Full Paper

Synthesis and Biological Evaluation of New Tetra-aza Macrocyclic Scaffold Constrained Oxadiazole, Thiadiazole and Triazole Rings

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A new series of *N,N′*-(benzene-1,3-diyldi-1,3,4-oxadiazole-5,2-diyl)bis{2-[(5-benzene-1,3-diyl-1,3,4-oxadiazol-2-yl)amino]acetamide}(macrocycle **1**), *N,N′*-(benzene-1,3-diyldi-1,3,4-thiadiazole-5,2-diyl)bis{2-[(5-benzene-1,3-diyl-1,3,4-thiadiazol-2-yl)amino]acetamide} (macrocycle **2**) and *S,S′*-[benzene-1,3-diylbis(4H-1,2,4-triazole-5,3-diyl)]bis{[(5-benzene-1,3-diyl-4H-1,2,4-triazol-3-yl)sulfanyl]ethanethioate}(macrocycle **3**) was synthesized from isophthalic dihydrazide (**4**) through a multistep reaction sequence. All the synthesized compounds were screened for their inhibitory effect against four different bacterial strains: *P. aeruginosa* ATCC-20852, *K. pneumoniae* MTCC-618, *S. aureus* ATCC- 29737, *S. typhi* MTCC- 3214. The synthesized compounds showed a significant zone of inhibition and the results were comparable with that of the standard drug ciprofloxacin. The synthesized compounds were further studied for their possible *in vitro* antioxidant effects by DPPH scavenging, total antioxidant capacity, total reductive capacity and H_2O_2 scavenging activity. The results indicated that the *in vitro* antioxidant activity for all the three molecules was efficient when compared to the standards. The DNA interaction behavior of macrocycles **1–3** with CT-DNA was investigated by the absorption spectra obtained (K_b constant for **1** is 4.53×10^4 M^{-1}, for **2** is 5.75×10^4 M^{-1} and for **3** is 5.86×10^4 M^{-1}). Based on the results it can be interpreted that the reducing power effect of the newly synthesized compounds demonstrates a direct effect on DNA binding and hence inhibiting the bacterial growth through their action on DNA by inhibiting DNA replication or DNA transcription.

Keywords: Antibacterial activity / Antioxidant activity / DNA binding / Macrocyclic

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Introduction

Numerous macrocyclic systems exist, which contain nitrogen, sulphur and oxygen donor atoms, either as a thiazole, oxazole, pyrrole or quinoline group or as an amine group [1–8]. However, it is somewhat surprising that there is a relative paucity of macrocycles containing five-membered heterocyclic groups [9], considering that there are many articles and reviews that ascertain that compounds bearing a 1,3,4-oxadiazole/thiadiazole and 1,2,4-triazole nucleus possess significant anti-inflammatory activity [10–13]. The development of synthetic macrocyclic methodology, containing five and six-membered heterocyclic rings as subunits has led to the preparation of a range of such compounds which have shown to possess very interesting biological properties in a variety of fields. These macrocycles were found to exhibit interesting host-guest complexation characteristics [14] and have shown antibacterial and antitumor activities [15, 16] in which the biological activity is highly dependent upon the side chain substitution pattern.

In order to demonstrate the potential antioxidant activity of heterocyclic compounds, the interactions of nitrogen,

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sulfur and oxygen containing molecules with DNA are of major biochemical and biological importance [17]. It has been suggested that macrocyclic systems can chelate Fe(II) or Fe(III) and prevent free radical production in the Fenton reaction [18] and themselves can also intercalate in the DNA duplex and react with free radicals in order to protect DNA from oxidative damage [19, 20].

A literature survey shows that there is evidence that antitumor activity is due to the intercalation between the base pairs of DNA and interference with the normal functioning of the enzyme topoisomerase II, which is involved in the breaking and releasing of DNA strands [21]. The antitumor drugs that intercalate in DNA are of growing interest in the field of anticancer derivatives. Generally, they are characterized by planar chromospheres, which are often constituted by three or four condensed rings, which can intercalate between base pairs. Results of these various binding studies have been useful in designing new and promising anticancer agents for clinical use [22, 23]. DNA binding and cleavage studies of macrocyclic molecules have been recently reported in the literature [24, 25].

In view of the above biological importance and in continuation of our previous work [26–28], we demonstrate a new strategy to introduce oxadiazole or thiadiazole or triazole rings into macrocycles employing the novel precursors **1**, **2** and **3** (Fig. 1) without using high dilution conditions. In support to their biological activity, antibacterial activity was screened. Further to unmask and understand the mode of action against the bacterial strains, we further evaluated the antioxidant property, and ds-DNA interacting ability of the synthesized compounds.

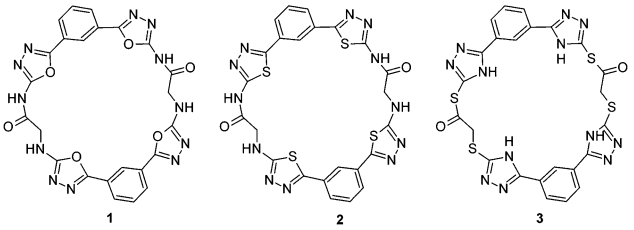
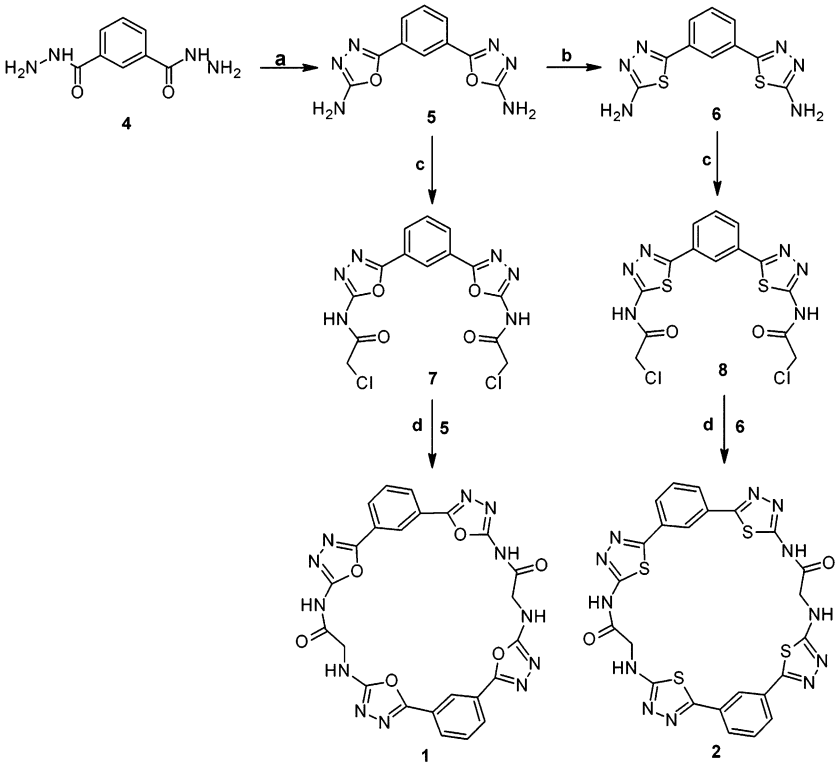


Figure 1. Structures of macrocycles **1–3**.

