mM Tris –HCl/ 50 mM NaCl, pH = 7.2 at 25 °C).

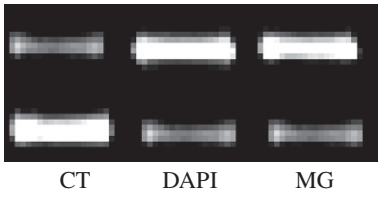


Fig. 8. Agarose gel electrophoresis pattern for the cleavage of pBR322 supercoiled DNA (300 ng) by **7**, in presence of minor groove (DAPI) and major groove binding agent (methyl green).

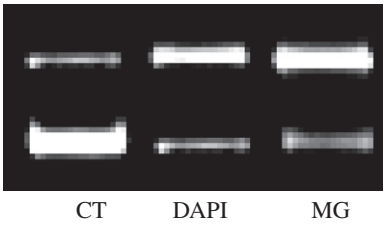


Fig. 9. Agarose gel electrophoresis pattern for the cleavage of pBR322 supercoiled DNA (300 ng) by **8**, in presence of minor groove (DAPI) and major groove binding agent (methyl green).

Table 1
Antibacterial activity of Tetrazines (**5**–**8**).

Compounds	Diameter of zone of inhibition (mm)				
	Gram positive bacteria		Gram negative bacteria		
	S. Pyogenes	*MRSA*[*]	*P. aeruginosa*	*K. pneumoniae*	*E. coli*
5	22.1 ± 0.2	19.4 ± 0.8	27.1 ± 0.6	18.6±.3	24.3 ± 0.5
6	21.3 ± 0.3	19.3 ± 0. 6	24.3 ± 0.3	18.4 ± 0.4	23.9 ± 0.3
7	20.5 ± 0.5	19.2 ± 0.6	24.1 ± 0.6	18.2 ± 0.2	22.7 ± 0.2
8	20.4 ± 0.5	18.5 ± 0.5	22.8 ± 0.4	17.8 ± 0.5	21.9 ± 0.5
Standard	23.0 ± 0.2	22.0 ± 0.2	32.0 ± 0.2	19.0 ± 0.3	27.0 ± 0.4
DMSO	–	–	–	–	–

Positive control (standard); ciprofloxacin and negative control (DMSO) measured by the Halo Zone Test (Unit, mm).
[*] Methicillin resistant *Staphylococcus aureus* (MRSA + Ve).

dismutase (SOD) as superoxide anion inhibitor as shown in Figs. 6 and 7. When the hydroxyl radical inhibitor DMSO and TBA were added to the reaction mixture no evident inhibition of the nuclease activity was observed, suggesting non involvement of hydroxyl radical in the cleavage process. Similarly, in the presence of radical scavengers like NaN_3 and SOD, the cleavage showed no inhibition in the presence of sodium azide with **7** and **8**, thus the presence of singlet oxygen can be ruled out where as in case of SOD the cleavage was not subdued therefore the presence of superoxide radical can also be ruled out in both the compounds. Therefore, DNA cleavage promoted by **7** and **8** might mainly (be or come) through the non-oxidative pathway thus it is very possible that the phosphodiester bond of DNA would have been cleaved by **7** and **8** via phosphoryl transfer reactions [10,15].

To probe the interacting site of **7** and **8** with pBR322 plasmid DNA, The DNA was treated with DAPI or methyl green [37,38] prior to the addition of **7** and **8**. DAPI (minor groove binder) was added to the reaction mixture, significant inhibition was observed in the cleavage pattern. Whereas in presence of methyl green (major groove binder), the cleavage was not suppressed (Figs. 8 and 9). Thus electrophoretic pattern demonstrates that both compounds **7** and **8** shows preferential affinity towards the minor groove of the DNA.

Table 3
Antifungal activity of Tetrazines (**5**–**8**) Positive control (greseofulvin) and negative control (DMSO) measured by the Halo Zone Test (Unit, mm). Diameter of zone of inhibition (mm).