Results and discussion

Chemistry

The first stride from the synthesis of macrocycle **1** and **2** can be achieved from the reaction of isophthalic dihydrazide **4** in dioxane with 2.2 equiv. of cyanogen bromide in water in the presence of 2.2 equiv. of sodium bicarbonate. The mixture was stirred at room temperature for 8 h, this workup afforded the 5,5'-benzene-1,3-diylbis(1,3,4-oxadiazol-2-amine) **5** (Scheme 1) with 77% yield [29] which was characterized from spectral



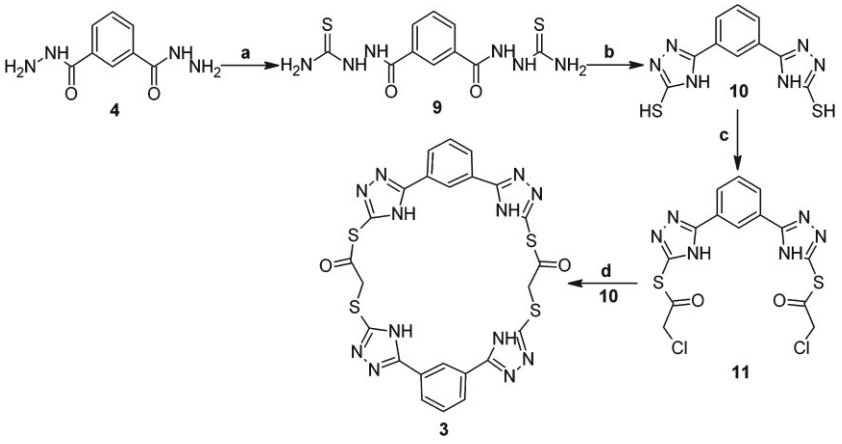
Scheme 1. Reagents and conditions: (a) 2.2 equiv. cyanogen bromide, $NaHCO_3$, dioxane, RT, 8 h, **5**, 77%, (b) 4.2 equiv. thiourea, THF, 120–150°C, 22 h, **6**, 73%, (c) 2.2 equiv. $ClCH_2COCl$, DMF, 0–10°C, 1 h, **7**, 89%; **8**, 84%, (d) triethylamine, 2.2 equiv. K_2CO_3, THF, reflux, 10–12 h, **1**, 68%; **2**, 62%.

and analytical data. Replacement of –O– by –S– in heterocycles was performed as previously reported, *viz.*, Bordners preparation of pyrroles from furan and the transformation of epoxides to episulfides by the action of thiocyanates or thiourea [30, 31]. The compound **5** was treated with twofold excess thiourea in tetrahydrofuran. The reaction mixture after workup gave a solid which was identified as 5,5′-benzene-1,3-diylbis(1,3,4-thiadiazol-2-amine) **6** (Scheme 1) with 73% yield. The probable mechanism involved in the formation of thiouronium salt which undergoes rearrangement to form the mesomeric oxouronium salt *via* the oxathiadiazepine derivative. Further, ring closure of the oxouronium salt led to formation of thiadiazole by the elimination of urea. The comparison of the IR spectra of compounds **5** and **6** showed that the peak at 1087 cm^{-1} can be assigned to ν(C–O–C) of the oxadiazole ring and the peak at 685 cm^{-1} can be assigned to ν(C–S–C) of the thiadiazole ring, thus the compounds were characterized from spectral and analytical data. Coupling of compound **5** or **6** with chloroacetyl chloride in DMF at 0–10°C stirred for 1 h afforded the bis chloroacetamide receptors **7** and **8** with 89% and 84% yield [32], respectively (Scheme 1). The ^{1}H NMR spectrum of **7** displayed the amide NH protons as singlet at 11.2 ppm and CH_2 protons as singlet at 4.9 ppm. The rest of the aromatic protons appeared in the usual region. Molecular ion $[M+H]^+$ peaks of compound **7** and **8** were observed at different intensities in positive ionization mode and confirmed the molecular weights. Compound **7** and **8** which have halogen atoms in their molecule show in their mass spectrum the characteristic peaks corresponding to isotopic distribution (^{35}Cl and ^{37}Cl isotopes). The structure of compound **8** was fully characterized by spectroscopic methods.

Two strategies were attempted for the synthesis of macrocycles **1** and **2**. In the first strategy, compound **5** or compound **6** was chosen as the starting material as described by Da-Ming Du and coworkers [33]. After the cyclization process, the yield of the target macrocycles **1** and **2** was 16–18%, we also observed (by TLC monitoring) the formation of unknown secondary products. This may be attributed to reaction (as by-products) as reported in the earlier literature. We next applied a second strategy as depicted above in Scheme 1.

The macrocyclization step in this method involves the reaction of compound **7** capping with compound **5** or **6** in THF which was completely solubilized with triethylamine under inert (N_2) atmosphere in the presence of K_2CO_3. Later the solution of **7** in THF was added dropwise for 30 min. The reaction mixture was refluxed for 12 h and after workup gave a solid which was identified as macrocycles **1** and **2** respectively in reasonable yields [34]. The IR spectrum of the compound **5** showed absorption bands at 3303 cm^{-1} due to the NH_2 group, which was absent in the IR spectrum of the macrocycle **1**. Similarly, the ^{1}H NMR spectrum of compound **5** showed a broad signal at 5.2 ppm attributed to the NH_2 group, which was not present in the spectrum of compound **1**. In the ^{1}H NMR spectrum of compound **1**, the signal corresponding to protons of the methylene group appeared as doublet at 4.36 ppm and signals due to the amide NH group appeared as singlet at 10.7 ppm. The attributions of the signals ^{13}C NMR of compound **1** resulted from the spectra. In compound **1**, the C=O signal is observed at ~168 ppm and the CH_2 group resonated at ~35 ppm. The C(3) and C(5) heterocyclic carbon resonated at ~153 and ~151 ppm, respectively, further confirming the formation of macrocycle **1**. The structure of macrocycle **2** was also confirmed through spectral studies.

As an extension of this study, 2,2′-(benzene-1,3-diyldiethene-1,1-diyl)dihydrazine carbothioamide **9** (Scheme 2) was obtained by the reaction of isophthalic dihydrazide **4** with potassium isothiocyanate. The cyclization of compound **9** in the presence of aqueous NaOH resulted in the formation of 5,5′-benzene-1,3-diylbis(4*H*-1,2,4-triazole-3-thiol) **10** [35]. The reaction of compound **10** with chloroacetyl chloride in the presence of dimethyl sulfoxide produced *S*,*S*′-[benzene-1,3-



Scheme 2. Reagents and conditions: (a) 2.2 equiv. KSCN, HCl, water, reflux, 6 h, **9**, 86%, (b) 4 N NaOH, water, reflux, 4 h, **10**, 68%, (c) 2.2 equiv. $ClCH_2COCl$, DMSO, RT, 14 h, **11**, 71%, (d) DMSO, RT, 26 h, **3**, 58%.

diylbis(4*H*-1,2,4-triazole-5,3-diyl)]bis(chloroethanethioate) **11**. The macrocyclization step in this method involves the reaction of compound **10** in dimethyl sulfoxide treated with compound **11** and then the reaction mixture was stirred at room temperature for 12 h, this workup gave a solid precipitate, which was identified as macrocycle **3** with 58% yield. The IR spectrum of the compound **3** showed strong absorption band at 1681 cm^{-1} characteristic of carbonyl group. The absorption due to the –NH group appeared at 3304 cm^{-1}, while the –SH peak at 2576 cm^{-1} was absent in the IR spectrum of the macrocycle **3**.

Similarly, the ^{1}H NMR spectrum of compound **10** showed a broad signal at 13.9 ppm attributed to the –SH group which was not present in the spectrum of compound **3**. The attributions of the ^{13}C NMR signals of compound **3** resulted from the spectra. In compound **3**, the C=O signal is observed at ~193 ppm and the CH_2 group resonated at ~34 ppm. The C(3) and C(5) heterocyclic carbon resonated at ~153 and ~152 ppm, respectively, this further confirmed the formation of macrocycle **3**. The structure of macrocycle **3** was fully characterized spectroscopically.

The synthesis of macrocycles **1**, **2** and **3** is of interest due to the presence of carbonyl functionalities in the annular cavity. Hence, its electron-deficient nature would alter the position of the charge transfer complexation band. Also functional group transformations can be carried out on the carbonyl group.

Biological activity

Antibacterial activity

The synthesized macrocyclic compounds were tested for their *in vitro* antibacterial activity against three gram negative and one gram positive standard strains of bacteria. The compounds were found to be effective against gram positive and gram negative strains based on the values obtained through relative zone of inhibition (Table 1). However, slightly varying levels of effect were observed among the synthesized compounds. The results were significant and were comparable with the standard antibiotic drug ciprofloxacin. On the basis of minimum inhibitory activity shown against the test bacteria, compounds **1** and **3** were more effective against *S. typhi* with inhibition zone diameter (IZD) of 15.2 ± 0.60 and 15.2 ± 0.44 respectively. *K. pneumoniae* was least susceptible to compound **1** (10.8 ± 0.17). Compound **2** showed moderate activity against all the test organisms with IZD ranging from 13.17 ± 0.44 to 13.8 ± 1.17. In addition, the above three compounds were found to be effective at different dilutions based on the activity. The MIC data was varying against different test organisms (Table 2). MIC of synthesized compound **1** was found to be 148 μg/mL for *K. pneumoniae*. It was comparatively lower against the other strains, *viz. P. aeruginosa*, *S. aureus* and *S. typhi*. The MIC of compound **2** was higher for all the strains. Compound **3** had MIC of 62 μg/mL and 64 μg/mL for *S. typhi* and *P. aeruginosa* respectively. It was found to be 82 μg/mL for both *K. pneumoniae* and *S. aureus*.

Table 2. Minimum inhibitory concentrations (in μg/mL) of compounds using macrodilution method.

Bacterial strains	Compounds			Ciprofloxacin
	1	2	3	
Pa ATCC-20852	82	122	64	2
Kp MTCC-618	148	132	82	2
Sa ATCC-29737	66	102	82	4
St MTCC-3214	54	122	62	2

In vitro antioxidant activity

In the DPPH assay for free radical scavenging the most potent molecule was found to be compound **1** which showed the maximum percentage inhibition of 88.9% at 100 μg/mL concentration. It was followed by compound **2** which also exhibited good scavenging activity ranging from 74.8% to 77.8% at different concentrations. Compound **3** demonstrated activity with values of 52.3% to 74.3% (Fig. 2).

The FRAP assay is to measure the reductive ability of antioxidant and it was evaluated by the transformation of Fe(III) to Fe(II) in the presence of the synthesized compounds. The results of reducing power assays of the synthesized macro-

Table 1. Antibacterial activity of synthesized compounds against standard bacterial strains (inhibition zone in mm).

Bacterial strains	Compounds			Ciprofloxacin
	1	2	3	
Pa ATCC-20852	13.50 ± 0.76*	13.50 ± 0.79*	14.83 ± 0.44*	20.00 ± 0.58
Kp MTCC-618	10.83 ± 0.17**	13.17 ± 0.44**	14.00 ± 1.04*	21.00 ± 0.87
Sa ATCC-29737	14.83 ± 0.44*	13.83 ± 1.17*	13.67 ± 0.88*	20.17 ± 1.01
St MTCC-3214	15.17 ± 0.60*	13.33 ± 0.88*	15.17 ± 0.44**	20.77 ± 0.54

The values are the means of three experiments ± S.E. *P <0.01 vs. standard.
Abbreviations: Pa, *Pseudomonas aeruginosa*; Kp, *Klebsiella pneumoniae*; Sa, *Staphylococcus aureus*; St, *Salmonella typhi*.