Compounds	CA	AF	TM	PM
5	19.1 ± 0.2	20.6 ± 0.2	18.5 ± 0.3	13.9 ± 0.4
6	18.5 ± 0.4	18.9 ± 0.2	18.4 ± 0.4	13.2 ± 0.2
7	17.2 ± 0.2	18.2 ± 0.4	17.1 ± 0.2	13.1 ± 0.3
8	16.2 ± 0.5	17.2 ± 0.2	16.0 ± 0.5	13.2 ± 0.1
Standard	30.0 ± 0.2	27.0 ± 0.2	24.0 ± 0.3	20.0 ± 0.5
DMSO	–	–	–	–

[*]CA; *Candida albicans*, AF; *Aspergillus fumigatus*, TM; *Trichophyton mentagrophytes*, PM; *Penicillium marneffei*.

Table 4
MIC and MFC results of Tetrazines (**5**–**8**) positive control, Greseofulvin.

Compounds	CA		AF		TM		PM	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
5	12.5	25	12.5	25	12.5	25	12.5	25
6	12.5	25	12.5	25	12.5	25	12.5	100
7	12.5	25	25	100	25	50	50	100
8	25	50	25	100	50	100	50	100
Standard	6.25	50	12.5	25	6.25	25	12.5	25

[*]MIC (μg/ml) = minimum inhibitory concentration, i.e. the lowest concentration of the compound to inhibit the growth of fungus completely.
[γ]MFC(μg/ml) = minimum fungicidal concentration, i.e., the lowest concentration of the compound for killing the fungus completely.

3.4. Antimicrobial activity

The *in vitro* antimicrobial activity of tetrazine-thiones derivatives (**5–8**) were tested using the bacterial culture of *S. pyogenes, MRSA, P. aeruginosa, K. peneumoniae, E. coli* and fungal culture of *C. albicans, A. fumigatus, T. mentagrophytes, P. marneffei*. The *in vitro* study results demonstrated that all the compounds were

Table 2
MIC and MBC results of Tetrazines (**5**–**8**) positive control, ciprofloxacin.

Compounds	Gram positive bacteria				Gram negative bacteria					
	S. Pyogenes		*MRSA*[*]		*P.aeruginosa*		*K. pneumoniae*		*E. coli*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
5	12.5	25	12.5	50	25	50	12.5	25	12.5	25
6	12.5	25	12.5	25	25	50	25	50	25	50
7	25	50	25	100	50	100	25	100	25	50
8	25	50	25	100	50	100	25	100	25	50
Standard	12.5	25	6.25	12.5	12.5	25	6.25	25	6.25	25

[γ]MBC (μg/ml) = minimum bacterial concentration, *i.e.*, the lowest concentration of the compound for killing the bacteria completely.
[*] MIC (μg/ml) = minimum inhibitory concentration, *i.e.* the lowest concentration of the compound to inhibit the growth of bacteria completely.

found to be active against all the tested microorganisms (Table 1–4). All the tested compounds **5, 6, 7** and **8** showed good inhibition against all the fungal strains. The MFC of synthesized compounds were found to be 2–4 times higher as compared to MIC.

4. Conclusion

A new class of Aryl-1,2,4,5-tetrazinane-3-thiones were synthesized by reacting acetophenone derivatives with thiocarbohydrazide in acetic acid and were thoroughly characterized. There *in vitro* DNA binding studies revealed that these compounds interact with DNA via non-intercalative mode of binding as well as the presence of —NH and —OH groups the hydrogen bonding plays a vital role. Furthermore, **7** and **8** were interacted with pBR322 DNA and the electrophoresis pattern was obtained which showed significantly good cleavage. The molecules exhibited preferential binding towards minor groove of the DNA helix. Consequently, they have shown the potential to act as good artificial nuclease mimics. In addition to this all the four compounds were subjected to examine their antimicrobial property. The results showed that these compounds possess notable antibacterial activity. Thus, these compounds possess significant potential to act as metal-free nucleolytic agents.

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