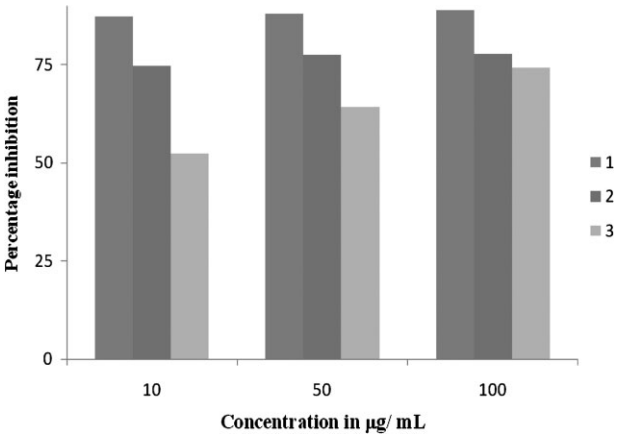


Figure 2. Free radical scavenging using DPPH.

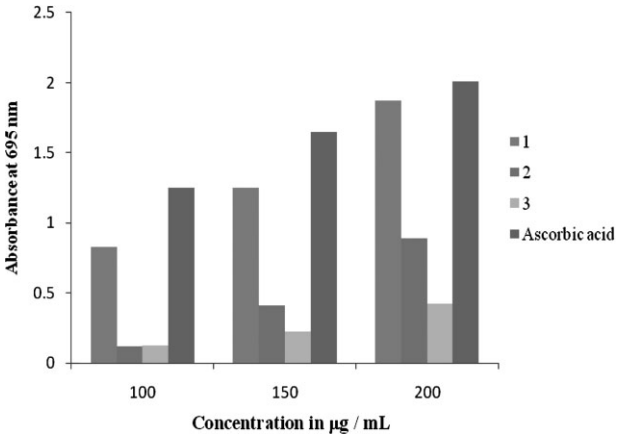


Figure 4. Phosphomolybdenum antioxidant assay.

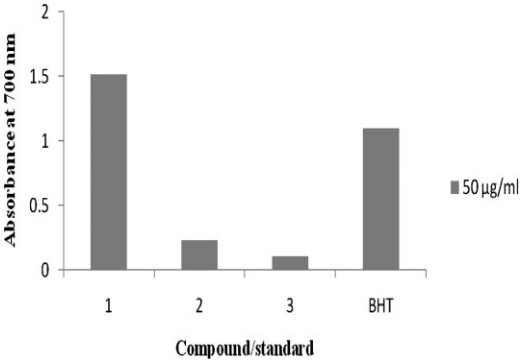


Figure 3. Ferric ion reducing/antioxidant power.

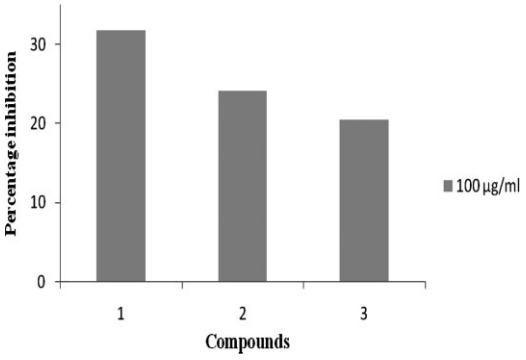


Figure 5. Hydroxyl radical scavenging.

cyclic compounds are summarized in Fig. 2. It is evident that the ability of compound **1** to reduce Fe(III) was better than that of the standard BHT at 100 μg/mL concentration. On the other hand, the compounds **2** and **3** were less effective in exhibiting reducing activity at the same concentration (Fig. 3).

The total antioxidant capacity of the compounds was evaluated at three different concentrations, *viz*. 100, 150 and 200 μg/mL. The results indicated that the compound **1** showed very good antioxidant ability, which was similar to the standard ascorbic acid. Compounds **2** and **3** demonstrated moderate activity (Fig. 4).

The hydroxyl radical scavenging activity was not appreciable at 100 μg/mL concentration of the compounds. Compound **1** exhibited efficient activity with 31.75%. The percentage inhibition was much lower for the other two synthesized compounds (Fig. 5).

DNA binding studies (absorption spectral studies)

The DNA binding studies of the macrocyclic compounds **1–3** (the structures of the compounds are shown in Fig. 1) were often characterized through absorption spectra followed by the changes in the absorbance and shift in the wavelength. The absorption spectra of macrocycles **1–3** in the absence and presence of CT-DNA are illustrated in Fig. 6a–c, respectively. As shown in Fig. 6, the interaction of macrocycles **1–3** with CT-DNA was monitored by the blue shift (the hypsochromic effect) in UV–visible spectra. The absorption maximum wavelength was shifted from 370 nm in the spectra of macrocycle **3** to 338 nm in the spectra of CT-DNA/macrocycle **3** (Fig. 6c). Progressive addition of DNA led to strong hypochromism in the absorption intensities in all the macrocycles studied. The hypochromicity, characteristic of intercalation [36], has been usually attributed to the interaction between the electronic states of the compound chromophores and those of the DNA bases [37].

The percentage hypochromicity for the macrocycles **1–3** was determined from $(\varepsilon_f - \varepsilon_b)/\varepsilon_f \times 100$, where ε_f is the extinction coefficient of the free compound and ε_b is the extinction coefficient of the bound compound. The percentages of hypochromism of macrocycles **1–3** were found to be 22.4, 21.3 and

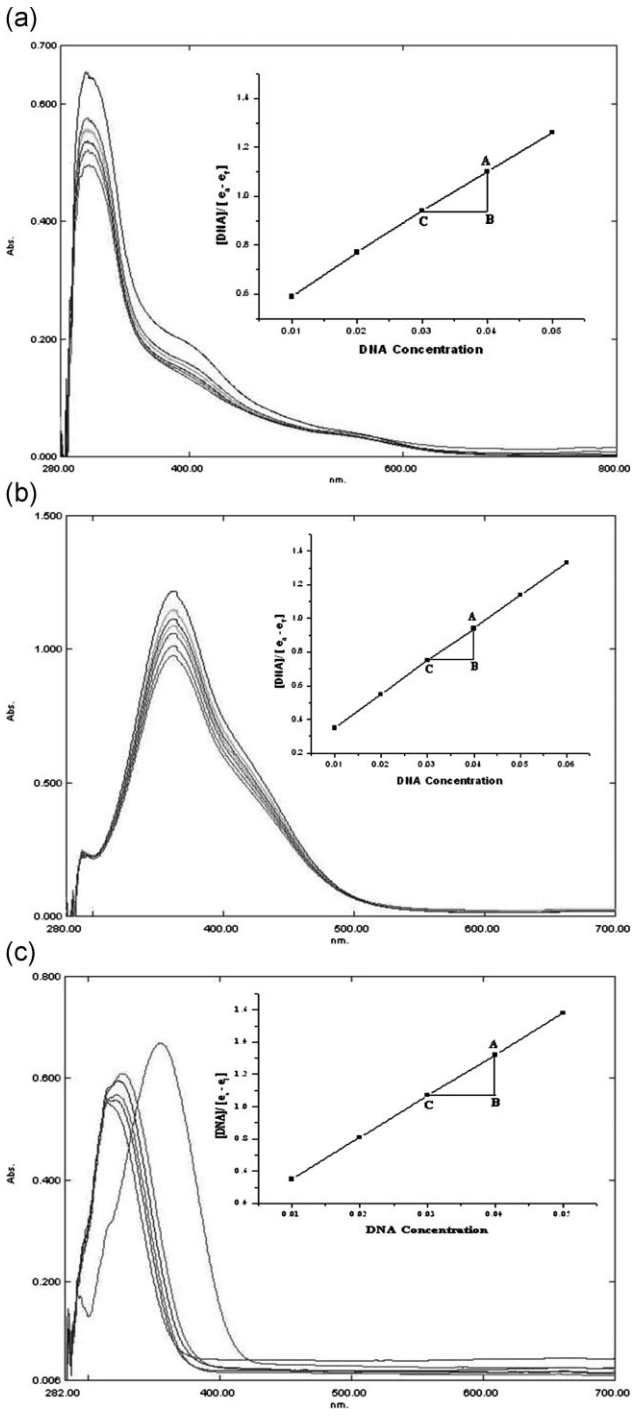


Figure 6. Absorption spectral traces of (a) macrocycle **1**, (b) macrocycle **2** and (c) macrocycle **3** in Tris-HCl buffer upon addition of CT-DNA. Inset: Plots of [DNA]/($\varepsilon_a - \varepsilon_f$) versus [DNA] for the titration of CT-DNA with macrocycles **1–3**.

18.1, respectively. The half-reciprocal plots for binding of macrocycles **1–3** with CT DNA were presented (Fig. 6a–c). The compounds exhibited similar absorption spectra pertaining to chromophore.

To further illustrate the DNA binding strength, the intrinsic binding constant K_b was determined for macrocycles **1–3** which were found to be $4.53 \times 10^4\ M^{-1}$, $5.75 \times 10^4\ M^{-1}$ and $5.86 \times 10^4\ M^{-1}$, respectively. The binding constants of these compounds were lower in comparison to those observed for typical classical intercalators (ethidium-DNA, $1.4 \times 10^6\ M^{-1}$) [38].

This investigation on biological activity of the newly synthesized macrocyclic compounds was by evaluating their effect against bacteria, antioxidant effect by free radical scavenging and reducing power effect. This reducing power effect is in direct corroboration with their effect on DNA binding. Thus it can be interpreted that the antibacterial effect elicited by the synthesized macrocyclic compounds is by binding to the DNA. Most of the antibiotics such as the fluoroqinolones (e.g. ciprofloxacin) inhibit procaryotic (not eucaryotic) DNA replication, and rifamycins inhibit bacterial (not eucaryotic) DNA transcription. The potential antibacterial and antioxidant activities of heterocyclic compounds by their interactions with DNA are of major biochemical and biological importance [17]. These chemical compounds that bind to DNA molecule can induce various effects on the microbial growth by inhibiting the binding of DNA dependant RNA polymerase to the DNA or cleaving the genome or forming DNA bulky adducts and thus hindering the growth of the bacteria. Further, it can also be interpreted that the reducing power effect of the newly synthesized compounds can demonstrate a direct effect on DNA binding and hence inhibiting the bacterial growth through their action on DNA by inhibiting DNA replication or DNA transcription.

Experimental

Chemicals and instrumentation

All chemicals used for the synthesis were of analytical grade and procured from HiMedia Laboratories Pvt. Ltd., and isophthalic dihydrazide was purchased from Sigma Chemical Co., U.S.A., E. Merck, Germany, Sarabhai Merck Company, India. Thin layer chromatography (TLC) was performed throughout the reaction to optimize the reaction for purity and completion of reaction on Merck silica gel GF254 aluminium sheets using a mixture of different polar and nonpolar solvents in varying proportions and spots were observed using iodine as visualizing agent. Melting points were measured on a Mel-Temp apparatus and are uncorrected. Mass spectra were recorded on an Agilent 6320 ion trap mass spectrometer. IR spectra were recorded on a Shimadzu IR-470 spectrometer. ^{1}H NMR spectra were recorded on a Bruker DRX-300 Avance spectrometer 300.13 MHz; chemical shifts (δ scale) are reported in parts per million (ppm). Signals were characterized as s (singlet), d (doublet), t (triplet), m (multiplet),

br s (broad signal) and Ar (aromatic). The ^{13}C NMR spectra were recorded at 75.47 MHz; chemical shifts (δ scale) are reported in parts per million (ppm). The elemental analyses were performed at Cochin University, Sophisticated Test and Instrumentation Center, Kochi, Kerala, India. All the final products are new compounds, which were characterized by IR, ^1H NMR, ^{13}C NMR spectra and mass spectral data.

Synthesis of 5,5′-benzene-1,3-diylbis(1,3,4-oxadiazol-2-amine) ***5***

To a stirring solution of compound **4** (1.44 g, 7.42 mmol) in dioxane (24 mL) sodium bicarbonate (1.25 g, 14.8 mmol) in water (16 mL) was added at room temperature. The mixture was stirred at room temperature for 5 min and cyanogens bromide (1.63 g, 15.4 mmol) was added. After 8 h water (60 mL) was added to the mixture and the precipitate was removed by filtration and recrystallized from ethanol to afford compound **5**.

Yield 77%; mp 137°C; IR (KBr): ν (cm^{-1}): 3303 (NH_2 stretch.), 3057 (aromatic CH stretch.), 1608 (C=N), 1489, 1462 (C=C ring stretch.), 1087 (C–O–C stretch); ^1H NMR (300 MHz, DMSO-d_6) δ (ppm): 8.06 (t, 1H, ArH), 7.89 (dd, 2H, ArH), 7.64 (t, 1H, ArH), 5.28 (bs, 4H, 2 NH_2); (LC-MS) *m/z*: 245 $[M+H]^+$. Anal. Calcd. for $C_{10}H_8N_6O_2$: C, 49.18; H, 3.30; N, 34.41. Found: C, 49.12; H, 3.33; N, 34.39.

Synthesis of 5,5′-benzene-1,3-diylbis(1,3,4-thiadiazol-2-amine) ***6***

A mixture of **5** (1.22 g, 5 mmol), thiourea (1.52 g, 20 mmol) dissolved in tetrahydrofuran (10 mL) was heated at 120–150°C in an oil bath for 22–26 h. After the reaction was completed, the reaction mixture was extracted with dichloromethane. The organic layer was washed with water, brine solution and dried over anhydrous Na_2SO_4. The resultant solid was recrystallized using methanol yielding compound **6**.

Yield 74%; mp 204°C; IR (KBr): ν (cm^{-1}): 3301 (NH_2 stretch.), 3077 (aromatic CH stretch.), 1597 (C=N), 1484 (C=C ring stretch.), 685 (C–S–C stretch); ^1H NMR (300 MHz, DMSO-d_6) δ (ppm): 8.14 (t, 1H, ArH), 7.94 (dd, 2H, ArH), 7.72 (t, 1H, ArH), 5.8 (bs, 4H, 2 NH_2); (LC-MS) *m/z*: 277 $[M+H]^+$. Anal. Calcd. for $C_{10}H_8N_6S_2$: C, 43.46; H, 2.92; N, 30.41. Found: C, 43.49; H, 2.88; N, 30.38.

General procedure for compounds ***7*** *and* ***8***

Compound **5** (1.22 g, 5 mmol) or compound **6** (1.38 g, 5 mmol) was dissolved in DMF (20 mL), and the solution was cooled to 0°C. To this cold solution, chloro acetylchloride (1.56 mL, 20 mmol) in dioxane (5 mL) was added dropwise during a period of 60 minutes, with vigorous stirring at 0–10°C. Compound **7** or compound **8** separated out as a solid from the reaction mixture. It was filtered, washed with water and dried, affording compound **7** or compound **8**, respectively.

N,N′-(Benzene-1,3-diyldi-1,3,4-oxadiazole-5,2-diyl)-bis(2-chloroacetamide) ***7***

Yield 89%; mp 176°C; IR (KBr): ν (cm^{-1}): 3397 (amide NH stretch.), 1652 (amide CO stretch.), 1601 (C=N), 1450 (C=C ring stretch.), 781 (C–Cl stretch); ^1H NMR (300 MHz, DMSO-d_6) δ (ppm): 11.2 (bs, 2H, 2 NH), 8.25 (t, 1H, ArH), 8.08 (dd, 2H, ArH), 7.96 (t, 1H, ArH), 4.9 (s, 4H, 2 CH_2); (LC-MS) *m/z*: 398 $[M+H]^+$, 400 $[M+H]^+$, 402 $[M+H]^+$. Anal. Calcd. for $C_{14}H_{10}Cl_2N_6O_4$: C, 42.43; H, 2.54; N, 21.16. Found: C, 42.45; H, 2.51; N, 21.19.

N,N′-(Benzene-1,3-diyldi-1,3,4-thiadiazole-5,2-diyl)-bis(2-chloroacetamide) ***8***

Yield 84%; mp 253°C; IR (KBr): ν (cm^{-1}): 3243 (amide NH stretch.), 1657 (amide CO stretch.), 1613 (C=N), 1464 (C=C ring stretch.), 791 (C–Cl stretch); ^1H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.7 (bs, 2H, 2 NH), 8.11 (t, 1H, ArH), 7.98 (dd, 2H, ArH), 7.78 (t, 1H, ArH.), 4.7 (s, 4H, 2 CH_2); (LC-MS) *m/z*: 430 $[M+H]^+$, 432 $[M+H]^+$, 434 $[M+H]^+$. Anal. Calcd. for $C_{14}H_{10}Cl_2N_6O_2S_2$: C, 39.17; H, 2.35; N, 19.58. Found: C, 39.19; H, 2.36; N, 19.55.

Synthesis of N,N′-(benzene-1,3-diyldi-1,3,4-oxadiazole-5,2-diyl)bis{2-[(5-benzene-1,3-diyl-1,3,4-oxadiazol-2-yl)-amino]acetamide} ***1***

Compound **5** (1.22 g, 5 mmol) in tetrahydrofuron (THF, 25 mL) was completely solubilized with triethylamine under inert (N_2) atmosphere. To this, K_2CO_3 (0.69 g, 5 mmol) was added. Later the solution of **7** (1.99 g, 5 mmol) in THF (25 mL) was added drop by drop for 30 min. The reaction mixture was refluxed for 10–12 h. The progress of the reaction mixture was monitored by TLC. The reaction mixture was then desolventized in a rotavapour and the compound was extracted in ethyl acetate. The ethyl acetate layer was washed with water and dried over anhydrous Na_2SO_4. The solid was obtained by further desolventation in a rotary evaporator at 50°C. The product was separated and purified by column chromatography, using mixture of ethyl acetate/hexane (80:20) and gave compound **1**.

Yield 68%; mp 105°C; IR (KBr): ν (cm^{-1}): 3286 (amide NH stretch.), 1647 (amide CO stretch.), 1589 (C= N), 1478 (C=C ring stretch.); ^1H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.7 (bs, 2H, 2 CONH), 8.15 (t, 2H, ArH), 7.94 (dd, 4H, ArH), 7.68 (t, 2H, ArH), 5.24 (s, 2H, 2 NH), 4.36 (d, 4H, 2 CH_2); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 168.37, 153.04, 151.26, 136.99, 128.74, 127.01, 124.86, 35.62; (LC-MS) *m/z*: 569 $[M+H]^+$. Anal. Calcd. for $C_{24}H_{16}N_{12}O_6$: C, 50.71; H, 2.84; N, 29.57. Found: C, 50.68; H, 2.87; N, 29.55.

Synthesis of N,N′-(benzene-1,3-diyldi-1,3,4-thiadiazole-5,2-diyl)bis{2-[(5-benzene-1,3-diyl-1,3,4-thiadiazol-2-yl)-amino]acetamide} ***2***

Similar to macrocycle **1** procedure, except compound **6** and **8** were used instead of compound **5** and **7**, which gave compound **2**.

Yield 62%; mp 158°C; IR (KBr): ν (cm^{-1}): 3152 (amide NH stretch.), 1659 (amide CO stretch.), 1602 (C=N), 1442 (C=C ring stretch.); ^1H NMR (300 MHz, DMSO-d_6) δ (ppm): 9.34 (bs, 2H, 2 CONH), 8.08 (t, 2H, ArH), 7.87 (dd, 4H, ArH), 7.62 (t, 2H, ArH), 5.29 (bs, 2H, 2 NH), 4.00 (s, 4H, 2 CH_2); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 167.79, 152.68, 150.49, 135.92, 128.43, 127.57, 125.04, 35.05; (LC-MS) *m/z*: 633 $[M+H]^+$. Anal. Calcd. for $C_{24}H_{16}N_{12}O_2S_4$: C, 45.56; H, 2.55; N, 26.56. Found: C, 45.59; H, 2.52; N, 26.53.

Synthesis of 2,2′-(benzene-1,3-diyldiethene-1,1-diyl)-dihydrazinecarbothioamide ***9***

A suspension of compound **4** (1.94 g, 10 mmol), potassium thiocyanate (30 mmol), hydrochloric acid (16 mL) and water (50 mL) was refluxed for 8 h. The solution was cooled and a white solid appeared. This was filtered and recrystallized from dimethyl sulfoxide/water (1:2) affording compound **9**.

Yield 86%; mp 187°C; IR (KBr): ν (cm^{-1}): 3246 (NH stretch.), 1682 (C=O stretch.), 1310 (C=S); ^1H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.2 (bs, 4H, 2 NH_2), 9.82 (s, 2H, 2 NH), 9.73 (s, 2H, 2 NH),

8.19 (t, 1H, ArH), 7.91 (dd, 2H, ArH), 7.84 (t, 1H, ArH); (LC-MS) *m/z*: 313 $[M+H]^+$. Anal. Calcd. for $C_{10}H_{12}N_6O_2S_2$: C, 38.45; H, 3.87; N, 26.90. Found: C, 38.49; H, 3.84; N, 26.86.

Synthesis of 5,5′-benzene-1,3-diylbis(4H-1,2,4-triazole-3-thiol) ***10***

A solution of compound **9** (3.12 g, 10 mmol) in 2 N NaOH was refluxed for 5 h. The resulting solution was cooled to room temperature and acidified to pH 3–4 with 37% HCl. The precipitate thus formed was filtered, washed with water and recrystallized from ethanol/water (1:1) giving compound **10**.

Yield 67%; mp 289°C; IR (KBr): ν (cm^{-1}): 3194 (NH stretch.), 2576 (SH stretch), 1599 (C=N stretch.), 1034 (N–N stretch); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 13.8 (bs, 2H, 2 SH), 11.4 (s, 2H, 2 NH), 8.33 (t, 1H, ArH), 8.09 (dd, 2H, ArH), 7.87 (t, 1H, ArH); (LC-MS) *m/z*: 277 $[M+H]^+$. Anal. Calcd. for $C_{10}H_8N_6S_2$: C, 43.46; H, 2.92; N, 30.41. Found: C, 43.48; H, 2.89; N, 30.43.

Synthesis of S,S′-[benzene-1,3-diylbis(4H-1,2,4-triazole-5,3-diyl)]bis(chloro-ethanethioate) ***11***

Compound **10** (1.38 g, 5 mmol) dissolved in dimethyl sulfoxide (30 mL) was treated with chloro acetylchloride (1.56 mL, 20 mmol) and the mixture was stirred at room temperature for 14 h. The reaction mixture was then poured into ice water. The precipitate obtained was filtered, washed with water and then with ethyl acetate. The compound was purified by recrystallization using ethanol giving compound **11**.

Yield 71%; mp 226°C; IR (KBr): ν (cm^{-1}): 3243 (NH stretch.), 1637 (C=O stretch), 1613 (C=N stretch.), 1018 (N–N stretch), 754 (C–Cl stretch); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 11.3 (s, 2H, 2 NH), 8.02 (t, 1H, ArH), 7.79 (dd, 2H, ArH), 7.54 (t, 1H, ArH), 4.4 (s, 4H, 2 CH_2); (LC-MS) *m/z*: 430 $[M+H]^+$, 432 $[M+H]^+$, 434 $[M+H]^+$. Anal. Calcd. for $C_{14}H_{10}Cl_2N_6O_2S_2$: C, 39.17; H, 2.35; N, 19.58. Found: C, 39.15; H, 2.38; N, 19.56.

Synthesis of S,S′-[benzene-1,3-diylbis(4H-1,2,4-triazole-5,3-diyl)]bis{[(5-benzene-1,3-diyl-4H-1,2,4-triazol-3-yl)-sulfanyl]ethanethioate} ***3***

Compound **10** (1.38 g, 5 mmol) dissolved in dimethyl sulfoxide (30 mL) was treated with compound **11** (2.15 g, 5 mmol) and the mixture was stirred at room temperature for 26 h. The reaction mixture was then poured into ice water. The precipitate obtained was filtered, washed with water and then with ethyl acetate. The compound was purified by recrystallization using ethanol giving compound **3**.

Yield 58%; mp 303°C; IR (KBr): ν (cm^{-1}): 3304 (NH stretch.), 1681 (C=O stretch.), 1587, (C=N), 1486 (C=C ring stretch.); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 10.7 (bs, 4H, 4 NH), 8.42 (t, 2H, ArH), 8.23 (dd, 4H, ArH), 8.07 (t, 2H, ArH), 4.38 (s, 4H, 2 –S–CH_2–CO); ^{13}C NMR (75 MHz, DMSO-d_6) δ (ppm): 193.28, 153.65, 152.78, 137.68, 129.57, 127.86, 125.43, 34.57; (LC-MS) *m/z*: 633 $[M+H]^+$. Anal. Calcd. for $C_{24}H_{16}N_{12}O_2S_4$: C, 45.56; H, 2.55; N, 26.56. Found: C, 45.51; H, 2.58; N, 26.58.

In vitro antibacterial activity of synthesized macrocycles 1–3

The macrocyclic compounds **1–3** were tested for antibacterial activity by the agar well diffusion method [39].

Test microorganisms

The standard microbial strains of *P. aeruginosa* ATCC-20852, *K. pneumoniae* MTCC-618, *S. aureus* ATCC-29737, *S. typhi* MTCC-3214 were used for antibacterial evaluation. The bacteria were maintained on agar slants at 4°C and sub-cultured into nutrient broth by a picking-off technique for 24 h before use.

Preparation of culture medium and inoculation

Nutrient agar (Hi Media, India) was used as the bacteriological medium. The media were sterilized by autoclaving at 120°C for 20 min. Under aseptic conditions, in the laminar air flow 15 mL of culture medium was dispensed into pre-sterilized petridishes to yield a uniform depth of 4 mm. After solidification of the medium, the microbial cultures were inoculated by spread plating technique.

Primary screening by agar well diffusion

Preliminary screening for all the macrocyclic compounds was performed at fixed concentrations of 10 mg/mL by dissolving in 10% DMSO. Pure DMSO was taken as the negative control and 10 mg/mL of ciprofloxacin (fluoroquinolone antibiotic) was used as the positive control. Wells were prepared in the agar plates using a sterile cork borer of 6.0 mm diameter. 1000 μg/100 μL of each compound were loaded in the corresponding wells. The plates were allowed to stand at room temperature for 1 h for extract to diffuse into the agar and then they were incubated at 37°C for 18 h. Subsequently, the plates were examined for microbial growth inhibition and the inhibition zone diameter (IZD) was measured to the nearest millimeter. The experiment was performed in triplicates.

Determination of minimum inhibitory concentration (MIC) of synthesized compounds

Minimum inhibitory concentration (MIC) is the lowest concentration of any compound at which it will inhibit the visible growth of microorganisms after overnight incubation. Minimum inhibitory concentrations are important in diagnostic laboratories to confirm the resistance of microorganisms to antimicrobial agents and also to monitor the activity of new antimicrobial agents. The MIC of the newly synthesized macrocyclic compounds was tested against bacterial strains through a microdilution tube method [40]. In this method, the test concentrations of chemically synthesized compounds were made from 148 to 0.25 μg/mL in the sterile tubes Nos. 1–11. Mueller Hinton Broth (MHB) medium was prepared and 100 mL sterile MHB was poured into each sterile tube followed by addition of 200 mL compound in tube 1. Twofold serial dilutions were carried out from tube 1 to tube 11 and excess broth (100 mL) was discarded from the last tube No. 11. 0.5 McFarland standard was made, which is visually comparable to a microbial suspension of approximately 1.5×10^8 cells/mL. To each tube, 100 mL of standard inoculum was added. Ciprofloxacin (antibacterial drug) was used as control. All the tubes were incubated for 24 h at 37°C.

Statistical analysis

The results of the antibacterial study are expressed as mean ± SEM of three replicates in each test. The data were evaluated by one-way analysis of variance (ANOVA) followed by

Tukey's multiple pairwise comparison tests to assess the statistical significance. The data were considered at $P < 0.01$.

In vitro antioxidant activity

The macrocycles **1–3** were evaluated for their antioxidant activity using assays such as DPPH scavenging, total antioxidant, ferric ion reducing and hydroxyl radical scavenging.

Determination of free radical scavenging using the DPPH radical

The DPPH free radical scavenging activity was assessed according to Okada & Okada method [41]. An ethanolic solution of DPPH (0.05 mM) (300 μL) was added to 40 μL of compound solution with different concentrations (0.01–0.1 mg/mL). The DPPH solution was freshly prepared and kept in the dark at 4°C. Ethanol 96% (2.7 mL) was added and the mixture was shaken vigorously. The mixture was left to stand for 5 min and the absorbance was measured using a spectrophotometer at 517 nm. Ethanol was used to zero the spectrophotometer. A blank sample containing the same amount of ethanol and DPPH was also prepared. All determinations were performed in triplicate. The radical scavenging activities of the tested samples, expressed as percentage of inhibition, were calculated according to the following equation [42]

$$\text{Percent of DPPH inhibition} = [(A_B - A_A)/A_B] \times 100 \qquad (1)$$

where A_A and A_B are the absorbance values of the test and of the blank sample, respectively. A percent inhibition versus concentration graph was plotted.

Ferric ion reducing/antioxidant power assay (FRAP)

Antioxidant activity was determined by ferric ion reducing antioxidant power assay (FRAP) as described by Oyaizu method [43]. 2 mL of compound **1**, **2** and **3** (100 μg/mL) were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and 0.1% potassium ferricyanide (2.5 mL). The mixture was incubated at 50°C for 20 min. Aliquots of 10% trichloroacetic acid (2.5 mL) were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and a freshly prepared ferric chloride solution (0.5 mL, 0.1%). The absorbance was measured at 700 nm. BHA was taken as standard.

Phosphomolybdenum antioxidant assay

The total antioxidant property of the synthetic compounds was evaluated by the phosphomolybdenum assay method [44] which is based on the reduction of Mo(VI) to Mo(V) by the compounds and subsequent formation of a green phosphate-Mo(V) complex in acidic condition. 0.3 mL (100 μg/mL) of macrocycles **1–3** were combined with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and the reaction mixture was incubated at 95°C for 90 min. Then the absorption of solution was measured at 695 nm using a UV visible spectrophotometer against blank after cooling to room temperature. The antioxidant activity was expressed as the number of gram equivalents of ascorbic acid.

Hydroxyl radical scavenging

This assay was determined according to the previously reported method [45] with a slight modification. The assay is based on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (the Fenton reaction). The reaction mixture contained, in a final volume of 1 mL, 2-deoxy-2-ribose (2.8 mM); KH_2PO_4–KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 μM); EDTA (100 μM); H_2O_2 (1.0 mM); ascorbic acid (100 μM) and the test compounds **1**, **2** and **3** at a concentration of 100 μg/mL. After incubation for 1 h at 37°C, 0.5 mL of the reaction mixture was added to 1 mL 2.8% TCA, then 1 mL 1% aqueous TBA was added and the mixture was incubated at 90°C for 15 min to develop the color. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All tests were performed six times. Percentage inhibition was evaluated by comparing the test and blank solutions.

UV-visible absorption studies

The concentration of CT-DNA per nucleotide [C(p)] was measured using its known extinction coefficient at 260 nm (6600 M^{-1} cm^{-1}) [46]. The absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) for CT-DNA were measured in order to check the purity level. The ratio A_{260}/A_{280} was found to be 1.8 to 1.9, indicating that CT-DNA was satisfactorily free from protein. Buffer (5 mM tris-(hydroxymethyl)aminomethane, pH 7.2, 50 mM NaCl) was used for the absorption experiments.

Absorption titration experiments were carried out by varying DNA concentration (0 to 100 μM) and maintaining the compound concentration constant (0.5 μM). Absorption spectra were recorded after each successive addition of DNA and equilibration (approximately 10 min). In order to obtain the intrinsic binding constant, K_b:

$$[\text{DNA}]/(\varepsilon_a - \varepsilon_f) = [\text{DNA}]/(\varepsilon_b - \varepsilon_f) + 1/K_b(\varepsilon_b - \varepsilon_f) \qquad (2)$$

where ε_a, ε_f and ε_b are the apparent, free and bound compound extinction coefficients, respectively. A plot of [DNA]/(ε_a–ε_f) versus [DNA] gave a slope of 1/(ε_b–ε_f) and an intercept y equal to 1/K_b(ε_b–ε_f), where K_b is the ratio of the slope to the intercept y [47].

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